MIP-1α: A Structure - Function Study

by

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Abstract

The observation that MIP-1α can inhibit the proliferation of transiently engrafting haemopoietic stem cells was first reported more than ten years ago. However, very little has since emerged about the molecular mechanism underlying stem cell inhibition. The work presented in this thesis therefore aimed at shedding some more light on the molecular mechanism and on how the structural properties of MIP-1α relate to its function as a stem cell inhibitor.

Two properties of MIP-1α, which it shares with most other chemokines, were first of all considered, its ability to self-aggregate and its interaction with proteoglycans. It has already been demonstrated that the aggregation of murine MIP-1α is influenced by the pH and the ionic and hydrophobic strength of the buffer. In addition, three acidic residues in the carboxy terminal region of murine MIP-1α have been previously shown to be involved in self-association as their neutralisation generates aggregation-incompetent mutants. Since the aggregation of human MIP-1α has previously been found to be concentration-dependent, similar experiments were carried out for murine MIP-1α which established that its self-association is also controlled in a comparable way which allowed the isolation of differentially aggregated murine MIP-1α by progressive dilution. However, the forces that stabilise the oligomers appear to be stronger in human MIP-1α as compared to murine MIP-1α. In the recent determination of the crystal structure of murine MIP-1α, two calcium ions were found in association with the tetramer that were proposed to mediate the formation of higher order aggregates. However, experiments carried out as part of this thesis demonstrate that this is not the case since the removal of these ions by the addition of EDTA and EGTA has no influence on the aggregation process.

Secondly, investigations were made into the way murine MIP-1α interacts with the glycosaminoglycan heparin. More specifically, experiments were aimed at establishing whether there is a link between the aggregation state of MIP-1α and its affinity for heparin since it is known for some chemokines, such as PF4, that the individual heparin binding sites in the monomers display positive cooperativity upon aggregation, resulting in a higher affinity of the tetramer for heparin. This may also have implications for a possible interaction of MIP-1α with proteoglycans in the bone marrow microenvironment. In a first approach, three aggregation-incompetent murine MIP-1α mutants, a monomer, dimer and a

tetramer, were analysed for their binding to a heparin matrix. Surprisingly, the tetramer exhibited the lowest affinity for heparin, followed by the dimer and then the monomer with the highest affinity. One possible interpretation of this observation is that the heparin binding site becomes progressively occluded upon aggregation, thereby decreasing heparin binding in MIP-1α oligomers. Alternatively, the neutralisation of the negative charges during the generation of these aggregation mutants may have altered the strength with which they bind to heparin since glycosaminoglycan - protein interactions are predominantly determined by electrostatic forces. In order to resolve this question, differentially aggregated murine MIP-1α was prepared by progressive dilution (see above) and the different oligomers tested for their heparin binding affinity. No difference was observed in the strength with which the different aggregates bound to immobilised heparin which was also confirmed with stably cross-linked murine MIP-1α oligomers. This suggests, that the aggregation state of murine MIP-1α has no impact on its affinity for heparin which is instead controlled by its overall charge.

Attention was then turned to the interaction of murine MIP-1α with the receptor on haemopoietic stem cells through which it mediates its inhibitory effect, as demonstrated in an *in vitro* assay, known as the CFU-A assay. It was first of all established that this inhibitory receptor is none of the four known murine MIP-1α receptors (CCR1, CCR3, CCR5 and D6) for the following reasons; (1) none of the other chemokines tested, which included other ligands for the four MIP-1α receptors as well as for all of the other known CC chemokine receptors, displayed any activity in the CFU-A assay, (2) the four known human MIP-1α variants, which show differential binding to murine CCR1, CCR5 and D6, have indistinguishable potencies as stem cell inhibitors, and (3) stem cells from $CCR1^{-/-}$, $CCR3^{-/-}$, $CCR5^{-/-}$ and $D6^{-/-}$ mice were still inhibited in their proliferation by MIP-1α, even when a chemokine analogue, which has the capacity to displace MIP-1α from all of its four receptors, was included in the assays. All of these data suggest that MIP-1α's inhibitory signal is conveyed by a novel, as yet uncharacterised receptor.

As the crystal structure of murine MIP-1α has recently been solved, attempts were made at identifying a region within MIP-1α that is responsible for its inhibitory activity. This was achieved by creating chimaeras in which domains of murine MIP-1α were exchanged with equivalent domains in the highly related CC chemokine RANTES. The chimaeras were generated by Overlap PCR, expressed in the Baculovirus System, purified in a number of chromatography steps and analysed for proper folding in calcium flux assays. They were

then each analysed for stem cell inhibition in the CFU-A assay. The results demonstrate that the main determinants for haemopoietic stem cell inhibition reside within the main body of murine MIP-1α, between the second and the fourth cysteine residue. Further mutagenesis studies are currently underway with the aim of narrowing down this region even further to a possible inhibitory motif on the basis of which an inhibitory peptide may be designed.

In summary, the structure - function studies presented in this thesis have established that the aggregation of murine MIP-1α is concentration-dependent and that the presence of calcium ions is not essential for that process to take place. It was also demonstrated that aggregation does not enhance MIP-1α's affinity for heparin. Previous mutagenesis studies have shown that aggregation and heparin binding are not required for the direct inhibition of haemopoietic stem cells. However, they may nevertheless be relevant for the production, secretion and immobilisation of MIP-1α in the context of the bone marrow microenvironment. Data is also presented that suggests that MIP-1α mediates its suppressive effect via a novel receptor. Studies with MIP-1α/RANTES chimaeras localise the inhibitory domain to the region between the second and fourth cysteine residue of murine MIP-1α. This information may be crucial in the search for the inhibitory receptor which is likely to shed light on the regulation of haemopoietic stem cells and may possibly offer an explanation for the breakdown of this control in leukaemia.

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Declaration

I declare that all work presented in this thesis was performed by me personally unless acknowledged otherwise

Dedication

This thesis is dedicated to my family,
Diese Doktorarbeit ist meiner Familie gewidmet,
Ingeborg Ottersbach
Reiner Ottersbach
Thomas Ottersbach
Phillip Ottersbach
Ernestine Müller

Abbreviations

AIDS	acquired immune deficiency syndrome
AOP	aminooxypentane
b	baculovirus-expressed
BCA-1	B cell-activating chemokine-1
BFU-E	burst-forming unit erythroid
Bl-CFC	blast colony-forming cell
BM	bone marrow
bp	base pair
BSA	bovine serum albumin
C	carboxy
CAFC	cobblestone area-forming cell
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CFC	colony-forming cell
CFU-A	colony-forming unit agar
CFU-E	colony-forming unit erythroid
CFU-GEMM	colony-forming unit granulocyte-erythroid-monocyte-megakaryocyte
CFU-GM	colony-forming unit granulocyte-macrophage
CFU-S	colony-forming unit spleen
CHO	chinese hamster ovary
CM	conditioned medium
CNS	central nervous system
CRU	competitive repopulating unit
CTAP III	connective tissue-activating peptide III
DARC	Duffy antigen receptor for chemokines
DC	dendritic cell
DCCK-1	dendritic cell chemokine 1
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylenebis(oxyethylenenitrilo)tetraacetic acid
ELC	Epstein-Barr virus-induced receptor ligand chemokine
FACS	fluorescence-activated cell sorting
FL	flt3 ligand
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
GRO	growth-related oncogene
h	human
HCC	hemofiltrate CC chemokine
HEK	human embryonic kidney
Hepes	*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid
HIV	human immunodeficiency virus
HLA-A2	human leukocyte antigen-A2

HPP-CFC	high proliferative potential colony-forming cell
HRP	horseradish peroxidase
HSC	haemopoietic stem cell
IEC	ion exchange chromatography
IFN	interferon
Ig	immunoglobulin
IL	interleukin
INT	2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT)
IP10	interferon-inducible protein 10
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobases
kD	kiloDaltons
KSHV	Kaposi's sarcoma-associated herpesvirus
L-Broth	Luria Broth
LTBMC	long-term bone marrow culture
LTC	long-term culture
LTC-IC	long-term culture initiating cell
LTR	long-term repopulating
m	murine
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony-stimulating factor
MCP	monocyte chemotactic protein
MDC	monocyte-derived chemokine
MDR	multidrug resistance
MEM	minimal essential medium
MHC	major histocompatibility complex
Mig	monokine induced by interferon-γ
MIP-1α	Macrophage Inflammatory Protein-1α
MIP-1β	Macrophage Inflammatory Protein-1β
ML-IC	myeloid-lymphoid initiating cell
MMP	matrix metalloproteinase
MOI	multiplicity of infection
MPIF	myeloid progenitor inhibitory factor
M_r	relative molecular weight
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
N	amino
NAP-2	neutrophil-activating protein-2
NK	natural killer cell
NMR	nuclear magnetic resonance
NOD	nonobese diabetic
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PF4	platelet factor four
pfu	plaque-forming unit
r	recombinant
rATP	ribosomal adenosine triphosphate
RANTES	regulated on activation normal T cell expressed and secreted
rpc	reversed phase chromatography
SCF	stem cell factor

SCID	severe combined immunodeficient
SDF-1	stromal derived factor-1
SDS	sodium dodecyl sulphate
SLC	secondary lymphoid-tissue chemokine
SP	side population
SR buffer	Stuber-Roos buffer
STR	short-term repopulating
TECK	thymus-expressed chemokine
TEMED	tetramethylenediamine
TFA	trifluoroacetic acid
TGFβ	transforming growth factor β
T_h	T helper
TNFα	tumour necrosis factor α
Tris	tris(hydroxymethyl)aminomethane
U	units
uv	ultraviolet
v/v	volume for volume
w/v	weight for volume
WECHE	weird chemokine
wt	wild type
X gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

all protein structures were prepared in RasMol

Chapter 1: INTRODUCTION

1.1. The Haemopoietic System

The haemopoietic system is characterised by a high turnover of mature blood cells from as many as eight different lineages (see Fig. 1.1) which are constantly, and throughout the entire life of the organism, replenished from a pool of multipotential stem cells that resides in the bone marrow. This complex system has a hierarchical structure as reflected in the fact that this small number of stem cells is sufficient to give rise to the vast quantity of circulating blood cells via a process of expansion and differentiation that involves a large, intermediate population of progenitors that become progressively more restricted in their differentiation and proliferation potential, a process that is also known as maturation. The following pages provide a brief overview of what is currently known about the origin and properties of haemopoietic stem cells and their regulation by stimulators, inhibitors, transcription factors and their environment.

1.1.1. The Stem Cell Compartment

Probably the most widely accepted definition of stem cells describes them as a rare (~1 in 10^5 total bone marrow cells) and largely quiescent population of cells capable of proliferation, self-renewal, production of a large number of differentiated, functional progeny, regenerating the tissue after injury and of being flexible in the use of these options (Potten and Loeffler, 1990). In the case of haemopoietic stem cells, the best test for whether a cell fulfils these criteria is provided by the transplantation assay in which mice that have received a lethal dose of radiation that destroys their haemopoietic system, are rescued by a transplant of stem cells that will repopulate all the different blood lineages.

However, from such studies it has become clear that haemopoietic stem cells do not just comprise a single population of cells, but should rather be considered as a heterogeneous compartment consisting of stem cells with varying degrees of differentiation and self-renewal capacities. It is nevertheless possible to subdivide the compartment into two types of stem cells according to the contribution they make during the rescue of an irradiated host. Two phases of repopulation following the transplant of bone marrow cells into irradiated animals have been observed which can be attributed to two different subsets of stem cells present in the transplanted bone marrow (Jones et al., 1990).

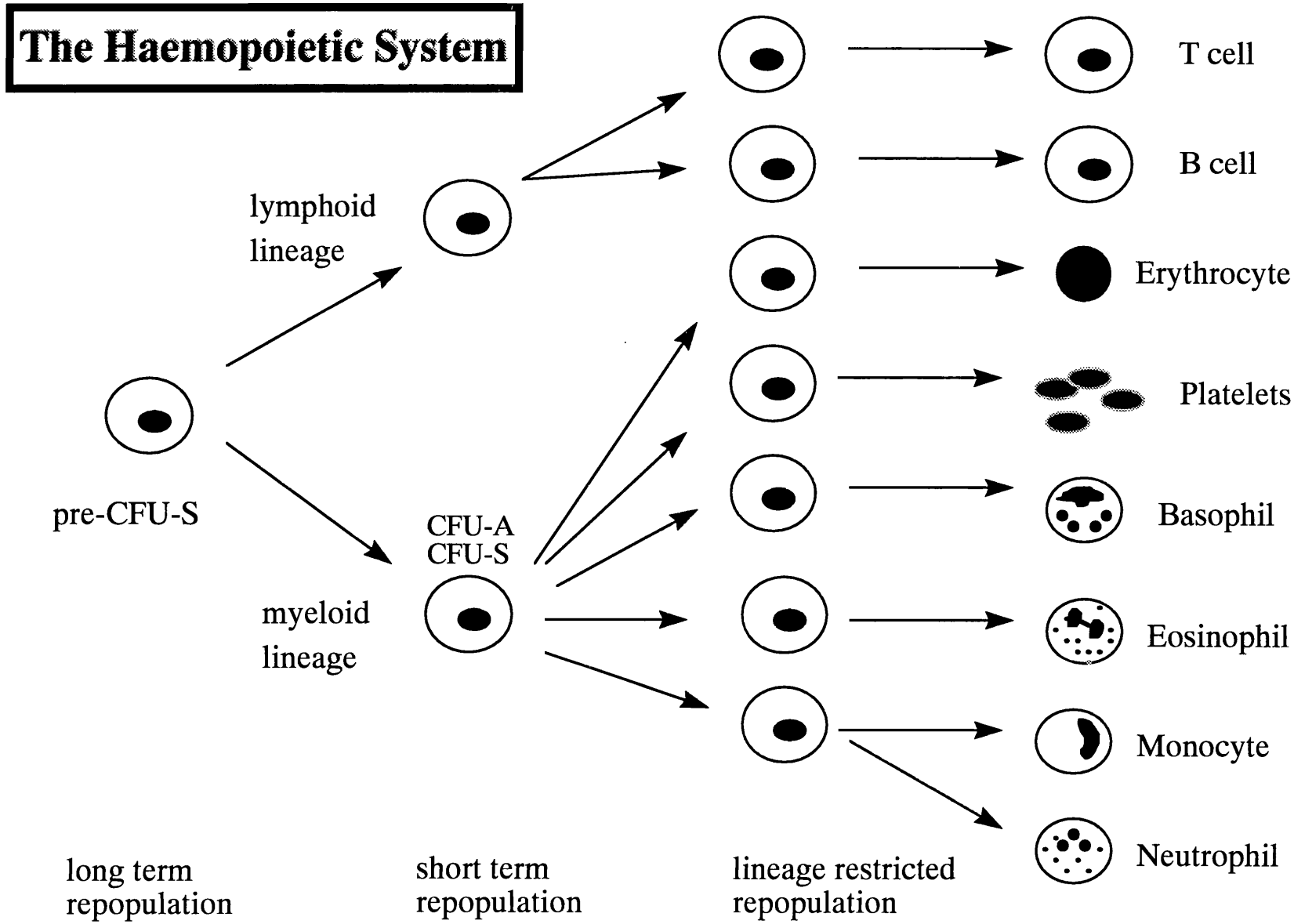


Figure 1.1: The Haemopoietic System

The first phase is accomplished by rapidly proliferating cells that represent the more mature members of the stem cell compartment which are unable to mediate long term repopulation of the haemopoietic system of the host, but which afford transient protection from the immediate and acute effects of radiation. These cells are therefore also known as short term repopulating (STR) cells or radioprotective cells.

The second phase of repopulation is more delayed since it involves cells that proliferate more slowly and that are thought to be dependent on the STR cells to establish a suitable environment into which these cells of the second wave can seed themselves. It is these cells that eventually mediate long term replenishment of the entire haemopoietic system of the host and accordingly are termed long term repopulating (LTR) cells. They are also referred to as competitive repopulating units (CRUs) on the basis of an assay in which genetically distinguishable bone marrow cells are injected together in order to compete for the repopulation of the host (Szilvassy et al., 1990).

For obvious reasons, such repopulation assays cannot be performed in humans. Therefore, in order to be able to test for human cells with repopulating activities, human haemopoietic stem cells are injected into irradiated immunocompromised non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice where human haemopoietic cells can be found to engraft and thus form a chimaeric human/murine haemopoietic system (Larochelle et al., 1996). All of these studies have established that the presence of both of these cell types (LTR+STR) is required to successfully rescue mice from the effects of lethal irradiation. Yet, even within these two subgroups, the stem cell populations are not homogeneous as has been demonstrated by a number of *in vivo* and *in vitro* assays. These assays (Graham and Wright, 1997) have traditionally been used to describe different types of haemopoietic stem cells and also more mature progenitor cells on the basis of their proliferative behaviour in response to different stimuli and the type of progeny they produce. In many cases it is still unclear how the different cell types detected in these assays relate to each other, especially since only slight variations in the assay conditions can markedly influence the outcome. Most of these assays involve the proliferation of stem/progenitor cells in either semisolid medium (agar or methylcellulose) where the progeny of the cells can be detected as colonies or on preformed supportive cell layers. The most common assays will be described in more detail below.

1.1.1.1. The CFU-S Assay

This assay, which was one of the first stem cell assays described (Till and McCulloch, 1961), assesses stem cell proliferation *in vivo* and is based on the observation that within two weeks following transplantation of bone marrow into lethally irradiated mice, clonogenic cells (=CFU-S cells) seed into the spleen where they form readily visible colonies that contain differentiating and differentiated haemopoietic cells. Furthermore, evidence of self-renewal of the CFU-S cells within colonies is evident in their ability to form secondary colonies when retransplanted. There are different types of CFU-S colonies which appear at different time points. The colonies that become visible at around day 8 are derived from more actively proliferating and therefore more mature and more committed erythroid progenitor cells, whereas the colonies visible at day 12 are derived from more immature and more multilineage cells. However, both types belong to the STR stem cell class which is why LTR cells are also sometimes called pre-CFU-S cells.

1.1.1.2. The CFU-A Assay

This is an *in vitro* clonogenic assay that detects the progeny of a cell thought to be equivalent to the CFU-S day 12 cell (Pragnell et al., 1988). It involves the plating of total murine bone marrow in the presence of stem cell factor (SCF), macrophage-colony stimulating factor (M-CSF) and granulocyte/macrophage (GM)-CSF in semisolid medium and will be described in more detail in the experimental section of this thesis as it is one of the major techniques performed as part of this work. A human equivalent to the murine CFU-A assay has also been developed (Holyoake et al., 1993).

1.1.1.3. Other Clonogenic Assays

Other *in vitro* colony-forming assays include an assay that detects so-called blast colony-forming cells (Bl-CFC) in mice (Nakahata and Ogawa, 1982) and in humans (Rowley et al., 1987) as cells giving rise to colonies containing mainly undifferentiated cells. The proliferating cells are similar to the more immature CFU-S cells and may even include some pre-CFU-S cells. An assay that detects a slightly more mature cell, the so-called high proliferative potential colony-forming cell (HPP-CFC) was developed for mouse and human cells (McNiece et al., 1989), and the colonies contain macrophages and granulocytes, erythrocytes and megakaryocytes. By varying the combinations of growth factors included in this assay, three different subclasses of HPP-CFCs can be detected with

varying degrees of maturity and with the most immature, HPP-CFC-1, being more or less equivalent to the cell detected in the CFU-A and CFU-S day12 assay (and possibly even slightly overlapping with the Bl-CFC). A cell overlapping with the slightly more mature members of the CFU-S compartment can be detected *in vitro* in the mixed colony-forming cell (Mix-CFC) assay in the mouse (Johnson and Metcalf, 1977) or in the CFU-GEMM assay in humans (Fauser and Messner, 1979) where the progeny of that cell can be recognised as colonies containing cells of the granulocytic, erythroid, monocytic and megakaryocytic lineages.

While all of the assays mentioned above detect various members of the stem cell compartment, clonogenic assays have also been developed for more mature progenitors which are named according to the type of progenitor they detect. For example, an assay that promotes the proliferation and colony formation from a progenitor that gives rise to the granulocytic lineage, is called colony-forming unit-granulocyte (CFU-G) assay. The general rule is that the more growth factors included in these assays, the more immature the stem/progenitor cell detected. Thus, if only one growth factor is included, a fairly mature progenitor usually only capable of giving rise to one lineage will be detected, whereas combinations of several different growth factors are required to induce the proliferation of stem cells since these cells are characterised by being mainly quiescent and more refractory to the stimulation by single growth factors.

1.1.1.4. Stem Cell Cultures on Stromal Cells

It is almost impossible to detect and maintain LTR cells *in vitro* in any of the clonogenic assays described above. These immature cells require contact to stromal cells, a reduced model of the bone marrow microenvironment, in order to survive and proliferate in culture. The first such culture capable of maintaining LTR cells was developed in the 1970s (Dexter et al., 1977) and involved the *in vitro* culturing of whole murine bone marrow for several months. During that time, an adherent layer of stromal cells formed onto which haemopoietic stem and progenitor cells were able to seed, proliferate and differentiate for a prolonged period. These long term culture (LTC) systems were further developed by first establishing an adherent stromal layer that was subsequently irradiated in order to eradicate any seeded haemopoietic cells (Ploemacher and Brons, 1989). This preformed stromal layer could then be used to test the seeding efficiency of different types of stem cells which established that LTRs are more readily seeded onto these layers than STRs. This culture system was also adapted for a limiting dilution assay that allowed the description of stem

cells that were termed cobblestone area-forming cells (CAFCs) on the basis of the morphological appearance of the colonies (Ploemacher et al., 1989). It was observed that stem cells with differing maturities appeared at different time points, with the more mature members (e.g. CFU-S day12) being detected earlier (day8) than the more immature stem cells (pre-CFU-S at day28). It seems to be a general rule of LTCs that the time of appearance correlates inversely with the degree of maturity of stem cells. LTRs detected in LTCs after 6-8 weeks are also often referred to as longterm culture-initiating cells (LTC-ICs) and were originally thought to be the most immature haemopoietic stem cell detectable in culture. However, since then, researchers have been able to extend the time of culture of LTCs even further, resulting in the detection of a possibly even earlier stem cell (Hao et al., 1996). Recently, another more complex variant of the LTC system has been developed (Punzel et al., 1999) in which stem cells can be tested for their ability to give rise to the myeloid as well as to the lymphoid lineage which is generally considered to be a characteristic of true haemopoietic stem cells. This system combines the 'classical' LTC-IC assay with an assay for lymphoid potential and allows cells that fulfil these criteria to be detected. This assay is referred to as the myeloid-lymphoid-initiating cell (ML-IC) assay.

1.1.1.5. Other Ways of Describing Stem Cells

Apart from their detection in the *in vitro* assays described above and the fact that they can regenerate the entire haemopoietic system and are largely protected from the effects of cytotoxic drugs due to their quiescence, researchers have been trying to find other and more direct ways of identifying haemopoietic stem cells. It is hoped that this may allow purification of this extremely rare population of cells for scientific as well as therapeutic purposes. One way of distinguishing early stem cells from other cells in the bone marrow is on the basis of their size (Jones et al., 1990). LTR stem cells have a small, lymphocyte-like appearance and can thus be separated from other, larger bone marrow cells by a technique called counterflow centrifugal elutriation, which sorts cells on the basis of size and density. This technique allowed, for the first time, the physical separation of STR cells from LTR cells (de Haan and Van Zant, 1999).

Another method of identifying multipotential haemopoietic stem cells capitalises on the observation that these cells stain poorly with the mitochondria-specific dye Rhodamine 123 or the DNA-specific dye Hoechst 33342. This weak staining was initially believed to be due to the stem cell mitochondria being smaller and less active, and a more condensed chromatin organisation in quiescent stem cells, but was finally linked to the expression of a

P-glycoprotein efflux pump of the ABC transporter type, members of which are involved in the drug resistance observed in certain tumours (Chaudhary and Roninson, 1991). An active pump of this type seems to be important for certain stem cell properties, since the overexpression of the ABC transporter MDR1 in bone marrow cells resulted in a myeloproliferative disorder (Bunting et al., 2000). However, the identity of the P-glycoprotein on haemopoietic stem cells that stain poorly for Hoechst 33342, is still unknown. FACS analysis of stem cells stained with Hoechst 33342 has identified a group of stem cells now termed side population (SP) cells (Goodell et al., 1996) which have LTR character. Populations of cells that exhibit a similar staining pattern have also been identified amongst other somatic stem cells, such as muscle cells (see below).

By far the most commonly employed method for identifying and purifying stem cells is based on the expression of relatively specific surface molecules (Spangrude et al., 1988). Probably the best known stem cell marker is the glycoprotein CD34, the exact function of which still remains unclear, but which has traditionally been one of the most useful ways of enriching for haemopoietic stem cells. However, in recent years, doubts have begun to emerge as to how useful this surface molecule is as a marker of mulitpotential stem cells. One of the first reports to this effect (Osawa et al., 1996) demonstrated that a single cell with a $CD34^-$ phenotype was able to repopulate the entire haemopoietic system of an irradiated mouse. The authors of this report proposed that CD34 marks STR cells, while LTR cells do not express this protein. A similar conclusion was drawn from another study in which STR cells were described as being positive for CD34 expression and negative for CD38, a marker normally thought to be present on mature cells, while LTR cells were found to have the opposite phenotype, $CD34^-$ $CD38^+$ (Zhao et al., 2000). This dispute over the usefulness of CD34 as a stem cell marker received further clarification by work showing that CD34 expression on haemopoietic stem cells is reversible and reflects the activation state of the cell rather than its developmental state (Sato et al., 1999). These authors demonstrated that under normal steady-state conditions, most of the cells that confer long term reconstitution in an irradiated recipient are $CD34^-$, but that expression of this surface molecule in these cells was rapidly induced after insult to the haemopoietic system or if these cells were numerically expanded *in vitro*. Both of these situations led to an activation of LTR cells which, as a result, converted to a $CD34^+$ state. In agreement with this observation, most of the stimulated $CD34^+$ LTR cells when transplanted into an irradiated recipient, reverted to a $CD34^-$ phenotype once the newly replenished haemopoietic system had reached steady-state conditions.

The tyrosine kinase receptor c-kit has also traditionally been regarded as a stem cell marker and has been used to enrich for haemopoietic stem cells. Its importance in haemopoiesis is stressed by the fact that mice which have a complete disruption of the gene coding for c-kit, display a severe defect in haemopoiesis early on in development (Russell, 1979). While the c-kit$^+$ haemopoietic stem cell fraction seems to encompass cells with STR as well as LTR abilities, evidence has emerged recently to suggest that these cells are recruited from an even more immature population of c-kit$^-$ haemopoietic stem cells, that do not proliferate in response to multiple growth factors and do not have radioprotective properties, but that will contribute to the longterm reconstitution of the haemopoietic system when injected in the presence of radioprotective cells, albeit with delayed reconstitution kinetics (Ortiz et al., 1999). Thus, it seems that reconstitutive haemopoiesis is maintained by c-kit$^+$ multipotential stem cells that are recruited from a more immature quiescent c-kit$^-$ stem cell population which represents a critical developmental stage in definitive haemopoiesis.

Other haemopoietic stem cell purification protocols include Sca-1 as a stem cell marker and select against the expression of Thy-1 and lineage markers expressed on differentiated blood cells (Spangrude et al., 1988). Recently, other haemopoietic stem cell markers have been reported such as aldehyde dehydrogenase (Storms et al., 1999), AA4 (Petrenko et al., 1999), the integrin GfIIb-IIIa for haemopoietic progenitors (Ody et al., 1999), CD27 for STR cells (Wiesmann et al., 2000) and the receptor tyrosine kinase Tie-2 for LTR cells (Hsu et al., 2000) which may well become part of future haemopoietic stem cell purification protocols.

1.1.2. The Regulation of Haemopoietic Stem Cells

The fact that haemopoietic stem cells, which are normally in a quiescent state, can be rapidly induced to enter the cell cycle following cytotoxic drug treatment and can subsequently return to a quiescent state once steady state conditions have been reestablished, suggests that the proliferation of these cells is under the control of stimulatory as well as inhibitory factors. A number of molecules have been identified as having positive or negative effects on the proliferation of different members of the stem cell compartment and some of these are described below.

1.1.2.1. Inhibitors of Proliferation

The search for inhibitors of early haemopoietic stem cells was not only driven by the hope that these factors would shed light on the processes regulating haemopoiesis under physiological as well as pathological conditions, but also that they may prove to be useful clinical agents. For example, current chemotherapy protocols employ doses of cytotoxic drugs that are limited by the detrimental effects these agents have on normal cells, especially cells of the haemopoietic stem cell compartment. Therefore, protection of these cells by factors that will inhibit their cell cycle, would not only lead to reduced damage to the haemopoietic system and a faster recovery after chemotherapy, but would also allow administration of higher drug doses with a higher chance of complete removal of cancer cells. Clinical trials aimed at testing the usefulness of these inhibitors during chemotherapy schemes have therefore been initiated for some of the factors described below.

A **tetrapeptide** with the sequence NAc-Ser-Asp-Lys-Pro-OH (AcSDKP) was purified and shown to inhibit the proliferation of CFU-S day12 and earlier members of the stem cell compartment (Bonnet et al., 1995; Lenfant et al., 1989) as well as erythroid and granulocytic progenitors in the LTC system (Cashman et al., 1994). It has been proposed that AcSDKP is derived from the amino terminus of Thymosin-β4 by proteolytic processing of this precursor. Interestingly, Thymosin-β4 has also been demonstrated to possess stem cell inhibitory activites, which however map to the carboxy terminus of this protein (Bonnet et al., 1996), suggesting the AcSDKP sequence to be uninvolved. Several modes of action have been proposed for AcSDKP, including an interference of the peptide with the response of stem cells to growth stimulating factors (Robinson et al., 1993). However, other studies have indicated that AcSDKP requires the presence of accessory cells in the form of an adherent layer in order to be able to inhibit stem cell proliferation (Cashman et al., 1994). It was therefore suggested that it may carry out its function indirectly by inducing the release of an inhibitory factor from these accessory cells, with circumstantial evidence pointing to the chemokine MIP-1α as this factor.

The **pentapeptide** is an inhibitory peptide with the sequence pyroGlu-Glu-Asp-Cys-Lys (pEEDCK) produced by mature granulocytes and found as a normal constituent of the bone marrow milieu (Paukovits et al., 1998). It reversibly inhibits the proliferation of the more mature progenitors, CFU-GM, as well as that of early stem cells, CFU-S and pre-CFU-S, *in vitro* and *in vivo* and is thus able to protect these stem cells from the effects of cytotoxic drugs (Paukovits et al., 1990; Paukovits et al., 1993). This peptide may be an important

stem cell regulator *in vivo* since the addition of anti-pEEDCK antiserum to longterm bone marrow cultures or the injection of antiserum into mice significantly increases the numbers of CFU-GM in the bone marrow cultures and in the femora of the injected mice (Paukovits et al., 1999). Interestingly, the inhibitory activity of the pentapeptide is lost when this peptide forms dimers via a redox mechanism, thought to be mediated by activated granulocytes (Paukovits et al., 1998), which converts it into a stem cell stimulator (Paukovits and Paukovits, 1995). The mechanism by which pEEDCK exerts its inhibitory activity is unknown. It was, however, recently suggested that it interferes with the action of growth factors such as IL-11 by binding to them directly (Paukovits et al., 1999).

Transforming Growth Factor β (TGFβ) is a protein involved in many different developmental processes, including haemopoiesis (Fortunel et al., 2000). There are three highly homologous isoforms in mammals, TGFβ1, -β2 and -β3, all three of which have been shown to be potentially involved in the regulation of haemopoiesis. The effects of these proteins on haemopoietic stem cells *in vitro* was studied by adding them directly to clonogenic assays. This established that all three isoforms inhibit the proliferation of the very early members of the stem cell compartment, such as LTC-IC and HPP-CFC, with TGFβ2 displaying the lowest efficiency. In addition, both TGFβ1 and TGFβ2 stimulate the proliferation of late progenitors, an activity not shared by TGFβ3 (Jacobsen et al., 1991).

The inhibitory effects of TGFβ on early stem cells were also observed in long term bone marrow cultures, and antibodies against this protein were capable of prolonging or reactivating the proliferation of LTC-ICs in these cultures, indicating that TGFβ is actually produced and functions as an endogenous inhibitory factor (Eaves et al., 1991). The role of TGFβ as a regulator of haemopoietic stem cells was also confirmed *in vivo*. It was shown to protect haemopoietic stem/progenitor cells from the cytotoxic drug treatment in a reversible fashion (Grzegorzewski et al., 1994), and the local administration of TGFβ1 into the femur of mice, revealed a preferential growth-inhibitory effect of this factor on the earlier members of the stem cell compartment (Goey et al., 1989).

Knockout mice for all three TGFβ isoforms are also available, but difficult to interpret from an haemopoietic viewpoint because of the disruption of many other vital developmental processes that make all three knockouts lethal. However, some haemopoietic defects, such as reduced erythroid cell numbers in the yolk sac, were observed in TGFβ1 knockout mice (Dickson et al., 1995). Further evidence for a role of

these proteins in the regulation of haemopoietic processes is that abnormalities in TGFβ expression or in any other components of the TGFβ signalling cascade can contribute to defects such as neutropenia and leukaemia (Fortunel et al., 2000). There seem to be several levels at which TGFβ may exert its effects (Fortunel et al., 2000). It has been demonstrated to modulate the expression of several growth factor receptors, including c-kit (Heinrich et al., 1995), and was also proposed to induce apoptosis. However, several *in vitro* and *in vivo* studies have clearly shown that the inhibition of stem cell proliferation induced by TGFβ is a reversible process (Batard et al., 2000; Goey et al., 1989).

Macrophage Inflammatory Protein-1α (MIP-1α), described in more detail in the final section of this introduction, was the first chemokine to be shown to have an effect on the proliferation of haemopoietic stem and progenitor cells (Graham et al., 1990), that was distinct from its well-known function as a pro-inflammatory cytokine. Since then, several other chemokines have also been shown to regulate stem cell proliferation (see later section on Chemokines in Haemopoiesis, section 1.2.4.). Most of them, including MIP-1α, have a bidirectional effect similar to that observed with TGF-β, in that they inhibit the proliferation of earlier members of the stem cell compartment, while stimulating the proliferation of later progenitors. It remains to be shown what the relationship (if any) is between these effects on haemopoietic stem cells and the role of chemokines in the regulation and activation of mature leukocytes during inflammatory responses.

Other inhibitors of haemopoietic stem cells exist that have not been studied in as much detail as the ones described above. These include **all-trans retinoic acid** which again seems to be a bidirectional inhibitor that stimulates late committed CFU-GM and early myeloid progenitors (CFU-Blast), but inhibits the very early CAFCs, possibly through increasing soluble forms of adhesion molecules (Sammons et al., 2000). **Interferon-γ (IFNγ)** and **Tumour Necrosis Factor-α (TNFα)** have also both been reported as bidirectional inhibitors, with IFNγ being a selective inhibitor of very early haemopoietic stem cells (Snoeck et al., 1994). The fact that TNFα regulates haemopoietic processes at the stem cell level was mainly deduced from studying the phenotype of mice deficient for the p55 subunit of the TNFα receptor (Rebel et al., 1999; Zhang et al., 1995). These mice had increased marrow and peripheral blood cellularity of the myeloid compartment, accompanied by a decrease in the actual numbers as well as the self-renewal ability of haemopoietic stem cells.

Thus, several inhibitors of haemopoietic stem cell proliferation have been identified, however, their importance for steady state haemopoiesis and how their actions relate to each other *in vivo* is still unclear. For example, although it has been demonstrated that MIP-1α and TGFβ have overlapping target cells and that the expression of TGFβ is upregulated by MIP-1α (Maltman et al., 1996), an inhibitor of TGFβ does not abrogate MIP-1α-induced stem cell inhibition, thus demonstrating that MIP-1α works independently of TGFβ. It is also doubtful whether any of these inhibitors will have a clinical value. As will be described in a later section, phase I and phase II clinical trials that were carried out to assess MIP-1α as a potential haemopoietic stem cell protective agent during chemotherapy, did not produce the expected results. And although TGFβ is a very powerful stem cell inhibitor, its crucial role in a number of other developmental processes and its related toxicity prevent any possible clinical applications of this protein.

1.1.2.2. Stimulators of Proliferation

An extensive repertoire of haemopoietic growth factors, including SCF, Interleukin (IL)-1, -3, -4, -6, -11, -12, G-CSF and GM-CSF, has been identified over the years that influence the survival, proliferation and differentiation of haemopoietic cells (Graham and Wright, 1997; Ogawa, 1993). These can be roughly categorised into three groups: (1) late-acting lineage-specific, (2) intermediate acting nonlineage specific and (3) factors affecting the kinetics of cell cycle dormant immature stem cells.

In the regulation of immature cell proliferation, most of these growth factors will only act in synergy which often makes it difficult to exactly define the effects of a single growth factor. For example, SCF, IL-3 and GM-CSF will individually maintain the viability of early haemopoietic stem cells, but require the presence of synergising factors such as IL-6 or IL-11 to induce those cells to proliferate (Graham and Wright, 1997; Kollet et al., 1999). SCF and flt3 ligand (FL) is another pair of growth factors that can synergise with each other as well as a whole range of other growth factors to support the proliferation and differentiation of a number of different components of the stem cell compartment (Borge et al., 1999; Graham and Wright, 1997). There is a great deal of interest in potential stimulators of stem cell proliferation from a clinical point of view, and some of the factors mentioned above are routinely included in protocols for the *in vitro* or *ex vivo* expansion of stem cells for transplantation. However, the answers to several important questions remain to be determined. For example, it is not yet known whether the expansion of stem cells observed in the presence of some of these factors is a true process of self-renewal, or

whether it is always accompanied by a certain degree of differentiation or phenotypical alteration. It is known that haemopoietic stem cells cannot divide indefinitely, but have a finite life span, and that serial transplantation will compromise and eventually exhaust the stem cell pool.

Related to this debate is the question of how adult stem cells divide at a single cell level, whether they can undergo symmetric as well as asymmetric divisions and how this may be controlled. In theory, a stem cell should have the following options: (1) it could give rise to two identical, undifferentiated daughter stem cells in a true self-renewal and expansion process, (2) it could give rise to two identical daughter cells that have undergone differentiation during the process (i.e. proliferation coupled to differentiation) or (3) it could give rise to two cells that can behave differently, with one of them staying dormant while the other one continues to proliferate and differentiate. While the existence of symmetric stem cell divisions can be inferred from the fact that a single pluripotent haemopoietic stem cell can repopulate the haemopoietic system in transplantation assays, asymmetric divisions have recently been detected at a single cell level (Ema et al., 2000; Huang et al., 1999). In one of these reports (Huang et al., 1999), the fluorescent dye PKH26 which incorporates into membranes was used to trace stem cell divisions since the decrease in fluorescence that occurs when the dye becomes equally distributed among the membranes of the progeny of a cell, is directly proportional to the number of cell divisions. The observation of single PKH26-stained $CD34^{+}CD38^{-}$ cells by a time-lapse camera system revealed that in addition to multiple, synchronous and symmetric divisions, single cells also gave rise to a daughter cell that remained quiescent for up to eight days, whereas the other daughter cell proliferated exponentially. Further experiments described in this report also suggest that although lineage commitment and cell proliferation can be skewed by extrinsic signals, symmetry of early divisions is probably under the control of intrinsic factors.

Related to this is yet another important question which is whether differentiation of stem cells is an instructive, permissive or even stochastic process, with experimental evidence supporting a role of growth factors in instructing as well as permitting the differentiation process. For example, in a recent report, the reprogramming of lymphoid progenitors to progenitors that give rise to cells of the myeloid lineage in response to IL-2, was achieved by the enforced expression of the receptor for IL-2 (Kondo et al., 2000). On the other hand, while the growth factors IL-7 and M-CSF have also been associated with differentiation along specific lineages, it has recently been demonstrated that defects observed in mice that

are deficient in the genes of either of these proteins, can be reversed by overexpressing Bcl-2, a blocker of apoptosis (Akashi et al., 1997; Lagasse and Weissman, 1997; Maraskovsky et al., 1997). This implies that these two factors, rather than being instructive to the differentiation process, act as 'survival' or 'happiness' factors that prevent the cells from undergoing apoptosis and allow them to undergo differentiation spontaneously or in response to intrinsic factors.

These intrinsic factors are most likely to be transcription factors as a great number of them have now been associated with the differentiation along specific blood cell lineages (Amatruda and Zon, 1999; Blobel, 2000; Enver and Greaves, 1998). However, despite the fact that the knockouts of certain transcription factors in mice result in a phenotype that implicates this factor in determining the fate of uncommitted progenitors, a picture has now emerged that resembles that of growth factors, in that a combination of transcription factors will determine cell fate rather than individual factors on their own. It was even observed that prior to differentiation, components of competing or alternative lineage pathways are simultaneously active at the level of chromatin accessibility and gene expression in varying patterns or combinations reflecting considerable intrinsic heterogeneity and developmental plasticity (Enver and Greaves, 1998; Papayannopoulou et al., 2000). A multipotential stem cell therefore seems simultaneously to have several options open to it, and the decision towards a specific lineage is likely to involve several steps of activation where a certain threshold has to be overcome at every single step that then results in the shutdown of alternative pathways and the increased accessibility of components that drive differentiation further along the chosen path. It is therefore the relative balance of positive and negative regulators that will determine the outcome.

1.1.3. The Bone Marrow Microenvironment

As mentioned above, combinations of soluble cytokines and growth factors can never mimic the environment that the haemopoietic stem cell finds itself in within the bone marrow which explains why only long term bone marrow cultures can detect the earliest stem cells which can not be scored in any other *in vitro* clonogenic assay. In the bone marrow, haemopoietic stem cells are in close contact with stromal cells and other components of the marrow extracellular matrix and thus form defined niches or 'hematons' (Blazsek et al., 2000) that act as protective and regulatory microenvironments (Greenberger, 1991; Whetton and Graham, 1999). Stroma in several other tissues have a similar composition and include macrophages, fibroblasts, osteoblasts, adipocytes,

epithelial cells and endothelial cells, yet only the haemopoietic microenvironment can support haemopoiesis which is likely to be due to the expression of specific growth factors and adhesion molecules.

There are several components in the microenvironment that contribute to stem cell maintenance and regulation. Stromal cells have been shown to secrete a number of important haemopoietic mediators such as IL-1, -6, -11, several CSFs, SCF, FL, TNFα and TGFβ (Sensebe et al., 1997). Some of these factors may also be presented on the surface of these cells in a membrane-bound form. Equally important is the interaction between haemopoietic stem cells and stromal cells via adhesion molecules both for the retention of stem cells in the bone marrow as well as for the migration and homing to the bone marrow during development and after transplantation. For example, knockout studies have revealed that β1 integrins mediate the adhesion of haemopoietic stem cells to vascular endothelial cells, and that in their absence seeding of the bone marrow during development and after stem cell transplantation by haemopoietic stem cells does not occur (Potocnik et al., 2000). These adhesion molecules may also have a role in regulating the proliferation and differentiation of haemopoietic stem cells. For example, PSGL-1 binds to P-selectins on stem cells and mediates the suppression of stem cell proliferation (Levesque et al., 1999), and CD9, which interacts with β1 integrins on haemopoietic stem cells, has been implicated in regulating their differentiation (Aoyama et al., 1999). Other adhesion molecules thought to be involved in stem cell regulation in the microenvironment are α4 integrins (Arroyo et al., 1999) and laminins (Gu et al., 1999).

Gap junctions, and especially connexin 43 within these junctions, have also been shown to be a crucial component of a functional haemopoietic stem cell niche (Cancelas et al., 2000; Montecino-Rodriguez et al., 2000), with the loss of even one allele of connexin 43 affecting blood cell development.

A lot of evidence has also been accumulated to support the role of glycosaminoglycans in regulating haemopoietic stem cell function in the hematon. Not only were specific glycosaminoglycans found to bind to haemopoietic stem cells and influence the growth and differentiation characteristics of cultured $CD34^+$ cells (Madihally et al., 1999), but even the sulphation pattern and content in the glycosaminoglycan heparan sulphate (for a more detailed description of glycosaminoglycans, see section on chemokine-proteoglycan interactions, section 1.2.5.4.) determined whether it supported haemopoietic stem cell maintenance (Gupta et al., 1998) or differentiation along the erythroid lineage (Drzeniek et

al., 1999). In addition, sulphated polysaccharides were shown to be able to mobilise haemopoietic stem and progenitor cells, possibly through the disruption of glycosaminoglycan-mediated stem - stromal cell interaction or through the release of chemokines and cytokines that are often found in association with glycosaminoglycans (Sweeney et al., 2000).

Thus, haemopoietic stem cell regulation is a very complex process that involves a wide range of different components. In this complicated network, the relative contribution made by each one of these individual components is only just starting to emerge and the interaction between all of these regulatory molecules is still largely unknown. The fact that the environment of a cell is the primary determinant for its fate, is stressed even further in the following section.

1.1.4. Stem Cell Plasticity

Each type of tissue is likely to contain a population of stem cells that give rise to the different mature cells characteristically found in that particular tissue type. In some cases, where there is a high turnover or loss of mature cells, as for example in the haemopoietic system, the small intestine and the epidermis, this replenishment is a continuous process, while in other cases, for example in the central nervous system, liver and muscle, generation of new mature cells from a pool of proliferating and differentiating stem cells occurs more slowly and in response to injury. Until recently, it was believed that each tissue contained its own specific stem cell type and that these different types of somatic stem cells were restricted in their differentiation potential to the type of mature cells normally found in that tissue. However, this view of tissue-restricted stem cells has now been challenged by reports that suggest that somatic tissue-derived stem cells may have a remarkable degree of plasticity.

In the first of these reports (Ferrari et al., 1998), the process of muscle regeneration was closely examined since it was noticed that the pool of resident satellite cells, that are considered to be muscle stem cells and thought to give rise to committed myogenic precursors, is too small to account for the extensive repair observed after muscle damage, thus implying migration or recruitment of undifferentiated precursors from other sources. And indeed, it was demonstrated that cells from genetically marked whole bone marrow when injected into regenerating muscle, were able to contribute to the formation of new muscle fibres. Further support for the capability of haemopoietic stem cells to give rise to

muscle cells was provided by another report (Gussoni et al., 1999) in which it was shown that whole bone marrow or purified haemopoietic stem cells (SP cells, see above) cannot only reconstitute the haemopoietic system in lethally irradiated *mdx* mice, which serve as a model for the human disease Duchenne Muscular Dystrophy, but also partially restore dystrophin expression in the affected muscle. The authors were also able to identify cells within muscle that have a high degree of dye efflux and thus possess a similar SP cell phenotype. Furthermore, these muscle SP cells, when injected again into lethally irradiated *mdx* mice, not only gave rise to dystrophin-expressing muscle cells, but also reconstituted the haemopoietic system in these mice, albeit with lower efficiency than bone marrow-derived SP cells.

Thus, reprogramming of somatic stem cells can be observed in both directions, with the SP phenotype possibly serving as a general marker for cells with stem cell characteristics. In yet another report (Jackson et al., 1999), cells from disaggregated muscle tissue were cultured *in vitro* in a system not normally employed in a haemopoietic context. The haemopoietic differentiation potential of these cells was then tested in a competitive repopulation assay where they were injected in combination with bone marrow cells into lethally irradiated mice and shown to repopulate all blood lineages with a ten fold higher efficiency than bone marrow cells.

This observed plasticity is not just reserved for muscle and blood stem cells. Clonal or primary neural stem cells from either embryonic or adult murine forebrain were able to rescue the haemopoietic system of sublethally irradiated mice and contribute to all blood lineages, as demonstrated by a number of *in vitro* clonogenic haemopoietic assays (Bjornson et al., 1999). Sublethal, as opposed to lethal, irradiation was required because it was assumed that neural stem cells would need more time to adopt a haemopoietic stem cell phenotype. This assumption was supported by the fact that neural stem cells before transplantation do not produce colonies in *in vitro* haemopoietic colony forming assays and also show a delayed (by three weeks) repopulation of the immune system with weaker engraftment as compared to haemopoietic stem cells.

Finally, bone marrow stem cells were also demonstrated to participate in the production of hepatocytes, following liver injury (Petersen et al., 1999). Thus, the evidence described above suggests that haemopoietic stem cells are not restricted in their differentiation potential to the production of haemopoietic cells, but can also give rise to mature cells found in the muscle and in the liver. Furthermore, stem cells from other tissues, such as

brain and muscle, also seem to be endowed with a similar degree of plasticity and may be identified on the basis of high dye efflux.

Haemopoietic stem cells not only display plasticity in the type of tissue they produce, but also in their developmental potential. When mouse adult haemopoietic stem cells (lin^- c-kit^+ Sca-1^+) were injected into mouse blastocysts, they not only contributed to all blood lineages at different developmental stages in the developing embryo, but the erythroid progeny of the stem cells starts re-expressing embryonic- and foetal-type globin (Geiger et al., 1998). Similarly, the transplantation of embryonic or foetal progenitor cells into adult recipients causes an almost complete switch in their globin transcription to the adult type. Thus, haemopoietic stem and progenitor cells have a more extensive repertoire of developmental programmes open to them than originally assumed, and the final decision on which direction to take is predominantly made by the microenvironment the cell finds itself in.

1.2. Chemokines

Chemokines are members of a large family of chemotactic cytokines which were originally described as a subfamily of proinflammatory cytokines with a characteristic four cysteine motif that regulate the migration of leukocytes during inflammation. Recent years, however, have not only seen a dramatic expansion in the number of chemokines identified, concomitant with the characterisation of new subgroups, but it has also become clear that chemokines have a number of additional functions both within and outside the immune system.

As mentioned above, the majority of chemokines contain four conserved cysteine residues, the exact distribution of which forms the basis of their subdivision into chemokine families. Table 1.1 gives a complete list of all currently identified chemokines, their alternative names, the receptors they bind to and, in the case of CXC chemokines, the presence or absence of a three amino acid motif (ELR - see also section 1.2.5.3.). The two largest groups are the α or CXC chemokines, so called because of the presence of one intervening amino acid between the first two conserved cysteine residues, and the β or CC chemokines in which the first two cysteine residues are directly adjacent to each other. This subdivision is also reflected in the chromosomal location of these proteins, with most of the CXC chemokines forming a cluster on mouse chromosome 5 (human chromosome 4) and the CC chemokines forming a cluster on mouse chromosome 11 (human chromosome 17). Additional miniclusters are found both within and outside these two major clusters which represent further functional subdivisions within these groups. It has been suggested that most, if not all, chemokines arose from gene duplication of a single ancestral gene and that this diversification in chromosomal location likely reflects functional specialisation that has developed during the evolution of this superfamily (Zlotnik and Yoshie, 2000). These two major chemokine groups can be subdivided even further on the basis of functional differences, e.g. specific target cells, constitutive versus induced expression, angiogenic versus angiostatic properties, as well as on the basis of structural differences, e.g. presence or absence of specific sequence motifs, as will be discussed in the following sections.

Table 1.1: The Chemokine Family

CC Chemokines

Systematic Name	Common Names	Receptor(s)
CCL1	I-309, TCA3	CCR8
CCL2	MCP-1, MCAF, JE (mouse)	CCR2, D6
CCL3	MIP-1α, SCI, LD78α/LD78β (2 isoforms in humans)	CCR1, CCR3 (mouse), CCR5, D6
CCL4	MIP-1β, Act-2	CCR5, D6
CCL5	RANTES	CCR1, CCR3, CCR5, D6
CCL6	C10 (no human homologue known)	Unknown
CCL7	MCP-3	CCR1, CCR2, CCR3, D6
CCL8	MCP-2	CCR1, CCR2, CCR3, CCR5, D6
CCL9/CCL10	MRP-2, CCF18, MIP-1γ, (no human homologue)	Unknown
CCL11	Eotaxin	CCR3
CCL12	MCP-5 (no human homologue known)	CCR2, D6
CCL13	MCP-4, Ckβ10, NCC-1 (no mouse homologue known)	CCR1, CCR2, CCR3, D6
CCL14	HCC-1, HCC-3, NCC-2, Ckβ1, MCIF (no mouse h.)	CCR1 (D6)
CCL15	HCC-2, NCC-3, MIP-5, MIP-1δ, Lkn-1 (no mouse h.)	CCR1, CCR3
CCL16	NCC-4, LEC, HCC-4, LCC-1, Ckβ12	CCR1
CCL17	TARC, ABCD-2	CCR4
CCL18	DCCK1, PARC, MIP-4, Ckβ7 (no mouse homologue)	Unknown
CCL19	ELC, MIP-3β, exodus-3, Ckβ11	CCR7, CCR11
CCL20	LARC, MIP-3α, exodus-1, Ckβ4	CCR6
CCL21	SLC, 6Ckine, exodus-2, TCA4, Ckβ9	CCR7, CCR11
CCL22	MDC, ABCD-1	CCR4
CCL23	MPIF-1, MIP-3, Ckβ8 (no mouse homologue)	CCR1
CCL24	MPIF-2, eotaxin-2, Ckβ6	CCR3
CCL25	TECK, Ckβ15	CCR9, CCR11
CCL26	eotaxin-3, MIP-4α (no mouse homologue)	CCR3
CCL27	ESkine, CTAK, skinkine	CCR10
CCL28	MEC	CCR10

CXC Chemokines

Systematic Name	Common Names	Receptor(s)
CXCL1 (ELR+)	GROα, MGSA-α, NAP-3. MIP-2	CXCR2
CXCL2 (ELR+)	GROβ, MGSA-β, MIP-2α	CXCR2
CXCL3 (ELR+)	GROγ, MGSA-γ, MIP-2β	CXCR2
CXCL4 (ELR-)	PF4	Unknown
CXCL5 (ELR+)	ENA-78, LIX	CXCR2
CXCL6 (ELR+)	GCP-2, CKA-3	CXCR1, CXCR2
CXCL7 (ELR+)	PBP, CTAPIII, β-TG, NAP-2, LA-PF4 (no mouse h.)	CXCR2
CXCL8 (ELR+)	IL-8, NAP-1, GCP-1 (no mouse homologue known)	CXCR1, CXCR2
CXCL9 (ELR-)	Mig	CXCR3
CXCL10 (ELR-)	IP10	CXCR3
CXCL11 (ELR-)	I-TAC, IP9 (no mouse homologue known)	CXCR3
CXCL12 (ELR-)	SDF-1, SDF-1α/SDF-1β (2 human isoforms)	CXCR4
CXCL13 (ELR-)	BLC, BCA-1	CXCR5
CXCL14 (ELR-)	BRAK, bolekine	Unknown
CXCL15 (ELR+)	WECHE, lungkine	Unknown
CXCL16 (ELR-)	CXCL16	CXCR6

C Chemokines

Systematic Name	Common Names	Receptor
XCL1/XCL2	Lymphotactin, SCM-1α/SCM-1β (2 human isoforms)	XCR1

CX_3C Chemokines

Systematic Name	Common Names	Receptor
CX3CL1	Fractalkine, neurotactin	CX3CR1

In addition to CC and CXC chemokines, two further subfamilies have recently been described with, so far, only one member identified per group. Lymphotactin is the sole member of the γ or C chemokine subfamily and contains only two instead of the usual four cysteine residues. Fractalkine, also known as Neurotactin, the only representative of the δ or CX_3C chemokines, has its first two cysteine residues separated by three amino acids and also has the unusual feature of possessing a transmembrane domain and potentially existing in a soluble as well as a membrane-anchored form (Bazan et al., 1997; Pan et al., 1997). Only one other chemokine, CXCL16, with a similar transmembane domain is known to date (Matloubian et al., 2000).

The numerous functions of chemokines are mediated through seven-transmembrane G protein-coupled receptors (GPCRs), most of which employ the Pertussis Toxin-sensitive G_i class of heterotrimeric G proteins (Kuang et al., 1996). Binding of a chemokine to its specific receptor induces dissociation of receptor-G protein complexes and, in turn, dissociation of α from βγ G protein subunits. βγ then signals to downstream effectors, notably phospholipase C β2 which is expressed selectively in haemopoietic cells. This enzyme catalyses the hydrolysis of plasma membrane phospholipids to diacylglycerol, which activates protein kinase C, and inositol triphosphate, which induces release of Ca^{2+} from intracellular stores. A common assay for the functional interaction of chemokines with their receptors measures the rise in intracellular Ca^{2+} in chemokine receptor-transfected cell lines which have been loaded with a calcium-sensitive dye (as described in more detail in the Materials and Methods section of this thesis). Other signalling pathways such as the MAP Kinase pathway have also been shown to be activated in response to chemokine signalling (for a review of chemoattractant signalling see Bokoch, 1995).

Eleven receptors for the CC chemokines (CCR1-11) and six for the CXC chemokines (CXCR1-6) have been identified to date and one each for the C (XCR1) and CX_3C (CX_3CR1) chemokine family. Within the CC and CXC family of chemokines and receptors, a great deal of promiscuity exists which is reflected in the fact that several chemokines can bind to more than one receptor and several receptors can bind more than one chemokine. For example, murine MIP-1α has been found to bind to as many as four receptors, CCR1, CCR3, CCR5 and D6 (Nibbs et al., 1997) and the promiscuous chemokine receptor D6 has been found to bind at least nine CC chemokines (Nibbs et al., 1997). Furthermore, there is one chemokine receptor that can bind chemokines of the CC as well as of the CXC family (Horuk et al., 1994), while all other cellular chemokine receptors known so far are restricted to their own subfamily of chemokines. The reason for

this apparent redundancy is not entirely clear, however, in a recent review (Mantovani, 1999), Mantovani suggests that such promiscuity provides the immune system with a high degree of robustness. This concept seems to be further supported by the observation that chemokines are also redundant in their action on target cells and that certain cells concomitantly produce several chemokines with an overlapping spectrum of action. Furthermore, several knockout mice have been generated which are deficient for certain chemokines and their receptors. In most cases, these mice lacked an overt phenotype, although they do display occasional deficiency in response to certain pathological situations. There is, however, one chemokine, SDF-1, for which there is an absolute requirement since it is known to play important roles in development (see below).

1.2.1. Chemokines and Leukocyte Trafficking

It has become clear in recent years that chemokines and their receptors are involved in the control of the migration of leukocytes both during the development of these cells and their subsequent trafficking in immune surveillance as well as during a humoral immune response (for reviews on this subject, see Cyster, 1999; Kim and Broxmeyer, 1999; Melchers et al., 1999; Sozzani et al., 1999). This resulted in the categorisation of these proteins into "constitutive" chemokines and "induced/inflammatory" chemokines. ELC, SLC, BCA-1, SDF-1, TECK and MDC are examples of the former class of chemokines which are constitutively expressed at defined locations in the primary and secondary lymphoid organs and which regulate the basal trafficking of cells that express the corresponding receptors to these sites. Examples of inflammatory chemokines are MIP-1α, RANTES, MIP-1β and the monocyte chemoattractant proteins (MCPs), whose expression is induced during the course of an immune response and which serve to attract leukocytes to the sites of infection.

Progenitors of lymphocytes initiate T lymphopoiesis mainly in the thymus and B lymphopoiesis in the bone marrow. Within these primary lymphoid organs, the progenitors colonise specific niches for proliferation and development into mature naïve lymphocytes. Knockout mice that are deficient either in SDF-1 or its receptor CXCR4 show amongst other defects a complete absence of B lymphopoiesis. This, however, does not represent an actual developmental block, but rather an inability to home to the specific niches in the bone marrow in which B lymphopoiesis occurs since B cell progenitors are found in the peripheral blood of these mice (Nagasawa et al., 1996; Tachibana et al., 1998; Zou et al., 1998). It has recently been shown that these early B cell progenitors also respond to TECK

(Bowman et al., 2000), a chemokine which until then had only been implicated in T cell development in the thymus and homing of gut-specific T lymphocytes to the intestine (Kunkel et al., 2000; Zabel et al., 1999).

Concomitant with the exit of B lymphocytes from the bone marrow, they display an upregulation in their responsiveness towards BCA-1 (Bowman et al., 2000), which together with its receptor CXCR5 is essential for the homing of B cells to B cell rich areas in the spleen and in Peyer's Patches (Gunn et al., 1998). This homing has been formally demonstrated in CXCR5 null mice which displayed an absence of germinal centres in these tissues (Forster et al., 1996). CXCR5 is also found to be upregulated in antigen-activated $CD4^+$ T cells which can then migrate to B cell areas in order to mount T cell-dependent antibody responses (Ansel et al., 1999).

Another chemokine receptor that is very important for the homing of lymphocytes and dendritic cells (DCs) to lymphoid organs is CCR7, knockout mice for which were recently generated (Forster et al., 1999). It had previously been shown that immature DCs, i.e. cells that had not encountered antigen, express receptors for proinflammatory chemokines, such as CCR5, which allows them to be attracted to sites of infection. Following activation through contact with antigen, DCs downregulate CCR5 expression and at the same time start expressing CCR7, the receptor for SLC and ELC, which allows them to migrate to lymphoid tissues where they become antigen presenting cells and serve to activate lymphocytes (Iwasaki and Kelsall, 2000). SLC is expressed in the endothelium of lymphoid organs and mediates the homing of T lymphocytes and DCs to these tissues. There has been some controversy over whether SLC also induces B lymphocyte homing since data by Warnock (Warnock et al., 2000) seems to suggest that B lymphocytes utilise a different entry mechanism. B lymphocytes do, however, respond to SLC, and results from the CCR7 knockout studies indicate that, despite the possibility that B cells enter the lymphoid organs via a different route, their expression of CCR7 may keep them for a defined period of time in close contact with T cells in the T cell rich areas in order to allow effective B cell-T cell interactions to take place. There is also evidence for an increase in responsiveness of B cells to SCL and ECL after activation which induces them to move towards T cell rich areas. At the same time, they secrete MIP-1β, MIP-1α and MDC which attract activated T cells (Cyster, 1999).

On the basis of the findings mentioned above, different subsets of leukocytes can now be distinguished according to the chemokine receptor repertoire expressed on their surfaces

and, consequently, the chemokines they respond to. This does not only depend on their developmental stage, but also on their tissue specificity and the subtype of these cells. For example, Sallusto (Sallusto et al., 1999) report the existence of two different subsets of memory T cells which apparently circulate around the body by different pathways and mediate the memory response in different ways. One subset does not express CCR7 and is regarded as an "effector" memory T cell that does not migrate through the secondary lymphoid organs but instead surveys the peripheral tissues for its specific antigen. The other subset is termed "central" memory T cell and is characterised by the expression of CCR7. It can, however, rapidly convert to an "effector" subtype once it encounters its specific antigen in secondary lymphoid tissues upon which it loses CCR7 expression and migrates to the site of infection. Chemokine-mediated tissue specificity of lymphocytes was recently demonstrated by work by Campbell *et al* 1999 (Campbell et al., 1999) who showed that CCR4 mediates the adhesion of skin-specific memory T cells.

The mechanism by which circulating leukocytes leave the blood and migrate into tissue in response to a chemokine gradient is a multi-step process. It involves in the first instance selectin-mediated rolling of leukocytes along vessel walls and, subsequently, following chemokine-mediated activation, firm adhesion through integrins present on the endothelial cells as well as on the leukocytes themselves. The arrested cells will then leave the blood flow and migrate through the endothelial cell layer along the chemokine gradient in a process called diapedesis.

In order to be able to maintain a stable chemokine gradient on the luminal surface of endothelial cells that is not washed away by the blood flow, chemokines have to be tethered in a way that allows them to be presented to passing leukocytes. One way this could be achieved is by binding of chemokines to proteoglycans present on cell surfaces and in the extracellular matrix. Most if not all chemokines have the capacity to interact with sulphated proteoglycans such as heparin and heparan sulphate via a number of basic residues usually arranged in clusters (discussed in more detail in a later section). A recent report by Pelletier (Pelletier et al., 2000) also seems to implicate the adhesion protein fibronectin in the tethering of chemokines to vessel walls and in their presentation to chemokine receptors on passing leukocytes. They found that SDF-1α bound to fibronectin with a relatively high K_d of 20 nM and that the presented chemokine was more efficient in stimulating directed migration of responsive cells which also showed a clustering of CXCR4 at their leading edge. In fact, the authors go so far to suggest that continuous directed movement of leukocytes does not actually require the presence of a chemokine

gradient. Instead, they propose that the initial encounter of tethered chemokine at a certain threshold concentration is sufficient to induce the polarisation of these cells and that they then simply continue migrating in the direction in which they first polarised.

A mechanism has recently been proposed by which chemokines that are produced within the tissue at the site of an infection reach the other side of the endothelial cell layer where they may form a gradient. Until then it was thought to be accomplished by chemokine diffusion through the intercellular gaps. However, work by Middleton (Middleton et al., 1997) demonstrated that IL-8, when injected intradermally, binds to the abluminal surface of the endothelial cell layer of postcapillary vessel walls, undergoes internalisation and incorporation into calveolin-containing vesicles, is transcytosed across the endothelial cell layer and is eventually found on the apical side of these cells. This process of receptor-mediated transcytosis has been proposed to involve the promiscuous chemokine receptor DARC (Duffy antigen receptor for chemokines) which binds to CC as well as CXC chemokines. Knockout mice for this receptor have very recently been generated (Dawson et al., 2000) which have a normal phenotype under non-pathological conditions, but show an exaggerated inflammatory response to a challenge with bacterial lipopolysaccharide which suggests that DARC modulates the intensity of inflammatory reactions and thus acts as a regulatory sink for chemokines.

1.2.2. Subversion and Exploitation of the Chemokine System by Pathogens

It is now clear that chemokines are essential for mounting an effective immune response which is why they are a common target for exploitation by pathogens that attempt to evade the immune system (for reviews on this subject see Lalani et al., 2000; Murphy, 2000; Pease and Murphy, 1998). Pathogens have evolved a number of diverse strategies for corrupting the chemokine system. One way of exploiting the system has been achieved by the Human Immunodeficiency Virus (HIV)-1 and the parasite *Plasmodium vivax* which causes Malaria. These two pathogens use chemokine receptors for entry into host cells; in the case of the Malaria parasite it is DARC (Horuk et al., 1993) whereas HIV-1 employs a number of CC and CXC chemokine receptors (CXCR4, CCR2b, CCR3, CCR5, CCR8 and CX_3CR1 - depending on the strain) in combination with the T helper cell marker CD4 (Clapham and Weiss, 1997). It was also demonstrated that activated $CD8^+$ T lymphocytes from HIV-infected individuals secrete a soluble activity that suppresses HIV infection and which was shown to contain a number of chemokines, including RANTES, MIP-1α, MIP-

1β (Cocchi et al., 1995), and MDC (Pal et al., 1997) which compete directly with the virus for chemokine receptors. Also, individuals who are homozygous for a CCR5 deletion are largely protected from HIV infection (Doranz et al., 1996).

HIV also encodes in its genome the glycoprotein gp120 which is a chemokine mimic that mediates neuronal apoptosis via CXCR4 (Hesselgesser et al., 1998) and the Tat protein which is a CC chemokine mimic that acts as an agonist (for CCR2 and CCR3) (Albini et al., 1998). Chemokine homologues are found in a number of other viruses where they serve different functions. The genome of Kaposi's sarcoma-associated herpesvirus (KSHV), for example, encodes three CC chemokine homologues vMIP-I, vMIP-II and vMIP-III which carry out a wide range of different functions. All three of them have been demonstrated to possess angiogenic properties (Dittmer and Kedes, 1998; Stine et al., 2000), which may be important for the blood supply of the lesions produced by this virus. In addition, vMIP-II has also been shown to display antagonistic effects on CCR1, CCR2, CCR3, CCR5 and CXCR4 (Kledal et al., 1997; Murphy, 2000), while all three of them display chemoattractant properties towards T_H2 cells either through CCR8 (vMIP-I, vMIP-II) or through CCR4 (vMIP-III) both of which are expressed on T_H2 cells. The reason for this chemoattractant effect is not entirely clear, but may serve to change the balance of the immune response away from a virally-targeted T_H1 towards a T_H2 response. KSHV, like a number of other viruses, also encodes a chemokine receptor homologue which is constitutively active, but which retains chemokine binding properties (Gershengorn et al., 1998), and which also acts as an angiogenic factor (Bais et al., 1998). Another chemokine receptor homologue is encoded in the ORF U28 of the human cytomegalovirus. Its function seems to be the sequestration and internalisation of chemokines (Bodaghi et al., 1998).

Another way of manipulating the chemokine system was discovered in a number of poxviruses which encode in their genomes proteins that do not exhibit homology to any known host proteins but which are potent chemokine binding proteins and thus serve as broad spectrum chemokine scavengers. Interestingly, the genome of the Myxoma virus contains a gene that codes for a hybrid protein (M-T7) that binds chemokines with its carboxy terminus and has homology to the IFNγ receptor and therefore IFNγ binding properties in a different domain. Because M-T7 only binds to the rabbit form of IFNγ, but can bind to chemokines from different species, its clinical use as a selective chemokine blocking and therefore antinflammatory agent is currently being explored (Liu et al., 2000). This is a good example of how an understanding of the viral mechanisms used to perturb chemokine function might not only lead to the gain of insights about the normal function of

the chemokine system, but might also lead to novel therapeutic applications for the treatment or prevention of a wide spectrum of inflammatory or viral diseases.

1.2.3. Chemokines and Disease

Although an inflammatory response is necessary for the resolution of a pathogenic event, the toxic nature of many products secreted by various cells implicated in these events can cause significant damage to tissues (Perry et al., 1995). Chemokines and their receptors are instrumental for various aspects of an inflammatory response and have therefore been implicated in numerous inflammatory disorders that are characterised by aberrant immune inflammatory responses, such as allergies and autoimmune diseases. In addition, tumours have been shown to exploit several functions of chemokines in order to promote the proliferation and spread of cancer cells. And recent findings in the study of neurological disorders seem to suggest that pathogenic actions of chemokines can sometimes arise from the inappropriate extensions of their normal physiological functions (see Asensio and Campbell, 1999 and below).

1.2.3.1. Allergy and Asthma

During recent years the prevalence of allergic diseases has increased rapidly in developed countries. There are basically two types of hypersensitivity reactions that result in allergic responses. The first type involves an immediate hypersensitive reaction that is initiated by antigen binding to IgE molecules, followed by a late phase reaction that is characterised by an accumulation of basophils, neutrophils, eosinophils and T_H2 cells. This type of reaction is responsible for allergic diseases such as asthma. The second type is a delayed-type hypersensitivity reaction, such as found in contact allergic responses, and involves $CD4^+$ T lymphocytes of the T_H1 class. Chemokines contribute to these responses through their ability to recruit and activate leukocyte populations, induce degranulation, and cause the release of inflammatory mediators from effector cells such as basophils, mast cells, neutrophils and eosinophils (Lukacs et al., 1999). The close and intriguing association of chemokine receptor expression with the T_H1 or T_H2 cell phenotypes, with CCR3, CCR4 and CCR8 expressed by T_H2 cells and CCR5 and CXCR3 found on T_H1 cells (Zlotnik and Yoshie, 2000), suggests that chemokines may participate in these hypersensitivity responses by not only influencing the migratory patterns of these cells but also by induction of differentiation towards the T_H1 or T_H2 phenotypes (Homey and Zlotnik, 1999; Lloyd et al., 2000). In the research into the causes of asthma, attention has also been focused on

chemokines that have the ability to attract eosinophils, as it is these cells that are thought to be primarily responsible for the induction of bronchial mucosal injury and are associated with bronchial obstruction during asthmatic responses (Folkard et al., 1997; Holgate et al., 1997). It was found that the movement of these cells through the vessel wall into the lung interstitium, and subsequently into the airway, during the early stages of asthma is dependent upon RANTES and MIP-1α, whereas eotaxin is necessary for the eosinophil accumulation during chronic stages of the response (Campbell et al., 1999; Campbell et al., 1998). It is therefore eotaxin and its receptor CCR3 that have been the main focus in the search for therapeutic treatments for asthma (Sabroe et al., 2000). Indeed, an amino terminally altered form of Ckβ7 has proved to be a very effective antagonist of CCR3 (Nibbs et al., 2000).

1.2.3.2. Cancer

There are three ways in which chemokines can potentially influence the growth of tumours: by their angiogenic or angiostatic properties, by initiating tumour immunity or by regulating their metastasis (Oppenheim et al., 1997). CXC chemokines can be subdivided into two groups according to the presence or absence of a conserved three amino acid motif consisting of glutamic acid, leucine and arginine, ELR, which directly proceeds the first cysteine residue (see Table 1.1). These three amino acids are not only crucial for ligand/receptor interactions on neutrophils (Clark-Lewis et al., 1993; Hebert et al., 1991 and see below), but were also demonstrated to confer angiogenic properties, while ELR$^-$ CXC chemokines were found to act as angiostatic factors (Strieter et al., 1995). As tumours progress, their demand for sufficient blood supply increases and, as a result, the growth of new blood vessels into the tumours is intitiated in a process termed angiogenesis which depends on the relative balance of angiostastic and angiogenic factors (Belperio et al., 2000). Angiogenic chemokines such as GROα have been implicated in promoting the growth of tumours since they were found to be expressed in human melanomas and their depletion resulted in a marked reduction of tumour-derived angiogenesis and, as a consequence, tumour growth was inhibited (Luan et al., 1997). ELR$^-$ CXC chemokines, on the other hand, have the ability to suppress tumours due to their angiostatic properties. For example, IP10 has been demonstrated to impair angiogenesis in Burkitt's lymphoma cell lines to the extent that severe damage was caused in established tumour vasculature, resulting in tissue necrosis (Sgadari et al., 1996).

Evidence exists that some chemokines are expressed inside tumours where they induce infiltration of leukocytes that can lead to a temporary tumour regression (Negus et al., 1997; Rollins and Sunday, 1991; Zhang et al., 1997). In other cases, transfection of genes encoding chemokines, such as IP10, RANTES and I-309, into tumour cells promoted the development of immunity in mice to tumours that overexpress these chemokines (Laning et al., 1994; Luster and Leder, 1993; Mule et al., 1996). Furthermore, mice injected with tumours expressing these chemokine genes reject the tumours and develop specific resistance to subsequent challenge with nontransfected parental tumour cells. There have been recent reports stating that not only CXC chemokines but also CC chemokines have direct effects on angiogenesis where MCP-1 was shown to be angiogenic (Salcedo et al., 2000) whereas SLC displayed angiostatic qualities (Vicari et al., 2000). In the latter report, SLC also seemed to cause an infiltration of leukocytes that resulted in tumour regression.

There have been a few reports of chemokines being directly involved in the migration of tumour cells and potentially in their metastatic spread to specific organs (Oppenheim et al., 1997; Youngs et al., 1997). However, this has not been clearly demonstrated in an *in vivo* situation.

1.2.3.3. Neurological Disorders

There is increasing evidence that chemokines play a significant role in the central nervous system (CNS) under both pathological as well as physiological conditions (for recent reviews see Asensio and Campbell, 1999; Mennicken et al., 1999). For example, mice lacking the gene for either CXCR4 or its ligand SDF-1 display defects in neuronal patterning during development (Nagasawa et al., 1996; Tachibana et al., 1998; Zou et al., 1998). In the adult brain, the specific localisation of certain chemokines, such as fractalkine, and the fact that chemokine receptors show some homology to neurotransmitter receptors suggests that chemokines and their receptors might have an important role in physiological cellular communication within the adult mammalian CNS (Asensio and Campbell, 1999). In addition, work by Araujo (Araujo and Cotman, 1993) and Robinson (Robinson et al., 1998) indicates that CXCR2 and its ligands may promote the growth and survival of neural cells.

As in the immune system, the expression of chemokines and their receptors in the CNS is upregulated by inflammatory mediators which then allows them to mediate leukocyte infiltration of the CNS. This is an important step in response to diverse challenges,

including infection, trauma and stroke but, as in the immune system, can lead to inflammatory disorders when the normal regulation is disturbed. One such example is multiple sclerosis (MS) which is a human chronic inflammatory and demyelinating disease that induces subsequent neuronal death. Brain lesions of MS patients were found to be enriched in $CCR5^+$ and $CXCR3^+$ T cells (Balashov et al., 1999; Sorensen et al., 1999) and their corresponding ligands MIP-1α and RANTES (for CCR5) and IP10 and Mig (for CXCR3). And studies on CCR2 $^{-/-}$ mice have implicated this receptor and its ligands in Experimental Autoimmune Encephalomyelitis which serves as an animal model for MS (Fife et al., 2000). Other chemokine-associated neurological diseases include HIV dementia, Alzheimer's disease and CNS tumours (Asensio and Campbell, 1999; Mennicken et al., 1999).

1.2.4. Chemokines in Haemopoiesis

Since the first discovery of a chemokine as an haemopoietic stem cell (HSC) inhibitor by Graham (Graham et al., 1990), a number of chemokines have now been shown to act as negative or positive regulators of different types of haemopoietic stem or progenitor cell proliferation. There exists, however, a certain degree of controversy since it is often difficult to compare and reconcile the results from different groups as different haemopoietic stem and progenitor assays and different conditions therein are employed to investigate the regulatory activities of chemokines within the HSC compartment. It is nevertheless likely that research into this area of chemokine activity will continue to expand, fuelled by the hope that some members of the chemokine family will prove to be valuable therapeutic agents in clinical applications, such as chemotherapy and HSC expansion (see below).

A number of functions have now been proposed for chemokines in the regulation of the haemopoietic system. As mentioned above, a large number of chemokines (more than 15 in the CC chemokine subfamily and at least 8 of the CXC chemokines) have now been demonstrated to reversibly suppress the proliferation of early progenitors as measured in a number of *in vitro* colony formation assays (Broxmeyer and Kim, 1999; Graham et al., 1990; Hromas et al., 1997; Maekawa and Ishii, 2000; Patel et al., 1997; Youn et al., 1995; Youn et al., 1998) as well as *in vivo* (Cashman et al., 1999; Dunlop et al., 1992). Although some of these chemokines display overlapping activities, with CFU-GEMM and CFU-GM cells being common targets, a number of studies now seem to suggest that different chemokines may have differential inhibitory effects on diverse haemopoietic cell lineages.

For example, in 1997 Patel et al (Patel et al., 1997) isolated two CC chemokines which were named myeloid progenitor inhibitory factors (MPIF) 1 and 2. As their names suggest, these two proteins exhibit suppressive activities on haemopoietic progenitors in addition to more characteristic chemotactic activities on mature leukocytes. However, while MPIF-1 targets the proliferation of low proliferative potential colony-forming cells which represent committed progenitors that give rise to granulocyte and monocyte lineages, MPIF-2 suppresses the colony formation by the high proliferative potential colony-forming cell, a multipotential haemopoietic progenitor. More recently, a CXC chemokine, termed WECHE, was isolated from an AGM (a mesodermally derived region containing the dorsal aorta, genital ridge/gonads and pro/mesonephros, where the first definitive haemopoietic stem cells arise) - derived endothelial cell line and shown to inhibit the formation of the erythroid lineage at a very early stage in haemopoietic development (Ohneda et al., 2000). The observation that chronic myeloid leukaemia progenitors are often refractory to inhibition by chemokines such as MIP-1α and MCP-1 (Cashman et al., 1998; Eaves et al., 1993) opened up the possibility of using these chemokines during chemotherapy as they were shown to protect normal progenitors from the effects of cytotoxic drugs by preventing them from entering the cell cycle, while leukaemic cells remained unprotected. However, such protective agents could be used for ameliorating the side effects of chemotherapy in any type of cancer (discussed in more detail in the section on MIP-1α).

Not only negative but also positive regulation of the proliferation of haemopoietic stem and progenitor cells by chemokines has been observed (Broxmeyer and Kim, 1999; Maekawa and Ishii, 2000; VerFaillie, 1996). MIP-1α, for example, despite being an inhibitor of early haemopoietic progenitors, can have an enhancing effect on more mature progenitors, i.e. progenitors that only require stimulation with a single growth factor for colony formation (Broxmeyer et al., 1990). Chemokines may also play a role in the mobilisation and release of progenitors from the bone marrow into the peripheral blood as was seen for MIP-1α (see section on MIP-1α) and IL-8 (Laterveer et al., 1996). The process by which IL-8 achieves progenitor mobilisation is thought to involve metalloproteinase gelatinase B (MMP-9), a protein imlicated in the degradation of extracellular matrix molecule, which is released from neutrophils following IL-8 activation and may then induce stem cell mobilisation by cleaving matrix molecules to which stem cells are attached (Pruijt et al., 1999).

The mechanism by which chemokines suppress haemopoietic stem and progenitor cells is not well understood. In a number of cases, it is not even known whether they inhibit the proliferation of these early cells or just the differentiation into certain haemopoietic

lineages (Ohneda et al., 2000). It has also been suggested that analogous to their effect on mature leukocytes whose integrin-dependent adhesion to vascular endothelium is triggered by chemokines, these proteins may also increase the integrin-mediated adhesion of progenitors to stroma which can induce inhibition of proliferation (Hurley et al., 1995). The signalling events involved in suppression also still remain largely unknown. Work by (Aronica et al., 1995) on the human growth-factor-dependent haemopoietic cell line MO7e which they showed to be inhibited in its proliferation by IP10 and MIP-1α, seems to implicate Raf-1 kinase-dependent pathways in proliferation. In other attempts to elucidate the signalling events following chemokine binding and leading to suppression of proliferation, mice lacking the genes for certain chemokines, chemokine receptors and signalling molecules have been examined for defects in haemopoietic development. For example, mice in which the phosphotyrosine phosphatase SHP-1 which is predominantly expressed in haemopoietic cells, is inactive display a number of severe haemopoietic and immune defects. In addition, their myeloid progenitors seem to be resistant to chemokines that suppress proliferation, thus implicating this signalling molecule in chemokine-induced suppression (Kim et al., 1999). Reid (Reid et al., 1999) analysed mice in which the gene for the chemokine receptor CCR2 was deleted and found that the bone marrow-derived myeloid progenitors showed increased cycling rates compared to progenitors found in wild type mice. However, there was no increase in total cellularity within the haemopoietic system which was explained by these investigators by an increase in apoptosis observed in these bone marrow cells, thus implicating CCR2 not only in myelosuppression but also in increase of cell survival.

The most striking evidence for the involvement of chemokines in haemopoiesis is provided by mice lacking the CXC chemokine SDF-1 (Nagasawa et al., 1996) or its receptor CXCR4 (Tachibana et al., 1998; Zou et al., 1998). Apart from neuronal and vascularisation defects, these mice also have striking haemopoietic defects, including absence of B lymphopoiesis as well as bone marrow myelopoiesis. SDF-1 is constitutively expressed in bone marrow-derived stromal cells where it serves to attract haemopoietic stem cells to their bone marrow niches as they migrate from the foetal liver to the bone marrow during normal development. This explains why myelopoiesis occurs normally in the foetal liver, but progenitors subsequently fail to migrate to the bone marrow and are instead found in the peripheral blood. The importance of SDF-1 and its receptor CXCR4 in homing of $CD34^+$ to the bone marrow microenvironment was supported by two recent publications by Peled (Peled et al., 1999; Peled et al., 2000) in which it was demonstrated that SDF-1 expressed on the bone marrow vascular endothelium stimulates integrin-

mediated arrest of $CD34^+$ cells that are also $CXCR4^+$. Furthermore, it also mediates the trans-stromal migration of these cells and their subsequent movement through the extracellular matrix to their respective "niche". This process is not only important for the movement of haemopoietic stem cells during development but also for the successful engraftment of donor haemopoietic stem cells into a recipient following transplantation. In addition to mediating stem cell homing, SDF-1 also regulates the proliferation of B cell progenitors.

Apart from SDF-1, a number of other chemokines have now been shown to induce chemotaxis in haemopoietic stem and progenitor cells, namely SLC and ELC which were demonstrated to chemoattract CFU-GM cells via their receptor CCR7 (Kim and Broxmeyer, 1999), and IP10 and Mig that induced chemotaxis in GM-CSF-stimulated $CD34^+$ cells (Jinquan et al., 2000). The significance of this in an *in vivo* situation is not entirely clear, especially since high concentrations of up to 2000 ng/ml of SLC are required to induce chemotaxis.

It is apparent from the evidence discussed above that chemokines are prominent players in almost all aspects of haemopoietic cell function, starting from the regulation of the proliferation and/or differentiation of the earliest haemopoietic stem cells up to the regulation of mature leukocytes in various situations. It is therefore not surprising that these proteins have been a major focus for research into possible therapeutic applications for various conditions ranging from AIDS and other infectious diseases to the treatment of allergies, cancer and the alleviation of the impact of chemotherapy and the improvement of tissue transplants. An important prerequisite to the clinical application of these proteins is an understanding of their functions in relation to their structures. A wealth of information is now available on their three-dimensional structures and on motifs and residues important for their various functions as will be discussed in the next section.

1.2.5. The Structure of Chemokines

A wealth of information has been accumulated about the three dimensional structure of chemokines, the mechanism by which they interact with their receptors, with proteoglycans, and also with themselves during the process of oligomerisation. Each one of these aspects will be discussed in the following sections.

1.2.5.1. The Tertiary Structure of Chemokines

The three dimensional structures have been solved by NMR and/or x ray crystallography for the following chemokines: PF4 (Mayo et al., 1995; St. Charles et al., 1989; Zhang et al., 1994), IL-8 (Baldwin et al., 1991; Clore et al., 1989; Clore et al., 1990), MGSA/GROα (Fairbrother et al., 1994; Kim et al., 1994), SDF-1 (Crump et al., 1997; Dealwis et al., 1998), MIP-2 (Shao et al., 1998), GROβ (Qian et al., 1999) and NAP-2 (Malkowski et al., 1995) in the CXC subfamily, MIP-1β (Lodi et al., 1994), RANTES (Chung et al., 1995; Skelton et al., 1995), MCP-1 (Handel and Domaille, 1996; Lubkowski et al., 1997), MCP-3 (Meunier et al., 1997), eotaxin (Crump et al., 1998), HCC-2 (Sticht et al., 1999), I-309 (Keizer et al., 2000) and MIP-1α (MacLean, J. unpublished results) in the CC subfamily, Fractalkine (Hoover et al., 2000; Mizoue et al., 1999), the only member of the CX_3C subfamily and vMIP-II (Liwang et al., 1999), a viral CC chemokine homologue. All of them were shown to possess a strikingly similar monomeric structure, the "chemokine fold" (see Fig. 1.2 for an example), which consists of an extended N terminal region, a triple-stranded, antiparallel β sheet, arranged in a Greek key motif, and a C terminal α helix which lies on top of the β sheet. This helix is of an amphipathic nature which allows it to pack closely on top of the β sheet via a number of conserved hydrophobic interactions, thus forming the hydrophobic core of the chemokines. The extended N terminal loop which, via a single helical turn, leads into the first β strand, consists of a collection of nonclassical turns and is anchored to the chemokine body via the two disulphide bridges. The extreme N and C termini are often disordered due to their highly flexible nature. Despite this very similar overall fold, there are certain structural differences within and between the different subfamilies which become apparent upon alignment of the monomeric units. These differences mainly reside in the more flexible parts of the molecules which are believed to be involved in receptor binding and activation (see below), especially in the residues preceding the first cysteine residue, but also in the remainder of the N terminal loop and the turns that connect the β strands. Other differences are found in the length and exact position of the C terminal α helix and surrounding the cysteine motif, since the introduction of one or three intervening residues in the CXC and CX_3C chemokines causes a bulge in the polypeptide chain which will also influence the conformation of the neighbouring residues.

Among the CC chemokines, there are three members, HCC-2, I-309 and SLC, that contain an additional, third disulphide bridge, the exact function of which is unknown. When the

three dimensional structures of HCC-2 and I-309 were solved, it was surprising to see that in the case of HCC-2, the additional disulphide bridge, which connects the base of the α helix with the helical turn preceding the first β strand, did not cause any major perturbations of the overall fold. On the contrary, it was suggested that it plays a role in stabilising the N terminus, thus compensating for the absence of a conserved tryptophan residue at position 53 that is thought to fulfil this function in other CC chemokines. In the case of I-309, however, the additional disulphide bond is found in a completely different position, connecting the first β strand with the C terminal end of the α helix. This results in the disruption of the helix, thus terminating it prematurely, and in the unique formation of a short β strand that runs perpendicular to the triple-stranded β sheet. Despite these differences, I-309 still retains the overall chemokine fold, however, it may explain why only one receptor is known for I-309 so far. Although the structure of SLC, the third chemokine to contain an additional disulphide bridge, has not been solved, sequence comparisons to HCC-2 and I-309 suggest that it will closely resemble HCC-2 in that the third pair of cysteines is not expected to have a great impact on the overall structure.

1.2.5.2. Chemokine Aggregation

Most chemokines can self-associate to form aggregates of varying molecular weights depending on concentration, pH and solvent ionic strength (Clark-Lewis et al., 1995) which can be detected by gel filtration chromatography or ultracentrifugation sedimentation equilibrium analyses. The exact orientation of the monomeric units within the oligomers was revealed in the NMR and crystal structures.

SDF-1 (NMR)

white - 1-10 extended loop
grey - 11-19 extended loop
yellow - 20-23 3_{10} helix
orange - 24-30 β1
red - 31-34 type III turn
green - 37-42 β2
cyan - 43-46 type I turn
blue - 47-51 β3
purple - 52-55 type I turn
violet - 58-65 α helix

cys9-cys34
cys11-cys50

Figure 1.2: 3D Structure of SDF-1

Above is shown the solution structure of the SDF-1 monomer ('ribbon-representation') as an example of the conserved chemokine fold. Different domains are represented by different colours and their names and boundaries (in residue numbers) listed in the box. The position of the two disulphide bridges is shown in the picture by 'stick-representations' of the cysteine residues and their residue numbers listed in the smaller box.

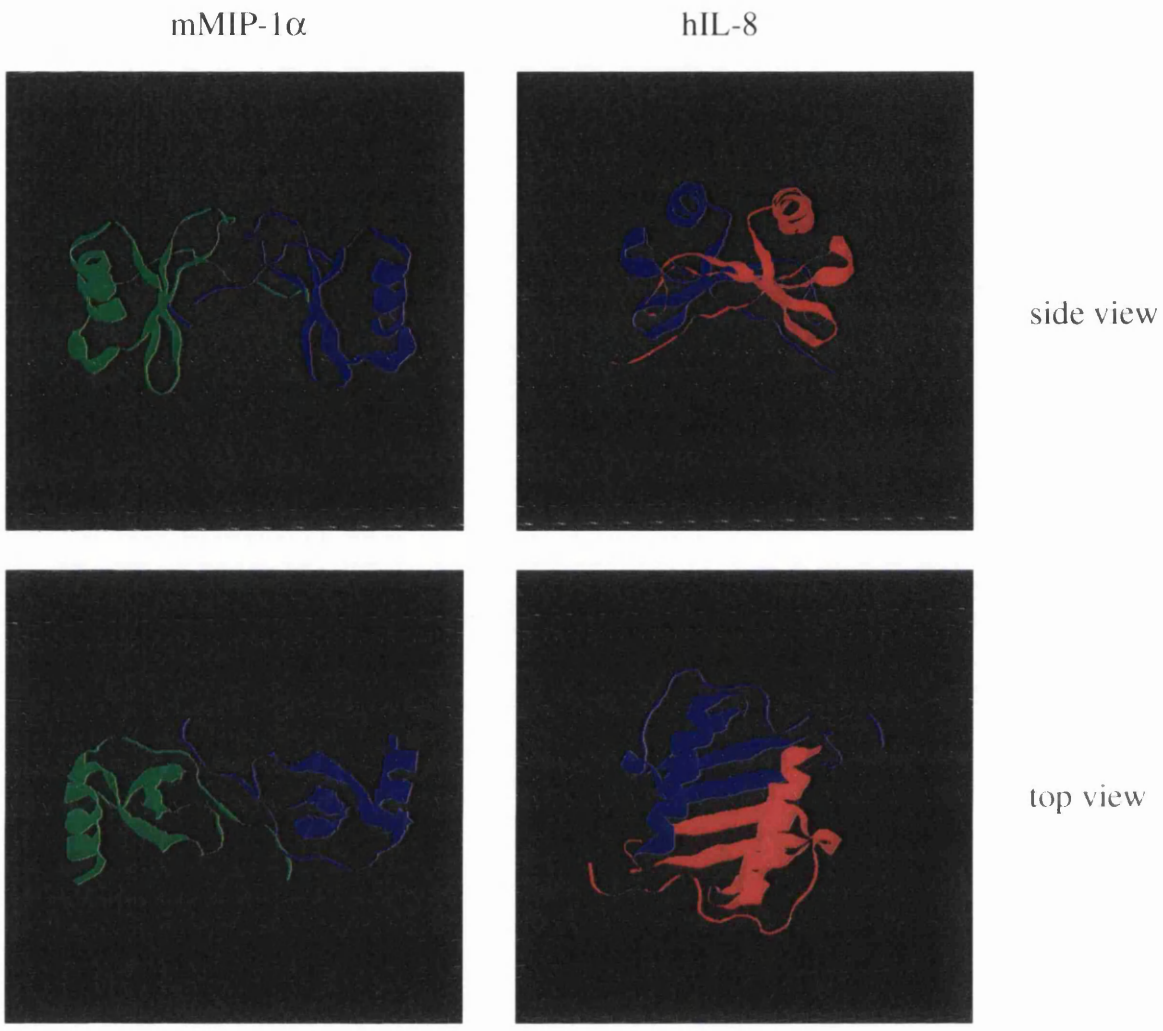


Figure1.3: Comparison of CC and CXC Chemokine Dimers

Above are shown 'ribbon-representations' of the murine MIP-1α dimer (a CC chemokine; two panels on the left hand side) and the human IL-8 dimer (a CXC chemokine; two panels on the right hand side). The different subunits are shown in different colours.

The three dimensional structures of PF4 and IL-8 were the first ones to be published and revealed the tertiary as well as the quaternary structures of these proteins. The mode of dimerisation proved to be exactly the same for both of them and involves the formation of a six-stranded antiparallel β sheet in which the two subunits lie side by side and interact via their first β strand. The two helices are located on the top of the β sheet, running antiparallel to each other with their C terminal ends making contact with the other subunit, thus helping to stabilise the dimer. This quaternary structure is highly reminiscent of the fold of the α1/α2 domains of the class I MHC antigen HLA-A2 (Clore et al., 1990) which led the authors to suggest that the mechanism by which HLA-A2 interacts with antigen and the T cell receptor may serve as a model for the way chemokines interact with their receptors. In that case, residues important for receptor binding and activation would be located in the region between and on the surface of the two α helices. However, mutagenesis studies have since established that this is not the case (see below).

While IL-8 is unable to aggregate past the dimeric state, PF4 is known to exist in a monomer-dimer-tetramer equilibrium in solution (Mayo and Chen, 1989). In the PF4 tetramer, as revealed by the crystal structure (St. Charles et al., 1989; Zhang et al., 1994), two dimers associate back to back, thus forming a β bilayer with the helices found on opposite sides of the complex.

Since IL-8 and PF4 were found to form identical dimers, it was assumed that this mode of dimerisation was conserved among all chemokines, and indeed some mutagenesis studies of MCP-1 were based on this assumption (Beall et al., 1992; Zhang et al., 1994). It therefore came as a surprise when the solution of the NMR structure of MIP-1β unveiled a completely different mode of dimerisation which is now believed to be conserved amongst almost all CC chemokines (see MCP-3 discussion below), whereas all CXC chemokines that form oligomers, do so via an IL-8-type dimer. In contrast to the globular CXC chemokine dimer, the CC chemokine dimer is more elongated and of a cylindrical shape, with the two helices located on opposite sides of the complex (see Fig. 1.3 for examples of CC and CXC - type dimers). This completely dissimilar design stems from a different dimer interface which in the case of CC chemokines is mainly formed by residues in the extreme N- terminus (residues 2-13 in MIP-1β) and which causes the creation of another, short, β strand which associates with the β strand in the other monomer in an antiparallel fashion. It has rarely been observed that proteins which are almost identical in their polypeptide fold, form such completely divergent oligomers. However, a number of

differences in the primary sequence and the secondary structure of CC as compared to CXC chemokines may help to explain this observation. On comparing the distribution of hydrophobic charges between CC and CXC chemokines (Clore and Gronenborn, 1995; Lodi et al., 1994), it becomes apparent that the strongest clusters of hydrophobicity are found at equivalent positions in the two chemokine families which explains the almost identical monomeric structure since these hydrophobic residues are involved in the packing of the hydrophobic protein core. However, the pattern of surface hydrophobicity is strikingly different in CC versus CXC chemokines which offers an explanation for the different dimerisation mode since these hydrophobic surface residues become buried upon oligomerisation. This is supported by the calculation of the solvation free energy of dimerisation (Clore and Gronenborn, 1995; Lodi et al., 1994) which suggests that the driving force for the formation and stabilisation of dimers is the burial of hydrophobic charges. However, hydrogen bonds and salt bridges have also been shown to contribute towards dimer formation. Further contributions to the divergent quaternary structures are thought to be made by the different conformation of the turn between strand β_2 and β_3 and of the first disulphide bond (due to the insertion of an amino acid between the first two cysteine residues in CXC chemokines), as well as by the completely different direction of the residues preceding the first cysteine residue and the longer helix that can make contacts with the other subunit in CXC chemokines.

In the three dimensional structures, only PF4 (Clore et al., 1989; Mayo et al., 1995; Zhang et al., 1994), NAP-2 (Malkowski et al., 1995), MCP-1 (Lubkowski et al., 1997) and MIP-1α (see below) have been detected as tetramers, while most of the other chemokines form dimers in the conditions employed for NMR studies or crystallisation. HCC-2 (Sticht et al., 1999) and I-309 (Keizer et al., 2000), both of which contain 3 disulphide bonds (see above), were detected as monomers when their structures were determined. HCC-2 was also determined to be a monomer over a wide range of concentrations (0.1 μM - 2 mM) by gel filtration studies at physiological pH which demonstrates that this chemokine does not oligomerise in solution, a finding which can at least in part be explained by a shorter N terminus and thus a shorter dimer interface (Sticht et al., 1999). Fractalkine (Hoover et al., 2000) and SDF-1 (Dealwis et al., 1998) were both shown to be in a dimeric state in the crystal structures, but since they were monomers in solution (Crump et al., 1997; Mizoue et al., 1999), it can be assumed that the dimerisation of these two proteins was a product of crystal packing which is supported by the finding that Fractalkine can form neither a CC-type nor a CXC-type dimer (Hoover et al., 2000; Mizoue et al., 1999).

Contrary to the theory that the dimerisation mode is conserved within the chemokine subfamilies, MCP-3, a CC chemokine, was found to form a dimer in the crystal structure (Meunier et al., 1997) that resembled the IL-8-type dimer more than the CC chemokine dimer. In that same study it was suggested that the MCP-3 monomer was unstable on account of a number of exposed core hydrophobic residues that become buried in the dimer. Yet again, the dimer observed in the crystal structure may well be a crystal artefact, since NMR and sedimentation equilibrium studies have shown MCP-3 to be monomeric at concentrations of up to 2 mg/ml (Kim et al., 1996). The fact that intersubunit interactions observed in crystal structures may not necessarily reflect native contacts is also demonstrated in the case of MCP-1. During crystallisation, two different forms of crystals were observed in the same droplet (Lubkowski et al., 1997), one containing a monomer, the other containing a dimer, which also differed somewhat from the previously published NMR structure of another MCP-1 dimer (Handel and Domaille, 1996). It can therefore be assumed that in a lot of cases the conditions employed for protein structure determination, such as high protein concentrations and nonphysiological pH and solvent ionic strength values, may favour intermolecular associations that are unlikely to occur *in vivo*. This has led to a controversy regarding the biological significance of chemokine oligomerisation with experimental data supporting a biological role for both monomers as well as oligomers.

When it was first discovered that CC and CXC chemokines form different dimers and that the type of dimer was largely conserved within the specific subfamily, it was proposed that these differences in quaternary structure may explain the lack of cross-reactivity of chemokines from one family with receptors from the other family (Clore and Gronenborn, 1995). This could either mean that the interaction with the receptor requires the presence of a dimer and that the receptors of one subfamily can only accommodate the chemokine dimer from the same subfamily. Alternatively, the same exposed hydrophobic surface residues that form the dimer interface and become buried upon dimerisation, may also be needed for interacting with the receptor. As the affinity of chemokines for their receptors is higher (nM range) than for their self-association (μM range), this would mean that the biologically functional unit is the monomer. However, this is more likely to be the case for CC chemokines, since in CXC chemokines the receptor binding site is remote from the dimer interface. Nevertheless, IL-8 dimers were detected on receptors by cross-linking (Schnitzel et al., 1994), but mutagenesis studies have also shown that only one functional subunit is required for the cross-linked dimer to be active. This supports a model in which IL-8 dimerisation does not interfere with receptor binding as cross-linked IL-8 can still

activate the receptor, but only one subunit at a time will interact with the receptor. This is reinforced by the fact that a mutant of IL-8 that can no longer self-associate, is nevertheless fully functional (Rajarathnam et al., 1994). However, a nonfunctional MCP-1 variant in which residues 2-8 had been deleted, was believed to have a dominant negative effect since it inhibited receptor activation by wild type MCP-1, but not by cross-linked wild type MCP-1, thus suggesting that dimerisation is required and that the MCP-1 mutant sequestered wild type MCP-1 into nonfunctional dimers (Zhang and Rollins, 1995). Yet, in another study (Paavola et al., 1998), this same mutant was shown to act as a competitive inhibitor that simply displaces wild type MCP-1 from its receptor. These authors also generated a monomeric mutant of MCP-1 that was nevertheless still active, thus lending further support to the suggestion that dimerisation is not required for MCP-1 function.

Despite the fact that the concentration of circulating chemokines lies below the dimerisation constant, one can envisage situations in which the local concentration of chemokines is increased, thus favouring oligomerisation. This could be achieved by binding of chemokines to matrix and cell surface proteoglycans (Clore and Gronenborn, 1995 and see section 1.2.5.4.) or by concentrating them in granules, as is the case for PF4 (St. Charles et al., 1989). Local environments may also be manipulated in a way that favours one aggregation state over another. For example, salt concentrations that were shown to shift the equlibrium towards a PF4 tetramer, resulted in the dissociation of the IL-8 dimer (Laurence et al., 1998). And in concentrations of 20 mM sodium phosphate and 150 mM sodium chloride, similar to the ionic strength of blood, MIP-1β was predominantly present as a dimer (Laurence et al., 1998). It may also be the case that the ligand binding site on the receptor favours dimerisation. In such a scenario, a low dimerisation constant would prevent inappropriate activation of leukocytes at low chemokine concentrations and result in a much steeper gradient towards high local concentrations (Zhang and Rollins, 1995). However, as described above, it appears that most chemokines are able to activate their receptors as monomers.

While most chemokines cannot aggregate past the dimeric or tetrameric state, MIP-1α, MIP-1β and RANTES can form high order aggregates at physiological conditions in a concentration-dependent manner (Lodi et al., 1994; Patel et al., 1993; Skelton et al., 1995). The MIP-1β aggregates can even be seen as helical fibrils (Lodi et al., 1994) in electron micrographs. This large scale aggregation seems to be largely controlled by electrostatic interactions (Patel et al., 1993), and acidic residues in RANTES have been identified that when mutated result in the disaggregation of RANTES. While these disaggregated mutants

display wild type receptor binding, activation and chemotactic activity, they were shown to inhibit HIV entry only at very high concentrations (>1000 nM), at which wild type RANTES actually stimulates viral entry (Czaplewski et al., 1999). Furthermore, these mutants were deficient in RANTES-mediated T cell activation via a Protein Tyrosine Kinase pathway (Bacon et al., 1995) for which high concentrations, and therefore possibly aggregation, of RANTES are required.

Other functions that have been suggested for chemokine aggregation include increased resistance against proteolysis and thus increased stability and half-lives of chemokines (Paolini et al., 1994), as well as a mechanism for limiting the active amount of circulating chemokine in the blood (Paolini et al., 1994; Skelton et al., 1995). While the evidence provided above seems to suggest that oligomerisation is not necessary for the induction of G protein-coupled receptor-mediated chemotaxis by chemokines since some of them only exist in a monomeric state and monomeric variants of others are still biologically active, it could be that the relevance of aggregation may only be revealed by the use of the appropriate assay system, as shown for the role of aggregated RANTES in T cell activation. Therefore, future experiments may shed further light on the possible relevance of chemokine oligomerisation *in vivo*.

1.2.5.3. Chemokine - Receptor Interactions

The way chemokines bind and activate their receptors has been studied by a number of techniques. Before the three-dimesional structure of chemokines was known, researchers had to resort to comparing their primary sequences in order to highlight functionally relevant regions that are expected to be conserved among functionally related proteins, but fairly divergent in more distant members. Residues selected by that method were then often mutated in order to confirm their involvement in receptor interactions. However, not only was this a rather inefficient approach, it was also impossible to distinguish between residues that make direct contacts with the receptor and residues that occupy structurally crucial positions since their alteration can often result in identical phenotypes. However, knowing the exact location and orientation of these residues within the overall fold allows one to make this distinction and thus provides one with a more rational approach to identifying and targeting regions likely to be involved in receptor binding and activation. In addition, the superposition of the tertiary structures has led to the identification of regions that adopt slightly different conformations in different chemokines and may thus account for differing receptor specificities.

Alternatively, such regions can be identified by exchanging domains between different proteins with the aim of generating chimaeric molecules that have acquired novel functions that can be attributed to the presence of the inserted domain. This has been successfully applied to chemokines as well as to their receptors (see below). However, there is always the risk that a foreign domain is not well tolerated within the original protein framework, thus potentially leading to disturbances in the overall structure that will make the entire approach invalid. This is less likely if domains are exchanged between more related proteins, however in those cases there is the potential risk of overlooking functional contributions made by shared determinants. In most mutagenesis studies on chemokines and their receptors, individual residues have therefore been targeted and either replaced by amino acids found in other chemokines at equivalent positions or by alanine residues in a method called alanine scanning mutagenesis. Alanine is chosen because it has got a short and nonpolar side chain that is unlikely to impose severe constraints on folding. It also represents the most common amino acid found in proteins where it can occupy buried as well as solvent-exposed positions. In combination with such mutagenesis studies, NMR spectra are occasionally obtained for these mutants in order to detect any structural disturbances caused by the newly introduced residue.

NMR studies were employed in yet another approach for investigating chemokine-receptor interactions (Clubb et al., 1994) where the authors monitored changes in the spectra of individual residues caused by the binding of the N terminal fragment of the corresponding receptor. This allowed the precise mapping of the binding site for this receptor fragment, but may not precisely reflect the interaction of chemokines with the native receptors since other extracellular regions of the receptors are known to contribute to ligand binding (see below) and the unique environment near the membrane is not taken into account. This latter approach, however, has the advantage of being able to characterise the entire receptor binding surface, whereas in mutagenesis studies only residues that contribute strongly towards receptor binding will be detected. In addition to these chimaeric and peptide approaches, truncated variants of chemokines and chemokine receptors have also been generated in an attempt to define the minimal functional unit. The following section describes some of the findings that were produced by these different methodologies which resulted in a fairly detailed description of how chemokines interact with their receptors.

The general picture that emerged from all of these studies is that the interaction between chemokines and their receptors is a two-stage process that involves more than one domain in the ligand as well as in the receptor. Binding and activation occurs via two distinct

regions that operate independently of each other, thus allowing the generation of potent antagonists by mutating the activation domain and creating a ligand that can compete for binding, but will no longer activate the receptor.

IL-8 of the CXC chemokines and MCP-1 of the CC chemokines are the two most intensively studied proteins that have come to serve as models for the other family members. From these studies, it has emerged that despite the completely different mode of oligomerisation of the two subfamilies, the way they interact with their receptors is remarkably similar. Initial docking to the receptor appears to be mediated in both subfamilies by a region termed 'N loop' which comprises the amino acids between the cysteine motif and the 3_{10} helical turn that leads into the first β strand (see Fig. 1.2). This region is highly variable and is thought to confer receptor specificity to chemokines. For example, by exchanging the N loop in GROα with the N loop in IL-8 and *vice versa*, Lowman (Lowman et al., 1996) achieved an exchange of receptor affinities between these two proteins. It proved to be important, though, to exchange an additional residue found at position 49 in IL-8 (leucine) and position 50 in GROα (alanine) since these make important contacts with the N loop and help position it in the correct orientation for receptor binding (Hammond et al., 1996; Hesselgesser et al., 1995; Lowman et al., 1996). This demonstrates the essential role played by residues that do not directly interact with the receptor but are part of the scaffold that is made up of the protein core and that supports the flexible N terminal region and presents it to the receptor in a fashion that optimises binding and activation (Crump et al., 1998). It has therefore been suggested that not only the sequence of the N loop itself, but also variations in the hydrophobic protein core which supports the conformation of the N loop, may contribute to the binding specificities of many chemokines (Mizoue et al., 1999). This scaffold is mainly held together in the right conformation by the two disulphide bridges which explains why changes made to any of the four conserved cysteine residues will render the protein completely inactive (Clark-Lewis et al., 1994).

Further support for a role of the N loop in tethering chemokines to their receptors prior to activation comes from studies in which the binding of a receptor-derived N terminal peptide to the corresponding ligand was investigated by NMR analysis. In two of those studies, a peptide derived from the N terminus of CXCR1, residues 1-40 (Clubb et al., 1994) and residues 9-29 (Skelton et al., 1999), was demonstrated to bind to a hydrophobic cleft between the N loop and the third β strand of IL-8, thus disturbing the NMR spectra of residues in the N loop, the turn preceding the third β strand and β_3 itself. Those same

regions, plus the 3_{10} helical turn following the N loop, were also shown to be contacted in eotaxin by residues 1-35 from the N terminus of CCR3, thus indicating that the mechanism of receptor recognition is conserved among members of the two subfamilies (Ye et al., 2000). A similar hydrophobic crevice was also detected in SDF-1 (Dealwis et al., 1998) which made the authors suggest that the pattern of a hydrophobic surface surrounded by a constellation of ionic residues may be a common characteristic of chemokines and the interaction with their receptors. A specific 'docking motif' has been identified in the N loop of SDF-1, consisting of the amino acids RFFESH and directly following the CXC motif, that is important for binding to CXCR4 (Crump et al., 1997). No such motif has been detected in any other chemokines so far.

There is a wealth of evidence pointing to the extreme N terminal residues, prior to the first cysteine, as being involved in receptor activation in both CC and CXC chemokines. In all neutrophil-activating CXC chemokines that display high affinity binding to CXCR2, a highly conserved three amino acid motif is found directly preceding the first cysteine, consisting of the sequence glutamic acid, leucine and arginine (see Table 1.1). This ELR motif, which also confers angiogenic properties (see above), is absolutely essential for receptor activation, as shown by the fact that mutating any one of the three residues will interfere severely with function (Hebert et al., 1991; Hesselgesser et al., 1995). It is not only the presence of these residues that is essential, but also their conformation and their orientation in relation to the rest of the molecule, since linear or circular peptides derived from the N terminus of IL-8 and including the ELR motif are inactive (Clark-Lewis et al., 1993). Therefore, residues that lie in close proximity to the ELR motif and help position it in the correct conformation, such as residues in the '30s loop' (connecting β_2 and β_3), in particular the cysteine that forms a disulphide bond with the cysteine following the ELR motif, are also indispensable for function (Clark-Lewis et al., 1994; Hebert et al., 1991; Qian et al., 1999).

The importance of the ELR motif in neutrophil activation is stressed even further by work showing that a simple replacement of the corresponding residues in PF4 (DLQ) with ELR endows this chemokine with neutrophil-activating properties and the ability to displace IL-8 from its receptor (Clark-Lewis et al., 1993). However, PF4 must otherwise be very closely related to IL-8, since the simple transfer of the ELR motif into IP10, another non-ELR CXC chemokine, does not confer neutrophil-activating abilities onto IP10, but instead requires almost the entire N terminal region (residues 4-22) and the two regions that help position the N terminal part (residues 30-35 and residue 49) of IL-8.

No other residues in the N terminus of IL-8 have the same importance as the ELR motif. In fact, deletion of all the residues preceding the ELR motif makes it even more active (Clark-Lewis et al., 1991). This is in sharp contrast to non ELR-containing CXC chemokines, such as SDF-1, in which the first two residues are crucial for receptor activation, and the entire CC chemokine family where no such conserved motif exists, but instead all N terminal residues (preceding the first cysteine) seem to be of more or less equivalent significance (Arenzana-Seisdedos et al., 1996; Crump et al., 1998; Gong and Clark-Lewis, 1995; Gong et al., 1996; Oravecz et al., 1997; Weber et al., 1996; Zhang et al., 1994). Removal of as few as one or two residues from the N terminus of MCP-1 will not only substantially lower its activity, but also turn it into an antagonist, in accordance with the two-site model of chemokine-receptor interactions (Gong and Clark-Lewis, 1995; Zhang et al., 1994). Interestingly, the glutamate residue at position one in MCP-1 spontaneously converts into pyroglutamate which is, however, not essential for chemokine function and can be replaced by any other nonpolar residue as long as the N terminus remains intact (Gong and Clark-Lewis, 1995).

It is not only truncations but also extensions of the N terminus that severely compromises the function of CC (Proudfoot et al., 1996; Simmons et al., 1997) as well as CXC (Malkowski et al., 1995) chemokines. When RANTES was produced in *E. coli*, it was found to be functionally inactive due to the retention of the initiating methionine at the extreme N terminus (Proudfoot et al., 1996), despite still being able to interact with its receptors. This prompted a search for similarly N terminally modified RANTES analogues that could serve as receptor antagonists, and resulted in the generation of AOP-RANTES, a RANTES derivative which has an aminooxypentane group attached to its N terminus (Simmons et al., 1997). This antagonist was incapable of inducing chemotaxis, but proved to be a very potent inhibitor of HIV infection (Simmons et al., 1997) with slightly altered receptor specificities (Proudfoot et al., 1999).

The fact that the activity of chemokines can be easily influenced by only slight alterations to their amino termini is exploited by the immune system *in vivo* as a means of regulating immune responses. Naturally truncated forms of chemokines were identified before it was known that some chemokines serve as substrates for a number of peptidases. For example, the dipeptidyl peptidase CD26 has a substrate specificity of NH_2-X-Pro and cleaves chemokines such as MCP-1, eotaxin, IP10 and RANTES with this N terminal sequence after the penultimate proline residue (Oravecz et al., 1997). In the case of RANTES, this proteolytic cleavage results in a 3-68 variant that retains signalling through CCR5, but

becomes an antagonist for CCR1 and CCR3 and hence a broad spectrum inhibitor for monocyte migration (Oravecz et al., 1997; Struyf et al., 1998). Members of the matrix metalloproteinase (MMP) family also recognise a number of chemokines as substrates. Processing of MCP-3, for example, by gelatinase A (MMP2), which is secreted by stromal cells, results in a 5-76 form that is incapable of signalling through CCR1 and CCR2 which has a dampening effect on inflammation (McQuibban et al., 2000). In contrast, cleavage of IL-8 by gelatinase B (MMP9) produces a 7-77 variant that is even more active than full length IL-8 and further stimulates the release of gelatinase B from neutrophils, thus resulting in a positive feedback loop that has an enhancing effect on inflammation (Van Den Steen et al., 2000). Matrix metalloproteinases can therefore serve as positive as well as negative regulators of inflammation. Other forms of proteolytic processing have been shown to convert PF4 into an inhibitor of endothelial cells (Gupta et al., 1995) and the CXC chemokines NAP-2 and CTAPIII into microbicidal proteins known as thrombocidins (Krijgsveld et al., 2000).

In contrast to the N terminus, alterations to the C terminal α helix do not produce such a dramatic effect on activity. Deletion of residues from the C terminus results in a progressive loss of activity (Clark-Lewis et al., 1991; Zhang et al., 1994), yet substantial activity is retained even in the absence of the entire α helix, thus demonstrating a role for this domain in stabilising the tertiary structure rather than interacting directly with the receptor (Clark-Lewis et al., 1991; Sticht et al., 1996). However, the α helix in CXC chemokines has been shown to contain important proteoglycan binding sites (see below). Other important residues that have been identified by mutagenesis studies include a conserved tyrosine residue in CC chemokines (position 28 in MCP-1) and another tyrosine or charged residue two positions further downstream which is always hydrophobic in CXC chemokines and which is thought to contribute to cell specificity (Beall et al., 1992; Lusti-Narasimhan et al., 1995). Other more extensive studies have pinpointed receptor specificities to individual residues and thus drawn a map of distinct, yet overlapping receptor binding sites within the same chemokine (Hammond et al., 1996; Pakianathan et al., 1997).

Other studies have concentrated on the receptors in order to dissect the interaction between chemokines and their receptors. Again, the importance of conserved cysteine residues in maintaining the overall structure and in bringing otherwise remote domains together for cooperative binding was highlighted (Hebert et al., 1993; Tournamille et al., 1997) and further evidence for the two-site model of chemokine-receptor interactions accumulated

(Monteclaro and Charo, 1997; Pease et al., 1998; Wu et al., 1996). From these studies it emerged that the main determinants for ligand specificity and for the initial binding of chemokines reside with in the N terminal extracellular domain of the receptor (Gayle et al., 1993; Lu et al., 1995; Monteclaro and Charo, 1997; Samson et al., 1997; Wu et al., 1996). Especially important seems to be a motif consisting of a combination of tyrosine and acidic residues found in the N terminus of CCR5 and other receptors (Farzan et al., 1998; Ye et al., 2000) which has also been found to be a site for posttranslational sulphation of the receptor (Farzan et al., 1999). Once the chemokine has been tethered to the receptor N terminus via its N loop, its flexible N terminal region can then make contact with other extracellular segments of the receptor, thus inducing the conformational change that results in G protein coupling and the initiation of downstream signalling events (Samson et al., 1997; Wu et al., 1996).

1.2.5.4. Chemokine and Proteoglycan Interactions

All chemokines are known to be able to interact with proteoglycans with varying degrees of affinity and specificity. The biological significance and precise details of this interaction are still largely unclear, and conflicting results have made this field, like in the case of chemokine aggregation, very controversial, as can be seen from the following section.

Proteoglycans consist of glycosaminoglycan chains which are linked to a protein core via serine residues (Park et al., 2000; Ruoslahti and Yamaguchi, 1991; Tyrrell et al., 1995). There are four main forms of glycosaminoglycans, (1) heparin and heparan sulphate, (2) chondroitin sulphate and dermatan sulphate, (3) keratan sulphate and (4) hyaluronic acid which are all composed of repeating disaccharide units of which one is always an amino sugar, whereas the other one is usually a uronic acid. These basic units are subsequently modified by a number of different enzymes in reactions that do not normally reach completion, thus making this a very heterogeneous group of molecules. It is especially the addition of sulphate groups to the amino sugar that determines and influences the interaction of proteoglycans with chemokines. It appears that these modifications are cell type specific since every cell has a specific repertoire of glycosaminoglycan chain modifying enzymes. The location of proteoglycans is determined by the core protein which in certain cases contains a domain that allows membrane anchorage. Proteoglycans are found in the extracellular matrix, on cell surfaces and in the secretory granules of various types of haemopoietic cells where they perform a wide range of different functions. Heparin, despite being much more restricted in its localisation, has been widely used as a

model glycosaminoglycan, and a lot of the observations described below have been made with heparin or heparan sulphate fragments.

Because of the high degree of sulphation of these molecules, protein - glycosaminoglycan interactions are mainly of an electrostatic nature and involve clusters of basic charges in the proteins which are either arranged in a short linear peptide stretch or in topologically close regions. However, specific sugar sequences have been shown to play a role in the interaction with certain proteins such as antithrombin III and FGF-2 (Park et al., 2000). Heparin binding sites have been identified in MIP-1α, MIP-1β, RANTES, MCP-1, IL-8, PF4 and SDF-1 and shown to be surprisingly different in their location and in the number of residues involved which partly accounts for the differences in binding affinity. MIP-1α, for example, which requires a salt concentration of 0.39 M NaCl (Kuschert et al., 1999) to be eluted from a heparin affinity column and is thus one of the weakest heparin binding chemokines, contains one heparin binding motif of positive residues in the loop connecting the second and third β strand and within β_3 and one additional positive amino acid in the N loop which come together as a positive cluster in the three dimensional structure (Kolset et al., 1996; Wagner et al., 1998). RANTES, on the other hand, has another such cluster of positive residues in the C terminal α helix in addition to the one also found in MIP-1α (Burns et al., 1998) and can thus bind heparin with a much higher affinity, requiring 0.9 M NaCl (Kuschert et al., 1999) for its elution. Heparin binding of IL-8 (Webb et al., 1993) and MCP-1 (Chakravarty et al., 1998) also seems be mostly due to basic residues found in the α helix, while in PF4 basic amino acids both within and outside the helix have been implicated in proteoglycan interaction (Mayo et al., 1995; Stringer and Gallagher, 1997). The heparin binding motif of SDF-1 is found in yet another location, mainly comprising residues found in the first β strand (Amara et al., 1999).

MIP-1β has a binding site very similar to the one found in MIP-1α, yet it binds heparin more avidly than MIP-1α (Koopmann et al., 1999) which suggests that other factors are also likely to affect proteoglycan binding. These might include the overall charge of chemokines, since MIP-1α is found to have a negative overall charge while most other chemokines are highly basic, or the possibility that the oligomerisation state has an influence on heparin binding. Studies carried out by Hoogewerf (Hoogewerf et al., 1997) seem to suggest that binding to glycosaminoglycans induces chemokine oligomerisation, thus displaying a biphasic chemokine binding curve at increasing chemokine concentrations that implies positive cooperation between binding sites from different

subunits. Further evidence comes from studies on PF4 (Mikhailov et al., 1999; Stringer and Gallagher, 1997) where the amino acids implicated in glycosaminoglycan binding are seen to form a ring of positive charges on the outside of the tetramer which is thought to induce the glycosaminoglycan chain to wrap around the tetrameric complex, running in a direction perpendicular to the α helices. A heparan sulphate fragment was isolated from fibroblasts which showed particularly strong binding to IL-8. Since it contained two highly sulphated blocks of six monosaccharide units separated by a stretch of at least 14 unsulphated monosacharide units, the high affnity for IL-8 was best fitted to a model in which the two sulphated units interact with the binding sites on the two α helices that are located on opposite sides of the IL-8 dimer with the unsulphated intervening stretch bridging the gap between the helices (Spillmann et al., 1998). This also demonstrates that in addition to oligomerisation, the degree and pattern of sulphation along the glycosaminoglycan chain and its actual length also contribute to proteoglycan binding affinities and specificities, an observation further supported by work from Witt (Witt and Lander, 1994).

A further level of specificity is added by the fact that chemokines have varying affinities for proteoglycans, depending on the type of glycosaminoglycan attached to the protein core (Kuschert et al., 1999). The complexity of this system therefore suggests that the interaction of chemokines with proteoglycans has an important function *in vivo*. However, mutants of MIP-1α (Graham et al., 1996; Koopmann and Krangel, 1997), SDF-1 (Amara et al., 1999), MIP-1β (Koopmann et al., 1999) and MCP-1 (Chakravarty et al., 1998) that have lost their ability to interact with heparin, are still functional chemoattractants and bind to and signal through their receptors as efficiently as their native counterparts. Although the heparin binding mutant of MIP-1α could no longer bind to CCR1 (Graham et al., 1996), this was explained by the fact that the actual mutation interfered with receptor binding, thus suggesting that their binding sites overlap, whereas they are clearly spatially distinct in SDF-1, MCP-1 and IL-8.

Another way of determining the contribution made by glycosaminoglycans to the function of chemokines involves their removal from cell surfaces either by treating the cells with enzymes that cleave off the sugar moiety or by using cell lines that are deficient in glycosaminoglycan production. While a number of studies showed that glycosaminoglycan removal abolished the HIV inhibitory activities of RANTES and MIP-1α (Oravecz et al., 1997), reduced RANTES-stimulated intracellular calcium release (Burns et al., 1998) and reduced the apparent affinity of IL-8, RANTES, MCP-1 and MIP-1α for their receptors

(Hoogewerf et al., 1997), others demonstrated that glycosaminoglycan-negative cells responded to SDF-1 as well as glycosaminoglycan-positive cells (Amara et al., 1999) and the affinity of MIP-1α for CCR1 was not altered in glycosaminoglycan-negative cells (Graham et al., 1996).

Conflicting results were also obtained when chemokines were tested in bioassays in the presence of soluble glycosaminoglycans. While the presence of soluble heparan sulphate seemed to enhance IL-8-induced calcium release and migration in neutrophils, it inhibited IL-8-stimulated elastase release from the same cells (Webb et al., 1993). This is in contrast to another study, where soluble heparin appeared to inhibit calcium release in neutrophils in response to IL-8 (Kuschert et al., 1999). Soluble glycosaminoglycans lacked any impact on the activity of SDF-1 (Amara et al., 1999) and on the binding of MIP-1α to CCR1 (Graham et al., 1996). The cause for these apparent discrepancies is not clear, but may be due to differences in bioassays, cell types and glycosaminoglycan composition, or the fact that other cell surface molecules, such as fibronectin, can immobilise chemokines (Pelletier et al., 2000).

It has been suggested that glycosaminoglycan expression is not necessary for the biological activity of MIP-1α, RANTES and MIP-1β, but that the presence of cell surface glycosaminoglycans does enhance the activity of low concentrations of these chemokines by a mechanism that appears to involve sequestration onto the cell surface (Ali et al., 2000; Oravecz et al., 1997). This may have the effect of raising the effective ligand concentration, and since the affinity of chemokines for glycosaminoglycans is usually in the μM range whereas the affinity for their receptors lies in the nM range, they will be readily transferred onto their receptors. Chemokines have been detected on endothelial cells (Tanaka et al., 1993) and also on cells that lack normal receptor expression (Amara et al., 1999) which can be attributed to the presence of proteoglycans and is thought to allow their presentation to passing leukocytes. This would not only prevent chemokines from being washed away by the blood flow (Rot, 1992), but would also ensure that they only act over a short range (Ruoslahti and Yamaguchi, 1991). This seems to be supported by findings that proteoglycan-immobilised MIP-1β induces homing of T cells (Tanaka et al., 1993), and that only IL-8 that has been secreted by endothelial cells, but not soluble IL-8, will activate neutrophil integrins for firm adhesion (Tanaka et al., 1993). Chemokines are also found in complex with glycosaminoglycans when secreted from macrophages and platelets (Kolset et al., 1996) and within the cytolytic granules of $CD8^+$ cytotoxic T cells (Wagner et al., 1998). And since no G protein-coupled chemokine receptor has been identified so far for

PF4, it was suggested that it may mediate its biological effects through surface proteoglycans (Petersen et al., 1998; Petersen et al., 1999). It therefore seems that different chemokines may have different dependencies on proteoglycans for their activities, however, further experimental data is still required in this field.

1.3. MIP-1α

MIP-1α was first described in 1988 (Wolpe et al., 1988) as a heparin binding protein with neutrophil-activating activities that was purified from an endotoxin-stimulated macrophage cell line and ran as a doublet of bands on SDS polyacrylamide gels. The two bands were later fractionated into two separate, but highly homologous proteins, the cDNAs of which were then obtained and renamed MIP-1α and MIP-1β (Davatelis et al., 1988; Sherry et al., 1988). Since then, MIP-1α has been shown to be produced by a wide range of cell types and capable of carrying out a number of functions both within and outside the immune system (see below).

1.3.1. Cellular Sources of MIP-1α and its Role as an Inflammatory Mediator

The fact that MIP-1α acts predominantly as a proinflammatory mediator is supported by the highly inducible nature of its production. Following stimulation with reagents such as bacterial endotoxins (Wolpe et al., 1988) and IL-3 (Li et al., 1996), MIP-1α is produced and secreted by a great number of cells, including macrophages, neutrophils, basophils, eosinophils, fibroblasts and T lymphocytes, although constitutive expression may occur in a very localised fashion and thus be below detection levels. In fact, Langerhans cells, the skin-specific antigen-presenting cells, have been demonstrated to express MIP-1α constitutively (Heufler et al., 1992), however this has been associated with MIP-1α's ability to inhibit keratinocyte proliferation (Parkinson et al., 1993) and may not actually occur in the context of an inflammatory response.

Although MIP-1α was originally described as a neutrophil-activating protein, it has since been established that its activity on neutrophils is rather poor (Zhang et al., 1999) and that its main targets for chemoattraction and activation include monocytes, specific subsets of T lymphocytes (Schall et al., 1993; Taub et al., 1993), eosinophils, Natural Killer cells and basophils. These findings have implicated MIP-1α in a range of infammatory disorders.

For example, the production of MIP-1α by stimulated basophils (Li et al., 1996) and the observation that it induces eosinophil attraction and activation (Rot et al., 1992) seems to suggest a role for this chemokine in allergic inflammation and parasitic infection. Elevated levels of MIP-1α have also been detected in patients with chronic inflammatory lung diseases (Smith et al., 1994; Standiford et al., 1993) and in the brains of patients with inflammatory neurological disorders such as Multiple Sclerosis where it is thought to cause leukocyte infiltration which is associated with these disease states. This was supported by the use of animal models in which mice stimulated with lipopolysaccharides (bacterial endotoxins) displayed MIP-1α-mediated recruitment of leukocytes into the lungs of these animals which caused severe lung injury (Standiford et al., 1995). A similar experimental model raised the possibility that MIP-1α, MIP-1β and RANTES produced by T cells, macrophages and astrocytes in the brain lead to infiltration of inflammatory cells into the central nervous system during the acute phase of experimental autoimmune encephalomyelitis, a mouse model for Multiple Sclerosis (Karpus et al., 1995; Miyagishi et al., 1997). A similar involvement has been shown for MIP-1α in rheumatoid arthritis (Koch et al., 1994), and it has also been implicated in mediating macrophage infiltration during the process of wound repair (DiPietro et al., 1998).

However, definite proof for the involvement of MIP-1α in disease states came from the generation of mice in which the gene for MIP-1α had been deleted (Cook et al., 1995). These mice, which are apparently normal under nonpathological conditions, are resistant to Coxsackievirus-induced myocarditis and exhibit reduced pneumonitis and delayed clearance of viral particles during infections with influenza virus which demonstrates that MIP-1α plays an important role as a mediator of virus-induced inflammation *in vivo*. More recently, MIP-1α was also identified as the osteoclast stimulatory factor that is found in the bone marrow of multiple myeloma patients and which may be responsible for the bone destruction associated with this disease (Clements et al., 1992).

1.3.2. MIP-1α in Haemopoiesis

Apart from its role as a proinflammatory mediator where its target cells mainly comprise mature leukocytes, MIP-1α can also modulate the activity of a number of more immature haemopoietic stem and progenitor cells. The exact effect that MIP-1α has on these cells very much depends on the type of cell, its maturation state, the presence of a number of other factors such as haemopoietic growth factors and the precise assay system used to

investigate these functions. Because of the fact that the study of haemopoietic stem and progenitor cells is still largely dependent on the use of *in vitro* assays and that only minor variations in the conditions of the assays can result in a completely different outcome, conflicting observations have been made as to the specific target cells of MIP-1α and the precise effect it has on these cells.

However, the overall picture that has emerged and been confirmed by a number of groups now, indicates that MIP-1α inhibits the proliferation of immature cells, such as the day 12 CFU-S cells that sit at the primitive end of the myeloid lineage, while it does not seem to have any effect on the most immature pluripotent stem cells (Keller et al., 1994). Once the cells have reached a certain maturation state when they can give rise to only one lineage of mature blood cells, their response to MIP-1α changes and instead of being inhibited, their proliferation is enhanced by this chemokine (Van Ranst et al., 1996), as illustrated in progenitor assays in which only a single growth factor is included for the promotion of colony formation from more mature progenitors (Clements et al., 1992; Graham et al., 1990). In addition, there have been reports showing that MIP-1α can cause an expansion of long term culture-initiating cells in *ex vivo* cultures (Verfaillie and Miller, 1995) as well as a rapid mobilisation of early haemopoietic progenitor cells (CFU-S and cells with marrow repopulating ability) from the bone marrow into the peripheral blood (Hunter et al., 1995; Lord et al., 1995).

The first data demonstrating MIP-1α's inhibitory activity were published in 1990 (Graham et al., 1990) when the active agent present in the conditioned medium of the murine macrophage cell line J774.2, that was shown to inhibit haemopoietic stem cells, was purified and identified as the chemokine MIP-1α. In that report, inhibitory activity of MIP-1α was demonstrated on CFU-S day 12, CFU-A (an *in vitro* equivalent of CFU-S day 12) and to a lesser extent on CFU-S day 8 cells, but not on more mature GM-CFC progenitors. Other stem cell types that have been shown to be inhibited include HPP-CFC in some cases (Eaves et al., 1993), but not in others (Keller et al., 1994), BFU-E (Maze et al., 1992; Quesniaux et al., 1993), CFU-GEMM in some studies (Cooper et al., 1994), but not in others (Keller et al., 1994), and CFU-GM (Maze et al., 1992), as long as the formation of colonies in these *in vitro* assays is stimulated with more than one growth factor. Interestingly, in a few cases, the inhibition induced by MIP-1α could be blocked by the addition of MIP-1β as an antagonist (Broxmeyer et al., 1993; Broxmeyer et al., 1991; Eaves et al., 1993) which seems to suggest that MIP-1β can bind to the stem cell inhibitory

receptor, but fails to activate it. Studies on the time course of inhibition established that MIP-1α acted within 3-24 hours (Maze et al., 1992), which could even be reduced to one hour at 4°C (Broxmeyer et al., 1993; Broxmeyer et al., 1991) and was reversible within 48 hours (Maze et al., 1992). The inhibition was shown to be caused by blocking the cell cycle prior to DNA synthesis as demonstrated by a suicide assay in which actively cycling cells undergoing DNA replication are killed by the addition of the base analogue cytosine arabinoside (Graham et al., 1990).

A later report not only detected the same activity in the human analogue of MIP-1α (LD78), but also confirmed the *in vivo* myeloprotective effect that these proteins have on haemopoietic stem cells since stem cells that were induced to proliferate after a cytotoxic insult to the haemopoietic system were protected from a second dose of the cytotoxic drug 5-fluorouracil in the presence of MIP-1α (Dunlop et al., 1992). This and the observation that haemopoietic stem cells from chronic myeloid leukaemia patients seemed refractory to the inhibitory effect of MIP-1α (Chasty et al., 1995; Eaves et al., 1993; Holyoake et al., 1993) led to the suggestion that MIP-1α may prove to be a useful agent given to patients who are undergoing chemotherapy since it would not only lessen the negative impact of the drugs on healthy haemopoietic stem cells followed by a faster recovery, but would also allow higher and more effective doses of chemotherapy drugs to be administered. The role of MIP-1α as a potential myeloprotective agent would not just be restricted to the treatment of leukaemia, but woul lessen the negative impact of chemotherapy treatment for any type of cancer. And, indeed, clinical trials were initiated with a non-aggregating variant of MIP-1α, called BB10010, that had improved pharmacological properties (Hunter et al., 1995). And although this variant showed haemopoietic stem cell mobilising activities in mice (Lord et al., 1995), myeloprotective properties in a chemotherapy mouse model (Lord et al., 1996) and was generally well tolerated in patients (Bernstein et al., 1997; Marshall et al., 1998), it failed to produce the expected results in human patients in stem cell mobilisation and chemoprotection (Bernstein et al., 1997; Clemons et al., 1998) in phase II clinical trials which resulted in the termination of the clinical trials.

A number of different sources for the production of MIP-1α in the bone marrow have been suggested. Immunohistochemical staining detected the expression of MIP-1α mRNA and production of the mature protein in eosinophilic myelocytes, the precursors of eosinophils, and osteoblasts (Kukita et al., 1997). Other proposed cellular sources for MIP-1α in the bone marrow include macrophages (Maltman et al., 1993) and stromal cells (Sensebe et al.,

1997). Expression was demonstrated to be induced by a number of factors, including GM-CSF and IL-3 (Jarmin et al., 1999), IL-1β (Sensebe et al., 1997) and neurokinin A, a neuropeptide that also stimulates the production of TGFβ (Rameshwar and Gascon, 1996). Interestingly, TGFβ is another haemopoietic stem cell inhibitor which has a target cell population that overlaps with the one inhibited by MIP-1α, but which is a generally more potent inhibitor that has more widespread effects (Keller et al., 1994; Mayani et al., 1995; Soma et al., 1996; Van Ranst et al., 1996). It was also demonstrated to inhibit the expression of MIP-1α and its receptors, while MIP-1α was shown to increase the production of TGFβ (Maltman et al., 1996; Maltman et al., 1993). This seems to suggest that TGFβ may be a physiologically more relevant stem cell inhibitor than MIP-1α, although neither TGFβ nor MIP-1α null mice have any overt defects in the proliferation of haemopoietic stem cells (see above). Nevertheless, the fact remains that MIP-1α has been shown to possess inhibitory activity in a number of different studies, a role that may be compensated for by other factors in the knockout mice, thus suggesting some degree of redundancy which, as mentioned above, may be a common feature of the chemokine family.

Very little is known about the signalling pathway employed by MIP-1α to exert its various functions on haemopoietic stem and progenitor cells, and the identity of the receptor through which MIP-1α mediates its inhibitory effects still remains elusive, as supported by experimental data provided elsewhere in this thesis. The main reason for the lack of information on the signalling pathways involved is the difficulties associated with the study of stem cells. As mentioned above, a lot of the studies carried out on MIP-1α's effects on haemopoietic stem cells have relied on *in vitro* colony forming assays, a system that makes the dissection of individual signalling pathways almost impossible. Cell sorting protocols for haemopoietic stem cells exist that rely on the presence or absence of certain cell surface markers, however these only result in a relative enrichment for stem cells which are still too heterogeneous to allow the study of individual signalling events.

In order to circumvent this problem, established haemopoietic cell lines have been used, yet these not only have the tendency to give variable results, it is also possible that they do not exactly behave like primary cells in their bone marrow microenvironment. One such cell line is the human haemopoietic cell line M07e which has been reported to be inhibited by MIP-1α and IP10 via a mechanism that involves the elevation of intracellular cAMP

levels, the inhibition of Raf-1 phosphorylation and activation of the MAPK pathway (Aronica et al., 1997; Aronica et al., 1995).

The most representative method for delineating MIP-1α-stimulated pathways so far has been the study of the response elicited by MIP-1α in the bone marrow cells obtained from mice that are deficient in components of the putative signalling pathway (see also in the Results section). In one such study it was shown that primitive myeloid progenitor cells obtained from mice that were deficient for CCR1 expression, still exhibited inhibition by MIP-1α, whereas more mature CFU-GM progenitors were no longer enhanced in their proliferation by MIP-1α and myeloid progenitor cells were no longer mobilised into the peripheral blood (Broxmeyer et al., 1999). This seems to suggest that while CCR1 does not mediate MIP-1α's inhibitory signal, it is nevertheless required for mobilisation and enhancement of proliferation. As illustrated in a later section, none of the other known MIP-1α receptors is involved in stem cell inhibition either.

1.3.3. Structural Features of MIP-1α

One of the first structural studies carried out on MIP-1α aimed at elucidating the processes involved in its aggregation (Patel et al., 1993). This report which relied on techniques such as analytical ultracentrifugation, circular dichroism and fluorescence spectroscopy showed that human MIP-1α, LD78, exists as soluble, heterogeneous, multimeric complexes of 100 to 250 kD at physiological ionic strength and high concentrations (0.5 mg/ml). These complexes were disrupted by varying the buffer conditions which established that a breakdown of complexes down to the tetrameric level can be achieved in conditions that disrupt ionic interactions whereas dimers and tetramers are mainly stabilised by hydrophobic forces. Another report also showed that the aggregation of MIP-1α is a dynamic and reversible process that is influenced by the concentration of the protein and the surrounding buffer environment (Graham et al., 1994). However, while this report seems to confirm that ionic interactions lead to high order aggregates since the neutralisation of a single acidic residue in the C terminal helix (glutamic acid at position 66 in murine MIP-1α to glutamine) led to a mutant that could not aggregate past the tetrameric state, the neutralisation of additional negative charges, E66Q + D64N and E66Q + D64N + E60Q, generated dimeric and monomeric mutants, respectively. Although this seems to contradict the previous report, it could be envisaged that the neutralisation of negative charges affects the overall charge distribution in such a way that it interferes with

oligomerisation. This is further supported by the fact that the neutralisation of two positive charges in the loop between the second and the third β strand also results in a mutant that is predominantly in a dimeric state (Graham et al., 1996). Another nonaggregating mutant of human MIP-1α that seems to be in a monomer-dimer-tetramer equilibrium was created by changing the aspartic acid residue at position 26 to an alanine residue (Hunter et al., 1995). All of these nonaggregating mutants, however, have biological activities that are comparable to the wild type protein, thus once again supporting the notion that aggregation is not required for normal chemokine function.

As mentioned in the earlier section on chemokine-proteoglycan interactions, the heparin-binding determinants of MIP-1α (for a picture of the heparin binding site within the three dimensional structure of MIP-1α, see Fig. 4.1) are located in the loop connecting β_2 and β_3 (residues Lys44 and Arg45 within a positive cluster) and in the N loop (Arg17). Mutations at these positions severely interfere with MIP-1α binding to heparin, however seem to leave its biological functions largely unaffected (Graham et al., 1996; Koopmann and Krangel, 1997). The mutant in which Lys44, Arg45 and Arg47 were changed to asparagine and serine, respectively, did lose its ability to interact with CCR1 which seems to suggest that this site is involved in the interaction with this receptor either directly or indirectly through its proximity to the N loop. Compared to other chemokines, MIP-1α is a relatively weak heparin binding protein with NaCl concentrations of only 0.39 M (Kuschert et al., 1999) required to elute it from a heparin column. This may be taken as another piece of evidence to suggest that proteoglycan binding properties are possibly not essential for its activity.

Very little is known about specific residues of MIP-1α that are involved in receptor interactions. One could assume that in agreement with findings in other chemokines, the N loop of MIP-1α (residues 13-20) may be important for its initial docking to the receptor whereas residues preceding the CC motif (residues 1-10) are involved in receptor activation. However, studies on MIP-1α and MIP-1β chimaeras seem to implicate a region encompassing the second and third β strand and the α helix and specifically lysine 37 and leucine 43 (human MIP-1α) in the second β strand in CCR1 binding (Crisman et al., 1999). As mentioned above, the two residues that are found in the adjacent loop and that are heparin-binding determinants, may also be involved in CCR1 interactions. In the human genome, two isoforms of MIP-1α are found, LD78α and LD78β, which differ in only three amino acids at position 2, 39 and 47. Despite their high degree of sequence identity, their

affinities for the known MIP-1α receptors are strikingly different (Menten et al., 1999; Nibbs et al., 1999). While LD78α is a better ligand for CCR1, LD78β has a much higher affinity for CCR5 and D6. These differences seem to be mainly determined by the presence or absence of a proline residue at position 2 which is further supported by receptor binding studies with truncated versions of both LD78α and LD78β which have the four most N terminal amino acids deleted (Nibbs et al., 1999). This once again stresses the sensitivity of the extreme N terminus of CC chemokines to modifications.

The three-dimensional structure of murine MIP-1α has been determined recently by x ray crystallography (MacLean, J unpublished results). This was brought about by the availability of monomeric, dimeric and tetrameric mutants of MIP-1α which facilitated its crystallisation which had up until then been impossible due to the formation of heterogeneous aggregates at higher concentrations. The tertiary structure of all three mutants displays the common monomeric chemokine fold of an extended N terminal region, a central β sheet and a C terminal α helix that lies on top of the β sheet. The dimeric structure is very similar to the ones found for other CC chemokines, whereas the tetramer is quite different from the only other known CC chemokine tetramer, MCP-1 (see above). In fact, two different tetramers were identified for MIP-1α occasionally even within the same droplet, which facilitated the distinction between "true" contacts and "crystal" contacts. However, the biological significance of the two tetramers is unknown. The quaternary structure confirmed that the majority of monomer-monomer and dimer-dimer interactions are of a hydrophobic nature. Yet, it also showed that Glu66 and Asp26, the mutation of which individually prevented the formation of high order aggregates, are part of two salt bridges that are crucial for intersubunit contacts, whereas Asp64 and Glu60, the other two residues implicated in the oligomerisation process, point away from the rest of the molecule and can therefore not directly be involved in intermolecular interactions, which supports the hypothesis that it is their charge, rather than their position that affects multimer formation. Interestingly, calcium ions were found to be associated with the tetramers and proposed to be important in mediating the oligomerisation process for three reasons, (1) they were found in almost exactly the same position in the two different tetramers, (2) the presence of calcium was necessary for the crystallisation of the tetramer, but not of the dimer or monomer, and (3) it could not be replaced by any other ion tested. Part of the work leading to this thesis dealt with the question of whether calcium was important for aggregation experimentally (see Results section). The three dimensional structure also revealed that the positive residues implicated in heparin binding come

together to form a so-called cationic cradle, a common heparin binding motif, and that they are also involved in intersubunit contacts as suggested from the mutagenesis studies (Graham et al., 1996).

The availability of the crystal structure of MIP-1α formed the basis for most of the work carried out during the course of this PhD since it allowed a rational approach to the investigation of the structure-function relationship in MIP-1α.

AIM

MIP-1α was the first chemokine to be implicated in the control of haemopoietic stem cells. However, the way in which MIP-1α inhibits the proliferation of these cells on the molecular level still remains largely unknown. This thesis therefore aimed at elucidating certain aspects of the relationship between the structure of MIP-1α and its function as an inhibitor of transiently engrafting haemopoietic stem cells (cells detected in the CFU-A assay). In the first instance, two well known properties of MIP-1α, its ability to bind to heparin and its tendency to aggregate at increasing concentrations, were considered. Although mutants of MIP-1α that have lost these properties retain full inhibitory capacity in the CFU-A assay, thus suggesting that aggregation and proteoglycan binding is not essential for stem cell inhibition *in vitro*, these characteristics may nevertheless play an important role *in vivo* in the context of the bone marrow microenvironment. For example, proteoglycans are essential components of the haemopoietic stem cell niche (see Introduction) where they may interact with regulatory molecules such as MIP-1α, possibly in processes of sequestration and presentation. Therefore, in order to increase our current knowledge of the mechanism by which MIP-1α self-associates and interacts with heparin and a possible relationship between the two processes, experiments were carried out to address these points and are described in the following chapters.

As mentioned in the Introduction, the receptor, through which MIP-1α exerts its inhibitory function, has not been characterised. Experiments were therefore undertaken in order to definitely rule out the involvement of any one of the four known murine MIP-1α receptors. Furthermore, in order to shed some light on the molecular mechanism underlying stem cell inhibition, experiments were carried with the aim of identifying the region within MIP-1α that confers stem cell inhibition. It is hoped that such information will contribute to the identification of the inhibitory receptor and the downstream signalling events which may enhance our current knowledge of the regulation of haemopoietic stem cells and how this control breaks down in proliferative disorders, such as leukaemia.

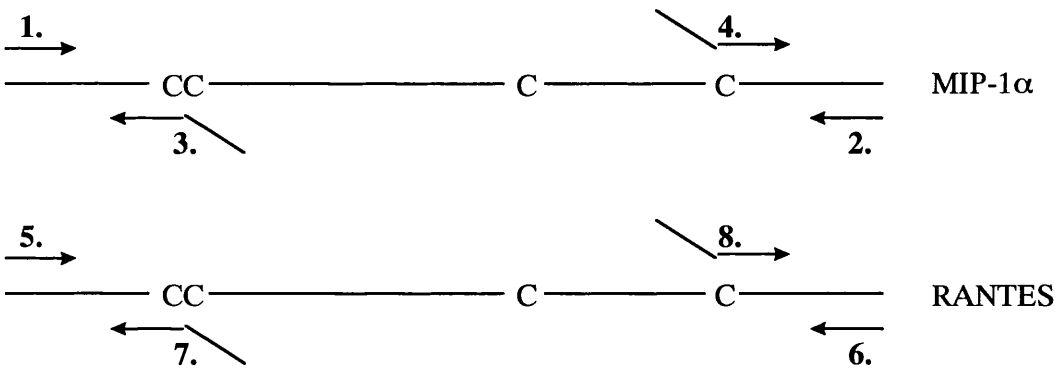
Chapter 2. MATERIALS & METHODS

2.1. Materials

2.1.1. Plasmids

Name	Insert	Source
pSK.mMIP-1α	murine MIP-1α cDNA 280 bp Xho1 / EcoRI	S. Wylie Beatson Institute Glasgow, UK
pSK.hRANTES	human RANTES cDNA 410 bp EcoRI / HindIII	S. Wylie Beatson Institute Glasgow, UK
pVL1392/1393 Baculovirus Transfer Vector	-	Becton Dickinson UK Ltd. Plymouth, UK

2.1.2. Primers for Overlap PCR Mutagenesis



Name	Description
M5' (1. on above figure) 5' ATGAAGGTCTCCACCACTGCC 3'	amplifies MIP-1α from the 5' end
M3' (2.) 5' CTCAGGCATTCAGTTCCAGGTC 3'	amplifies MIP-1α from the 3' end
R5' (5.) 5' ATGAAGGTCTCCGCGGCAGC 3'	amplifies RANTES from the 5' end
R3' (6.) 5' CTAGCTCATCTCCAAAGAGTTG 3'	amplifies RANTES from the 3' end

M-CC-R (3.) chimaeric internal primer; spans the CC motif; amplifies the N terminus of MIP-1α fused to the RANTES sequence C terminal to the CC motif

5' GGGCAATGTAGGCAAAGCAGCAGGCAGTCGGGGTGTCAGC 3'

R-CC-M (7.) chimaeric internal primer; spans the CC motif; amplifies the N terminus of RANTES fused to the MIP-1α sequence C terminal to the CC motif

5' CCGGCTGTAGGAGAAGCAGCAGGGTGTGGTGTCCGAGG 3'

R-C-M (4.) chimaeric internal primer; spans the final C motif; amplifies the C terminus of MIP-1α fused to the RANTES sequence N terminal to the C motif

5' CTAAGAGAAACCGGCAGATCTGTGCCAACCCAGAGAAGAAATGG 3'

M-C-R (8.) chimaeric internal primer; spans the final C motif; amplifies the C terminus of RANTES fused to the MIP-1α sequence N terminal to the C motif

5' CCCGAAAGAACCGCCAAGTGTGCGCTGACTCCAAAGAGACCTGG 3'

2.1.3. Tissue Culture Supplies

Supplier	**Material**
Beatson Institute, Central Services Glasgow, UK	Sterile phosphate buffered saline (PBS) Sterile PBS+EDTA Sterile Glassware and Pipettes
R2, Beatson Institute	L929 Conditioned Medium (CM) AF1 CM
Becton Dickinson, UK Ltd. Plymouth, UK	Falcon Tubes Tissue Culture Dishes Needles and Syringes 24 well plates Agarplaque Agarose Transfection Buffer A and B Set BaculoGold linearised Baculovirus DNA
Biowhittaker UK Ltd. Wokingham, UK	Insect Cell Culture Medium (TNM-FH and Protein-free medium)
Coulter Ltd. Luton, UK	Zapoglobin

Costar Cambridge, MA USA	Cell Scrapers
DIFCO Laboratories Detroit, USA	Agar Noble
Gelman Sciences Northampton, UK	Sterile acrodisc syringe filters (0.2 μm and 0.45 μm)
Gibco Life Technologies Paisley, UK	MEM alpha stock L-glutamine (200 mM) Sodium Bicarbonate (7.5%) Trypsin (2.5 %) Special Liquid Medium
Harlan Sera-Lab Ltd. Belton, UK	Foetal Calf Serum
Merck Poole, UK	Giemsa stain 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) microscope slides
Nalge Nunc International Roskilde, Denmark	Tissue Culture Flasks Cryotubes
Sigma Chemical Co. Poole, UK	Donor Horse Serum DMEM Fura-2-AM Neutral Red
Stem Cell Technologies Inc. British Columbia, CANADA	Methylcellulose (no growth factors)
Sterilin Ltd. Hounslow, UK	Culture Plates
Techne Cambridge, UK	Stirrer Culture Vessels

2.1.4. Cytokines and Antibodies

Supplier	**Material**
ARES Serono Geneva, Switzerland	recombinant human Fractalkine recombinant human HCC-4 recombinant human HCC-2 recombinant human LARC recombinant vMIP-II

Gryphon Sciences South San Franscico, USA	AOP-RANTES
Human Genome Sciences Maryland, USA	Recombinant murine C10 Recombinant human Ckβ-1 (HCC-1) Recombinant human Ckβ-4 (LARC) Recombinant human Ckβ-6 (MPIF2) Recombinant human Ckβ-12 (HCC-4)
Peprotech EC Ltd. London, UK	Recombinant murine Eotaxin Recombinant murine Exodus-2 (SLC) Recombinant human I-309 Recombinant murine MDC Recombinant human MIP-1α (full length LD78α) Recombinant human MIP-3 (MPIF-1) Recombinant human MIP-3α (LARC)
R & D Systems Europe Abingdon, UK	Recombinant anti-murine MIP-1α antibody Recombinant anti-human RANTES antibody Recombinant human DCCK-1 Recombinant human HCC-1 Recombinant mouse GM-CSF Recombinant human IL-8 (72aa) Recombinant human IL-8 (77aa) Recombinant human IP-10 Recombinant human MCP-1 Recombinant human MCP-2 Recombinant human M-CSF Recombinant human MIP-1α (full-length LD78β) Recombinant human MIP-1α (LD78α-4) Recombinant murine MIP-1α Recombinant human MIP-1β Recombinant human RANTES Recombinant murine SCF Recombinant murine SDF-1 Recombinant murine TECK
R2, Beatson Institute	PM1, PM2, PM3 (Graham et al., 1994) (mutants of murine MIP-1α) Recombinant human MIP-1α (LD78β-4) (Nibbs et al., 1999)
Sigma Immunochemicals Poole, UK	Anti-goat IgG (HRP-conjugated)

2.1.5. Molecular Biology

2.1.5.1. Bacterial Culture

Supplier	Material
Beatson Institute, Central Services Glasgow, UK	Luria-Broth (L-Broth)
DIFCO Laboratories Michigan, USA	Bactoagar
Fisher Scientific UK Ltd. Loughborough, UK	Ethanol Isopropanol DMSO Sodium Acetate
GIBCO BRL Paisley, UK	DH5α competent cells
Qiagen Inc. Chatsworth, USA	QIAGEN plasmid preparation kits QIAquick gel extraction kit
Sigma Chemical Co. Poole, UK	Ampicillin X gal, IPTG
Stratagene Ltd. Cambridge, UK	pCR-Script Amp SK(+) Cloning Kit

2.1.5.2. Enzymes and Nucleotides

Supplier	Material
GIBCO BRL Paisley, UK	all restriction enzymes and buffers
Promega Ltd. Southampton, UK	Deoxynucleotide Triphosphates
Stratagene Ltd. Cambridge, UK	Pfu DNA Polymerase
Transgenomic Ltd. Crewe,UK	T4 DNA Ligase Alkaline Phosphatase

2.1.5.3. Gel Electrophoresis

Supplier	Material
GIBCO BRL Paisley, UK	Agarose 1kb and 100bp DNA ladders DNA mass ladders
Sigma Chemical Co. Poole,UK	Bromophenol Blue Ethidium Bromide
Fisher Scientific UK Ltd. Loughborough, UK	EDTA, EGTA glycerol

2.1.6. Protein Studies

2.1.6.1. Kits

Supplier	Material
R & D Systems Europe Abingdon, UK	murine MIP-1α ELISA kit

2.1.6.2. Chromatography

Supplier	Material
Fisher Scientific UK Ltd. Loughborough, UK	Acetonitrile Sodium Chloride
Perkin Elmer Warrington, UK	Trifluoroacetic acid (TFA)
Pharmacia Biotech Ltd. St. Albans, UK	5ml HiTrap Desalting Column 1ml HiTrap Heparin Column Heparin Sepharose CL-6B MonoQ HR 5/5 column ProRPC HR 5/2 column Sephacryl S-200HR
Sigma Chemical Co. Poole, UK	Bovine Serum Albumin (BSA) Heparin-Agarose (attached through reductive amination) 12,000-200,000 molecular weight marker kit soluble heparin

2.1.6.3. Cross-linking

Supplier	Material
Sigma Chemical Co. Poole, UK	Glutathione-S-Transferase L-Histidine Tris(2,2'-bipyridyl)Ruthenium(II)chloride

2.1.6.4. SDS-Polyacrylamide Gel Electrophoresis and Silver Staining

Supplier	Material
Amersham Pharmacia Biotech Little Chalfont, UK	14,300-220,000 rainbow markers 10,000-250,000 rainbow markers
Fisher Scientific UK Ltd. Loughborough, UK	Acetic Acid Ammonium Persulphate Formaldehyde Glycine Methanol Sodium Carbonate (anhydrous) Sodium Dodecyl Sulphate Tris
Severn Biotech Ltd. Kidderminster, UK	Design-a-gel 30% (w/v) Acrylamide, 0.8% (w/v) bis-Acrylamide solution
Sigma Chemical Co. Poole, UK	Dithiothreitol (DTT) Silver Nitrate TEMED
TAAB Adermaston Reading, UK	glutaraldehyde

2.1.6.5. Western Blotting

Supplier	Material
Amersham Pharmacia Biotech Little Chalfont, UK	ECL Western Blotting Reagents
Calbiochem Novabiochem UK Nottingham, UK	NP-40
Kodak Scientific Imaging Systems Ltd. Cambridge, UK	X-OMAT AR X-ray film
Millipore Corp. Bedford, USA	Immobilon-P transfer membranes

Sigma Chemical Co. Poole, UK	ε-amino-n-caproic acid
Whatmann International Ltd. Maidstone, UK	3MM blotting paper

2.1.7. Solutions

2.1.7.1. Bacterial Culture

SOC medium	2% (w/v) bactotryptone 0.5% (w/v) yeast extract 10 mM NaCl 2.5 mM KCl 20 mM $MgCl_2$ 20 mM $MgSO_4$ 20 mM glucose

2.1.7.2. Calcium Flux Assay

SR buffer (pH 7.2):	136 mM NaCl 4.8 mM KCL 5 mM Glucose 20 mM Hepes 1 mM $CaCl_2$ 0.05% BSA

2.1.7.3. CFU-A Assay

2x medium (50ml)	21 ml αMEM 25 ml donor horse serum 1 ml L-glutamine (200 mM) 3 ml Sodium Bicarbonate (7.5%)

2.1.7.4. Chromatography

Heparin Affinity Chromatography buffers/: Ion Exchange Chromatography buffers (Mono Q)	A: 0.1 M NaCl / 0.02 M Tris pH 7.6 B: 2 M NaCl / 0.02 M Tris pH 7.6
Reversed Phase Chromatography buffers:	A: H_2O / 0.1 % TFA B: Acetonitrile / 0.1% TFA

2.1.7.5. Agarose Gel Electrophoresis

50x TAE	2 M Tris-acetate 50 mM EDTA

DNA loading buffer	30% glycerol Bromophenol blue to colour

2.1.7.6. SDS PAGE

17.5% Polyacrylamide gel (separating) (volumes for 2 gels)	3.0 ml H_2O 11.6 ml 30% Acrylamide mix 5.0 ml 1.5 M Tris (pH 8.8) 0.2 ml 10% SDS 0.2 ml 10% Ammonium Persulphate 8 µl TEMED
6.5% Polyacrylamide gel (stacking) (volumes for 2 gels)	5.5 ml H_2O 1.3 ml 30% Acrylamide mix 1.0 ml 1.0 M Tris (pH 6.8) 80 µl 10% SDS 80 µl 10% Ammonium Persulphate 8 µl TEMED
Running Buffer	200 mM Glycine 25 mM Tris 0.1% SDS (w/v)
SDS PAGE loading buffer (per 20 µl sample)	5 µl 20% SDS / 250 mM DTT 5 µl glycerol / Bromophenol blue

2.1.7.7. Western Blotting

Anode Buffer 1	0.3 M Tris / 20% methanol (pH 10.4)
Anode Buffer 2	25 mM Tris / 20% methanol (pH 10.4)
Cathode Buffer	40 mM ε-amino-n-caproic acid / 20% methanol (pH 7.2)

2.1.8. Cell Lines and Bone Marrow

Supplier	**Cells**
Becton Dickinson UK Ltd. Plymouth, UK	Sf9 Insect Cells
Dr. R.J. Nibbs Beatson Institute	Human Embryonic Kidney cells, stably transfected with hCCR5
Dr. D.N. Cook New Jersey, USA	$D6^{-/-}$ bone marrow

Dr. A. Humbles Boston, USA	$CCR1^{-/-}$, $CCR3^{-/-}$ bone marrow
Dr. W.A. Kuziel Texas, USA	$CCR5^{-/-}$ bone marrow

2.2. Methods

2.2.1. DNA Methodology

2.2.1.1. PCR Mutagenesis

PCR reactions were carried out in a final volume of 100 µl, containing 10 µl 10x Pfu buffer, 10 µl DMSO, 10 µl 50% glycerol, 4 µl dNTPs at 10 mM, 3 µl of each primer at 330 ng/ml, 1 µl of the template at 110 ng/ml and 1 µl Pfu polymerase (made up to the final volume with water). In a second round of PCR where the double-stranded product of the first round was included as an additional primer, the amount of this double-stranded primer and of the template added to the reaction was reduced to 10 ng, and the concentrations of the single-stranded extreme 5' and 3' end primers was 0.1 µM. The reactions were overlaid with 100 µl paraffin and products obtained after 25 cycles of a one minute denaturing step at 94°C, a one minute annealing step at 55°C and a one minute extension step at 72°C. After the 25 cycles, the reactions were left at 72°C for 10 minutes.

2.2.1.2. Restriction Digests

Restriction digests were carried out in a final volume of 20 µl, containing 2 µl of 10x reaction buffer, ~1 µg of plasmid DNA and 0.5 µl of each restriction enzyme (made up to the final volume with water). The digests were incubated at optimal temperature (depending on the restriction enzyme - usually 37°C) for 2 hours. Vectors that were prepared in this way for the ligation of inserts, were treated with 1 µl Alkaline Phosphatase for the last half hour of the incubation in order to block the reannealing of the vector.

2.2.1.3. Visualising DNA by Agarose Gel Electrophoresis

1.5% (for DNA analytical gels) and 1.2% (for DNA preparative gels) agarose gels were prepared by adding the required amount of agarose (w/v) to 1x TAE buffer and boiling until the agarose was dissolved. Upon cooling, Ethidium Bromide (10 mg/ml stock

solution) was added to a final dilution of 1 in 40,000 and the gel left to set in a gel cast. Samples to be analysed were prepared by adding one tenth of the total volume of DNA loading buffer. Size markers and the samples were loaded into the wells and the gel run in 1x TAE buffer at ~150 V constant voltage. DNA bands were visualised using a UV transilluminator, photographed and, in the case of preparative gels, the bands were excised and the cDNA purified (see below).

2.2.1.5. Purification of DNA from Agarose Gels

The DNA was extracted from gel slices using the QIAGEN QIAquick Gel Extraction Kit and following the manufacturer's instructions. Briefly, the excised gel slices were weighed and 3 volumes of Buffer QG added to 1 volume of gel (100 mg ~ 100 µl). The gel was dissolved by heating at 50°C for 10 minutes. One gel volume of isopropanol was added, the mix applied to QIAquick spin columns and centrifuged for 1 minute. Excess agarose was removed with 0.5 ml Buffer QG and the columns subsequently washed with 0.75 ml Buffer PE. The DNA was eluted into 50 µl of water and the yield determined by running a sample on an agarose gel in the presence of a DNA mass ladder.

2.2.1.5. Cloning of DNA fragments into pCR Script

Blunt-ended PCR products were cloned into pCR-Script using the Stratagene pCR-Script Amp SK(+) Cloning Kit and following the manufacturer's instructions. Briefly, the following components were mixed together in the following order: 1 µl of the pCR-Script Amp SK(+) cloning vector (at 10 ng/µl), 1 µl of pCR-Script 10x reaction buffer, 0.5 µl of 10 mM rATP, 4 µl of the blunt-ended PCR product (at 10 ng/µl), 1 µl of Srf I restriction enzyme (5 U/µl), 1 µl of T4 DNA ligase and 1.5 µl distilled water. The reaction was incubated at room temperature for 1 hour, subsequently heated to 65°C for 10 minutes and stored on ice until used for transformation.

For each transformation reaction, 40 µl of Epicurian Coli XL1-Blue MRF' Kan supercompetent cells were mixed with 0.7 µl of β-mercaptoethanol and incubated on ice for 10 minutes. 2 µl of the above cloning reaction were then added and the transformation reaction incubated on ice for a further 30 minutes. It was then heat pulsed at 42°C for 45 seconds and incubated on ice for 2 minutes. 0.45 ml of pre-warmed SOC medium was added to the transformation reaction which was then incubated at 37°C for 1 hour with shaking at 225 rpm. 50-200 µl of the transformation reaction were spread on ampicillin-

containing agar plates (1.5% bactoagar in L-Broth, containing 50 μg/ml ampicillin) in the presence of 40 μl X gal (at 20 mg/ml) and 40 μl IPTG (at 20 mg/ml). The plates were incubated overnight at 37°C and white colonies picked the following evening for further analysis.

2.2.1.6. Cloning of DNA fragments into the Baculovirus Transfer Vector pVL1392/1393

The ligation of DNA inserts into the vector was carried out in a reaction volume of 20 μl, containing 1 μl T4 DNA Ligase, 2 μl 10x Ligase Buffer and insert and vector DNA in molar ratios of 1:1 or 4:1, respectively. The ligation reactions were incubated overnight at 15°C.

100 μl of E. coli DH5α competent cells were thawed per transformation reaction. These were mixed with 1.7 μl β-mercaptoethanol (25 mM final concentration) and incubated on ice for 10 minutes. 1 μl of the above ligation reaction was then added to the cells and the reaction incubated on ice for a further 30 minutes. Following a heat shock at 42°C for 50 seconds, the reactions were returned to ice for 2 minutes. 0.9 ml of pre-warmed SOC medium was added to each reaction which were subsequently incubated at 37°C for 1 hour, with shaking at 225 rpm. After that incubation period, 100-200 μl of each transformation reaction was spread on ampicillin-containing agar plates and the plates incubated overnight at 37°C. The following evening, colonies were picked and grown in 4 ml of ampicillin-containing L-Broth overnight for further analysis and further production of plasmid.

2.2.1.7. Growth of Plasmids

Large amounts of plasmid were obtained from 250 ml transformed bacterial cultures using the QIAGEN Maxi Kit and following the manufacturer's instructions. Briefly, bacterial cells were collected by centrifugation for 10 minutes at 3000 rpm in a Sorvall GS-3 rotor. The cell pellet was resuspended in 10 ml of RNase-containing P1 buffer. 10 ml of buffer P2 was added to that and the mixture incubated at room temperature for 5 minutes. After the incubation, 10 ml of pre-chilled buffer P3 was added, the mixture incubated on ice for 20 minutes and subsequently centrifuged for 30 minutes at 10,000 rpm in a Sorvall SS-34 rotor. The supernatant was applied to a QIAGEN-tip 500 column that had previously been equilibrated with 10 ml buffer QBT. The column was washed with 2x30 ml buffer QC and the DNA eluted with 15 ml buffer QF. To the eluate were added 0.7 volumes (10.5 ml) of

isopropanol and the precipitated DNA collected by centrifugation at 10,000 rpm for 30 minutes. The pellet was resuspended in 300 µl of water. To the suspension, one tenth of volume (30 µl) of 3M Sodium Acetate and 3 volumes (900 µl) of ethanol were added and the precipitated DNA collected by centrifugation at 13,000 rpm for 10 minutes in a bench top microcentrifuge. The pellets were resupended in 800 µl distilled water and the yield determined by measuring the optical density at 260 nm wavelength.

2.2.2. Production of Recombinant Protein in the Baculovirus System

2.2.2.1. Growth and Maintenance of Sf9 Cells - Monolayers

Cells were grown in monolayers in TNM-FH medium and subcultured (cells were harvested using cell scrapers) 1:2 or 1:3 when they reached confluence (2-3 times a week). They are kept at 27°C.

2.2.2.2. Growth and Maintenance of Sf9 Cells - Suspension

Sf9 cells can be adapted to the growth in suspension cultures in spinner flasks that are stirred continuously at 60 rpm. They were initially seeded at $5x10^5$ cells/ml, but once they were adapted to suspension, this could be reduced to $1x10^5$ cells/ml on subculturing (when they reached a density of $2x10^6$ cells/ml). The cells tended to grow better and reach higher densities in suspension cultures.

2.2.2.3. Generation of Recombinant Baculoviruses by Co-Transfection

0.5 µg of linearised baculovirus DNA was mixed with 2-5 µg of recombinant Baculovirus Transfer Vector containing the gene of interest and incubated at room temperature for 5 minutes before adding 1 ml of Transfection Buffer B. The medium on a 60 mm plate seeded with $2x10^6$ cells was replaced with 1 ml of Transfection Buffer A. To this, the 1 ml of Transfection Buffer B/DNA solution was added drop-wise, and the plate incubated at 27°C for 4 hours. After the incubation period, the medium was removed and the plate washed once with 3 ml TNM-FH. 3 ml of fresh TNM-FH was then added and the plates incubated at 27°C for 5 days. At the end of the 5 days, the medium containing the recombinant viral particles was collected and stored at 4°C. An aliquot was tested in Western Blotting for the expression of the protein of interest.

2.2.2.4. Plaque Assay

This assay was used to obtain viral clones by plaque purification and to determine the viral titres of viral stocks. Sf9 cells were seeded the night before on 35 mm plates at a density of $1x10^6$ cells in an even monolayer. 10^{-4}, 10^{-5} and 10^{-6} dilutions of the viral stocks were prepared in TNM-FH. Once the old medium was removed from the culture plates, the cells were overlayed with 100 µl of the dilutions and the plates left at room temperature for 1 hour. After the incubation period, the virus inoculum was removed and the cells overlayed with 2 ml of a 1:1 mixture of 2% (w/v in protein-free insect cell medium) Agarplaque Agarose and TNM-FH. After the agarose had set, a 1 ml liquid overlay (TNM-FH) was added and the plates incubated at 27°C for 4 days. The plates were stained by adding 1 ml of 0.025% (w/v in PBS) Neutral Red for 4 hours, then the liquid overlay decanted and the colour developed overnight at room temperature in the dark. The viral titre (pfu/ml) was obtained by multiplying the number of plaques on a plate by the dilution factor.

2.2.2.5. Amplifying Virus

For an initial amplification of virus, $2x10^7$ cells were seeded on 15 cm plates and 0.1-1 ml of the low titre stock added. The plates were incubated at 27°C for 3 days, the supernatant was then harvested and its viral titre determined in the plaque assay.

2.2.2.6. Preparation of Large High-Titre Viral Working Stocks

These were prepared in suspension as this produces higher titres. $1x10^5$ cells/ml were seeded in a volume of 300 ml in a spinner flask and left stirring at 27°C until the cell density reached $5x10^5$ cells/ml (2-3 days). The cells were then infected with 0.1-0.2 pfu/ml and incubated for 7-9 days (aerated every 2-3 days). The supernatant was collected, stored at 4°C and its viral titre determined by plaque assay.

2.2.2.7. Large Scale Protein Production

Cells were seeded in suspension at a density of $5x10^5$ cells/ml in a volume of 300-500 ml. The cells were left until the density reached $1x10^6$ cells/ml and then infected with recombinant virus at an MOI (multiplicity of infection = no. of virus/no. of cells) of 5. The cells were left for 6 days (aerated every 2-3 days), the supernatant then collected and the

protein production assessed by Western Blotting. Prior to protein purification, the viral particles were removed by centrifugation at 18,000 rpm for 30 minutes.

2.2.3. Protein Detection

2.2.3.1. SDS Polyacrylamide Gel Electrophoresis

Gel mixtures were prepared according to the recipes described in the 'Materials' section. The 17.5% separating gel was poured first between two glass plates to within 2 cm from the top and overlayed with water-saturated isobutanol to ensure a smooth gel surface. Once the separating gel had set, the isobutanol was washed off with water. The 6.5% stacking gel layer was then poured on top of the separating layer and a well-forming comb inserted. 20 µl samples were mixed with 10 µl SDS/DTT loading buffer and heated at 100°C for 3 minutes. 5 µl of coloured molecular weight markers and 25-30 µl of the samples were then loaded into separate wells and the gels electrophoresed in SDS-containing running buffer in an Attoll minigel system at 50-100 mA constant current until the bromophenol blue band reached the bottom of the gel (for about 1 ½ hours). The gels were then either silver stained or Western blotted.

2.2.3.2. Silver Staining

Proteins in the gels were detected by silver staining. This involved soaking the gel for 10 minutes in 50% methanol/10% acetic acid, followed by 10 minutes in 10% ethanol/10% acetic acid and 10 minutes in 10% glutaraldehyde. The gels were then washed for 30 minutes in several changes of distilled water. Subsequently, they were soaked in 60 µM DTT for 10 minutes and 0.1% (w/v) silver nitrate for another 10 minutes. They were then briefly rinsed in water and the bands finally developed by rinsing the gels several times in 3% sodium carbonate/0.1% formaldehyde. When the bands had developed sufficiently, the gels were rinsed several times in water and then stored in fresh H_2O.

2.2.3.3. Western Blotting

Proteins to be detected by Western Blotting were transferred onto a PVDF membrane in a semi-dry blotting cassette as follows. Filter papers and membrane were cut to the same size as the gel. 6 pieces of filter paper were soaked in Anode Buffer 1 and placed at the bottom of the apparatus, next to the anode. On top of those were stacked three pieces of filter paper

soaked in Anode Buffer 2. The membrane was then soaked in methanol and placed on top of the anode stack. The gel was removed from the glass plates, briefly rinsed with water, the stacking gel removed and the separating gel placed directly on top of the membrane, followed by 9 pieces of filter paper soaked in Cathode Buffer. The apparatus was finally assembled by placing the Cathode on top of the stack and the proteins transferred for 1 hour at 50 mA constant current. After the transfer, nonspecific antibody binding sites in the membrane were blocked in several changes of BLOTTO (5% w/v milk powder/0.1% NP-40 in PBS) for 1 hour with shaking. After that, it was incubated with the primary antibody (diluted 1 in 2000 in BLOTTO) for 1 hour at room temperature, and then blocked again in several changes of BLOTTO for 1 hour, before being incubated with the secondary antibody (HRP-linked; diluted 1 in 2000 in BLOTTO) for 1 hour, again all with shaking. The membrane was then washed for 10 minutes in BLOTTO and subsequently for 1 hour in PBS/0.1% NP-40. The HRP enzyme reaction was initiated by adding ECL Western Blotting reagents for 1 minute and the bands detected by exposing the membrane to x ray film.

2.2.3.4. ELISA

Low concentrations of MIP-1α (picograms) were detected in a murine MIP-1α sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems), following the manufacturer's instructions. Briefly, samples had to be diluted (in chromatography buffer) to below 10 ng/ml to allow accurate readings. 50 µl of the assay diluent were then added to individual wells of a 96-well plate coated with an anti-murine MIP-1α antibody, followed by 50 µl of the diluted samples, and the plates then incubated at room temperature for 2 hours. At the end of the incubation period, the wells were washed 5 times with wash buffer, before adding 100 µl of an anti-murine MIP-1α antibody-HRP conjugate. The plates were again incubated for 2 hours at room temperature. Excess conjugate was then removed by another 5 washes with wash buffer and the enzyme substrate added to each well in a 100 µl volume. The colour reaction was then allowed to proceed for 30 minutes before it was terminated by the addition of 100 µl of stop solution per well. The optical density of each well was then determined at 450 nm. ELISAs were repeated with different dilutions of the samples in order to confirm the consistency of the results.

2.2.4. Chromatography

All chromatography was carried out using an FPLC system (Pharmacia Biotech, St. Albans, UK).

2.2.4.1. Heparin Affinity Chromatography - Analytical

The heparin binding affinity of MIP-1α at various concentrations, of MIP-1α mutants and of cross-linked MIP-1α was determined by using a 1 ml HiTrap Heparin Column which was run at a flow rate of 1 ml/min. Elution of MIP-1α was achieved by developing a continuous gradient either between 0.1 M NaCl/0.02 M Tris pH 7.6 and 2 M NaCl/0.02 M Tris pH 7.6 ('salt elution') or from 0 to 5 mg/ml soluble heparin in 0.1 M NaCl/0.02 M Tris pH 7.6 ('heparin elution'). The heparin affinity of various concentrations of MIP-1α and of cross-linked MIP-1α was also determined on a HR 5/5 (1 ml) column packed with Heparin-Agarose in which the heparin chains were end-coupled to the agarose beads by reductive amination (Sigma). The column was run at 0.5 ml/min and MIP-1α eluted with salt or with soluble heparin (as above). Protein elution was followed by either A_{280} measurement, Western Blotting or MIP-1α ELISA. Column runs were repeated, occasionally with slight variations in the elution conditions, in order to confirm the consistency of the results.

2.2.4.2. Heparin Affinity Chromatography - Preparative

For the purification of MIP-1α, RANTES and the different chimaeric proteins from large volumes of cell supernatant (300-500 ml), an XK50 column (Pharmacia Biotech) was packed with ~200 ml of Heparin Sepharose CL-6B and run at a flow rate of 5 ml/min. The samples were loaded with a peristaltic pump and elution of the proteins achieved by establishing step gradients or a continuous gradient between 0.1 M NaCl/0.02 M Tris pH 7.6 and 2 M NaCl/0.02 M Tris pH 7.6 (although individual steps were varied according to the requirements of the each recombinant protein as summarised in the 'Results' section). Elution of protein was followed by A_{280} and Western Blotting, and fractions containing recombinant protein were subjected to further purification.

2.2.4.3. Ion Exchange Chromatography (IEC)

Ion Exchange Chromatography was carried out using a 1 ml MonoQ column that was run at a flow rate of 1-2 ml/min. Protein elution was achieved by developing a continuous gradient between 0.1 M NaCl/0.02 Tris pH 7.6 and 2 M NaCl/0.02 M Tris pH 7.6 and monitored by A_{280} measurement and Western blotting. Positive fractions were again subjected to further purification.

2.2.4.4. Reversed Phase Chromatography (RPC)

Final purification was carried out on a 400 µl ProRPC HR 5/2 column which was run at 1 ml/min. The running buffers used were A: water/0.1% TFA and B: Acetonitrile/0.1% TFA, and protein eluted by a gradual increase in Acetonitrile concentration. Recombinant protein was detected by Western Blotting and the purity of the preparation confirmed by gel electrophoresis and silver staining. Sufficiently pure fractions were concentrated by freeze-drying and resuspending in PBS.

2.2.4.5. Gel Filtration Chromatography

The aggregation state of MIP-1α was analysed using an HR 10/30 column packed with Sephacryl S-200 and run at 0.5 ml/min with PBS/0.1% BSA. Different dilutions of MIP-1α were applied to the column in a volume of 0.5 ml and 1 ml fractions collected. MIP-1α was detected by analysing the fractions using a MIP-1α-specific ELISA, and the aggregates sized by comparison with molecular weight standards. For the analysis of the effects of calcium removal on the aggregation of MIP-1α, the running buffer also contained EDTA and EGTA (PBS/0.1% BSA/1 mM EDTA/2 mM EGTA). Sizing experiments were repeated on different columns in order to confirm the consistency of the results.

2.2.4.6. Desalt Column

The 5 ml HiTrap Desalt column contained Superfine Sephadex 25 and was run at a flow rate of 2 ml/min. It was employed during the experiments that looked into the effects of calcium on MIP-1α aggregation, and served to separate MIP-1α from acetic acid. MIP-1α was loaded in a volume of 100 µl and run in a buffer containing PBS/1 mM EDTA/2 mM EGTA. 250 µl fractions were collected, and MIP-1α was seen to elute over 2-3 fractions.

2.2.5. Disaggregation of MIP-1α

10 μg of MIP-1α was resuspended in 110 μl of 100 μM acetic acid in order to disaggregate it completely and to make the calcium ions accessible for removal. The acetic acid and the calcium ions were removed by desalting (see above).

2.2.6. Cross-linking of MIP-1α

The cross-linking experiments were based on a photoactivatable chemical cross-linking method described previously (Fancy and Kodadek, 1999) and were carried out in the presence of 0.125 mM Tris(2,2'-bipyridyl)Ruthenium(II)chloride ($Ru(bpy)_3Cl_2$) and 2.5 mM Ammonium Persulphate (APS). In preliminary experiments, different light sources, exposure times, dampening agents and concentrations of MIP-1α, its aggregation mutants and Glutathione-S-transferase (GST; as a positive control) were tested, and the reaction quenched by adding SDS/DTT gel loading buffer to the samples. The best results were obtained with a mercury lamp as a light source, an exposure time of 1 sec and 0.1 mM Histidine as a dampening agent. To test the heparin binding affinities of stable MIP-1α oligomers, 10 μg of murine MIP-1α was suspended in 100 μl PBS and cross-linked under a mercury lamp for 1 sec in the presence of 10 μl 1 mM histidine, 10 μl of 1.25 mM $Ru(bpy)_3Cl_s$ and 10 μl 25 mM APS. The sample was then immediately diluted by adding 970 μl PBS and directly run on a 1 ml HiTrap heparin affinity column (see above).

2.2.7. Calcium Flux Assay

HEK293 cells stably transfected with human CCR5 were grown in DMEM. Once they reached confluence, they were harvested by trypsinisation and resupended in 12 ml SR buffer per confluent 175 cm^2 tissue culture flask. To this, 25 μl of Fura-2-AM (at 4 mg/ml in DMSO) was added and the cells incubated at 37°C for 1 hour in the dark. The cells were then washed twice with SR buffer and resuspended in a final volume of 20 ml SR buffer. The fluorescence of the Fura-2-AM-loaded cells was determined in a fluorimeter at an excitation wavelength of 340 nm and an emission wavelength of 500 nm with measurements carried out every 0.1 sec under continuous stirring. Measurements were then carried out on 2 ml aliquots of cells that were allowed to equilibrate to 37°C before starting the time course. Once the time course was started, chemokine (at varying concentrations) was added in a volume of up to 100 μl after 50 sec and the fluorescence measured for ~200

sec in total. If desensitisation was assessed, different chemokines were added in 50 sec intervals during the same time course. Fluxes were repeated with different preparations of recombinant proteins and with different cell lines in order to confirm the consistency of the results.

2.2.8. CFU-A Assay

The murine CFU-A assay was basically carried out as described previously (Pragnell et al., 1988), with a few amendments as detailed below. In all of the CFU-A assays, the plates were set up in triplicates or quintuplicates for each point and the assays repeated several times in order to confirm the consistency of the results.

2.2.8.1. Preparation of Bone Marrow

Bone marrow was obtained from B6D2F1 mice (Harlan UK Ltd., Oxon, UK). The mice were killed by cervical dislocation and their femora removed and cleaned free from muscle. The ends of the bones were cut off, a needle (0.45x10 mm) attached to a syringe filled with PBS inserted at one end and the bone marrow flushed out. The cells were counted on a CASY 1 cell counter (Schärfe System) after first lysing the red cells with Zapoglobin. Each femur normally yielded about $3x10^6$ cells.

2.2.8.2. Two-layer CFU-A assay

This was the CFU-A assay employed for almost all of the experiments unless stated otherwise. 3 cm petri dishes were used and the appropriate chemokine added to the bottom of the plate in a volume of up to 200 µl. The under layer was then prepared by mixing equal volumes of 2x medium and 1.2% agarose (Agar Noble) and allowing the mixture to cool to 37°C before adding a tenth volume of L929 conditioned medium (source of M-CSF) and a tenth volume of AF1 conditioned medium (source of GM-CSF). In later experiments, the conditioned media were replaced by recombinant growth factors which were added at concentrations of 12 ng/ml for murine SCF, 6 ng/ml for human M-CSF and 0.2 ng/ml for murine GM-CSF. 1 ml of this underlayer mixture was then added to each dish and allowed to set at room temperature.

The upper layer was prepared by mixing equal volumes of 2x medium and 0.6% agarose and the mixture cooled to 37°C before adding bone marrow cells to a concentration of 5-

$10x10^3$ cells/ml of upper layer for fresh bone marrow or $5x10^4$ cells/ml of upper layer for defrosted bone marrow cells. 1 ml of this upper layer mixture was then added to each dish and the plates incubated at 37°C in a humid atmosphere of 5% O_2/10% CO_2 for 11 days to allow colonies to develop. These were stained overnight with 200 µl of INT solution (1 mg/ml in PBS) per plate, and colonies with a diameter of 2 mm and larger scored as CFU-A colonies.

2.2.8.3. One-layer CFU-A Assay

In order to reduce the amount of chemokine needed to detect inhibition, in some experiments a one-layer CFU-A assay was carried out in 24-well plates. Chemokine was added to the bottom of the wells in a volume of 6.25-25 µl and 250 µl/well added of a mixture containing 50% 2x medium, 50% 0.6% agarose, 3 ng mSCF, 1.5 ng hM-CSF and 0.05 ng mGM-CSF. The plates were incubated at 37°C in a humid atmosphere of 5% O_2/10% CO_2 for 11 days and the colonies stained and scored as above.

2.2.8.4. CFU-A Assay with Methylcellulose

The upper layer of a CFU-A assay was replaced with methylcellulose in order to be able to pick individual CFU-A colonies for cytospins and for counting the number of cells per colony. CFU-A assays were again set up in 3 cm petri dishes and underlayers prepared as described above. For the upper layer, methylcellulose was defrosted in a 37°C waterbath, mixed with enough fresh bone marrow cells to give a concentration of $1x10^4$ cells/dish and then 1 ml of the cell-containing methylcellulose plated on top of the underlayer in each dish. The plates were again incubated at 37°C in a humid atmosphere of 5% O_2/10% CO_2 for 11 days.

5-10 colonies per plate were then picked under a dissecting microscope, pooled in 500 µl ice-cold PBS and washed twice with 1 ml ice-cold PBS. The cells were then resuspended in PBS and counted in a Schärfe System CASY 1 cell counter. For cytospins, colonies were picked and washed as above and resuspended in 500 µl PBS. This suspension was cytospun at 500 rpm for 5 min onto polysine slides. The slides were air-dried, fixed in 100% methanol for 10 min at room temperature and then air-dried again. The cells on the slides were stained in 10% GIEMSA for 10 min at room temperature, washed briefly with water and allowed to dry. They were mounted under a cover slip with a drop of glycerol, and the cell types determined by inspection under a light microscope.

Chapter 3: RESULTS - Aggregation of MIP-1α

3.1. Introduction

Most chemokines have the ability to self-associate and form oligomers of varying sizes (see Introduction). While the majority of these cannot aggregate past the dimeric or tetrameric state, MIP-1α, MIP-1β and RANTES have been demonstrated to form higher order aggregates under certain conditions (Lodi et al., 1994; Patel et al., 1993; Skelton et al., 1995). A number of factors have been found to influence the aggregation process of chemokines and include protein concentration (Graham et al., 1994), salt concentration (Graham et al., 1992; Laurence et al., 1998), pH and hydrophobic environments (Graham et al., 1992; Patel et al., 1993). It has also been shown that aggregation is reversible and dynamic as it can be decreased or increased by varying the conditions listed above (Graham et al., 1994). It was determined that the formation of dimers and tetramers of MIP-1α is dominated by hydrophobic interactions, whereas aggregation past the tetrameric state is controlled by electrostatic interactions (Patel et al., 1993). Indeed, aggregation mutants of RANTES and MIP-1α were generated by mutating acidic residues (Czaplewski et al., 1999; Hunter et al., 1995, Graham, 1994 #218). In the case of MIP-1α, it was even possible to isolate different aggregation states by progressively neutralising acidic residues in the C terminal α helix. Thus, a mutant in which the glutamate residue at position 66 was neutralised (E66Q) could not aggregate past the tetrameric state, while mutants in which either two (E66Q + D64N) or three (E66Q + D64N + E60Q) acidic residues had been neutralised could not aggregate past the dimeric or monomeric state, respectively (Fig. 3.1). The effect of the latter two mutants seems to contradict the discovery that the forces stabilising the dimeric and tetrameric structures are exclusively of a hydrophobic nature. However, the alteration in the overall charge distribution of MIP-1α upon the neutralisation of two or three acidic residues may possibly interfere with self-association.

Figure 3.1: Sequence alignment of murine MIP-1α and human RANTES

Above are shown the primary amino acid sequences of murine MIP-1α and human RANTES. Basic residues are coloured in red, while acidic residues are coloured in blue. The numbers above the MIP-1α sequence highlight the residues that have been implicated in heparin binding (17, 44, 45, 47) and in aggregation (60, 64, 66).

A number of functions have been suggested for chemokine aggregation, including increased resistance to proteolysis (Paolini et al., 1994), a mechanism for limiting the active amount of circulating chemokine in the blood (Skelton et al., 1995) and the activation of specific signalling pathways at high chemokine concentration (Czaplewski et al., 1999). It was therefore decided to carry out a number of experiments that would further characterise the conditions that influence the aggregation of murine MIP-1α (as described in the following sections).

3.2. Effect of Dilution on MIP-1α Aggregation

Since the aggregation of human MIP-1α has previously been demonstrated to be influenced by its concentration (Graham et al., 1994), investigations were made into whether the self-association of murine MIP-1α is regulated in a similar way. The following dilutions of a 10 μg/ml stock solution (referred to as "neat" in later sections) of murine MIP-1α were prepared in PBS: 5 μg/ml ("1 in 2"), 1 μg/ml ("1 in 10"), 0.5 μg/ml ("1 in 20") and 0.1 μg/ml ("1 in 100"). The aggregation state of each dilution was analysed by gel filtration chromatography, using an HR 10/30 Sephacryl S200 column (fractionation range (M_r): 5,000-250,000) that was pre-equilibrated with PBS containing 1 mg/ml BSA. 0.5 ml of each dilution was applied to the column and the different aggregation states assessed by running the column at a flow rate of 0.5 ml/min in PBS containing 0.1% BSA, which was included in order to minimise non-specific interactions of MIP-1α with the gel matrix which had previously resulted in a poor resolution. 1 ml fractions were collected and analysed for the presence of MIP-1α using a murine MIP-1α-specific ELISA, as the presence of high BSA concentrations and low MIP-1α content in higher dilutions made it impossible to detect MIP-1α elution by absorbance measurements at 280 nm or Western Blotting. Fractions had to be substantially diluted in running buffer (500 fold for "1 in 2", 100 fold for "1 in 10", 50 fold for "1 in 20" and 10 fold for "1 in 100") in order to bring them into the detection range of the ELISA. Fig. 3.2 shows the results of the ELISA as measured at 450 nm. Peaks were assigned to the different aggregation states (MIP-1α monomer-8 kD, dimer-16 kD, tetramer-32 kD, octamer-64kD, dodecamer-96 kD etc.) in comparison to a linear standard curve that had been derived from the elution points of a range of molecular weight standards which are indicated at the top of the figure. It is apparent from Fig. 3.2 that the isolation of different MIP-1α aggregation states can be achieved by diluting the protein to varying degrees, and at concentrations below 0.5 μg/ml, murine MIP-1α is almost entirely monomeric.

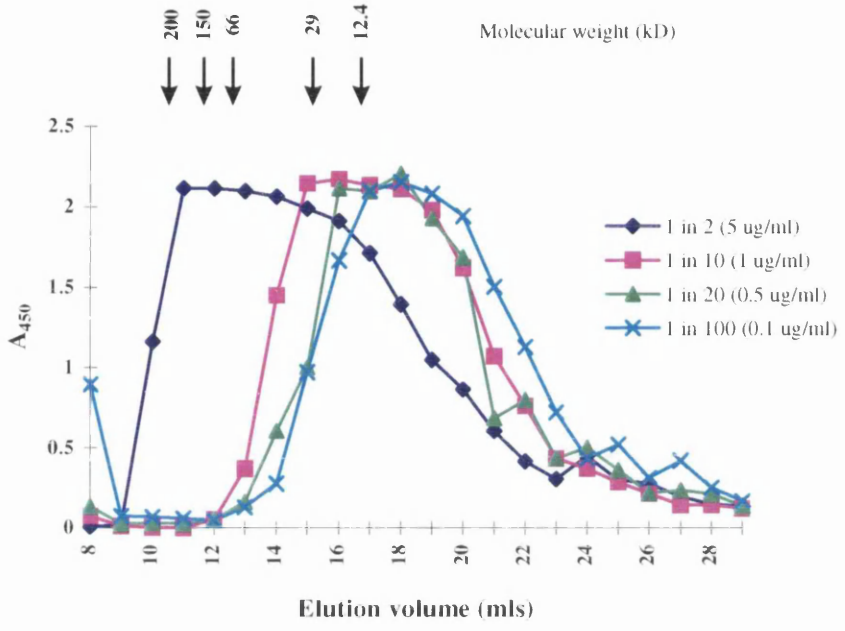


Figure 3.2: Effect of dilution on the aggregation of MIP-1α

Different dilutions (see legend) of murine MIP-1α were prepared in PBS and the aggregation state of MIP-1α within these preparations analysed by applying 0.5 ml of each dilution to a HR 10/30 Sephacryl S-200 gel filtration column which was run at a flow rate of 0.5 ml/min in PBS/0.1% BSA. 1 ml fractions were collected and analysed for their MIP-1α content in a murine MIP-1α-specific ELISA (A_{450}). Peaks were assigned to specific aggregation states by comparison to the elution profiles of a range of molecular weight standards (see top of the figure in kD).

This is a slightly higher value than the one previously reported for human MIP-1α, which disaggregates into monomers at around 0.1 μg/ml and therefore displays a slightly higher tendency for self-association than murine MIP-1α. These differences in aggregation are likely to be due to the charge differences between these two homologues, as human MIP-1α is more negatively charged. At concentrations of 1 μg/ml, murine MIP-1α is predominantly dimeric, while at 5 μg/ml it is mainly tetrameric with some evidence of higher order aggregates. At 10 μg/ml, murine MIP-1α exists in a range of higher order aggregates (data not shown).

However, the fact that the peaks are not entirely symmetrical and that there is a certain degree of overlap indicates that the starting populations may not be completely homogeneous populations of oligomers. The aggregation state of the samples may also have been slightly altered on their way through the column due to further dilution, since the protein is eluted over a volume of up to 10 times greater than the volume of the original sample. Therefore, it is not entirely clear how accurately the elution peaks represent the starting population. Nevertheless, Fig. 3.2 shows that the different aggregation states of murine MIP-1α can be isolated by making dilutions of defined concentrations, with 5 μg/ml providing a preparation of mainly tetramers, 1 μg/ml mainly dimers and 0.5 μg/ml or below mainly monomers. Above 5 μg/ml, higher order aggregates of murine MIP-1α can be obtained. Thus, like in the case of its human counterpart, the aggregation of murine MIP-1α is controlled by its concentration, with slightly weaker forces stabilising the murine oligomers due to the charge differences between the two homologues.

3.3. Effect of Calcium on MIP-1α Aggregation

Further insights into possible ways of regulating the aggregation of murine MIP-1α were derived from observations made during its crystallisation. As described in the Introduction, calcium ions were found in association with the MIP-1α tetramer in the crystal structure (see Fig. 3.3). One of these two ions is coordinated by the two backbone carbonyl groups on either side of Cys34 between two different tetramers. Its presence results in the loop being considerably more ordered in the tetramer and in a slightly different dimer. The second calcium ion is coordinated by Asp64 and Leu67 which causes the remaining residues (after Gln66) to adopt a nonhelical conformation. The interaction of the calcium ions with the tetramer was believed to be an interaction relevant for MIP-1α aggregation for the following reasons; (1) the presence of calcium was necessary for the crystallisation

of the MIP-1α tetramer (not the dimer or monomer), (2) it could not be replaced by any other ions, and (3) the position of the calcium ions was almost identical in the two different tetramers that were observed during the crystallisation of murine MIP-1α. The role of calcium ions in chemokine aggregation has so far not been investigated and would therefore represent a novel observation in this field. It may exemplify a novel mechanism by which the aggregation of chemokines is controlled by fluctuations in the local concentration of calcium ions. The self-aggregation of other proteins has, however, been shown to be influenced by the presence of calcium. For example, the self-aggregation of the surfactant protein SP-A is induced at 37°C and physiological ionic strength in the presence of trace amounts of calcium (Ruano et al., 2000). In order to determine whether calcium ions do indeed mediate oligomerisation of MIP-1α by an as yet undescribed mechanism, the effect of calcium removal on aggregation was tested.

The calcium ions were first made accessible by suspending 10 μg of murine MIP-1α in 110 μl of 100 μM acetic acid. It has previously been shown that a complete disaggregation of MIP-1α can be achieved through acidification with at least 10 μM acetic acid (Graham et al., 1992), and thus it can be assumed that MIP-1α, even at a concentration of as high as 0.1 mg/ml, will be completely disaggregated in the presence of 100 μM acetic acid. Disaggregated MIP-1α was then applied to a 5 ml Sephadex 25 Superfine Desalt column and run at a flow rate of 2 ml/min in PBS containing 1 mM EDTA and 2 mM EGTA. The desalting process will remove the acetic acid, and as EDTA and EGTA are both chelators of divalent cations with high affinities for Ca^{2+} (association constants at pH7 for EDTA is $\log_{10}K_a$=7.27 and for EGTA=6.68), they will effectively sequester any calcium ions in either MIP-1α or PBS. EDTA and EGTA, however, also complex other divalent cations, such as Mg^{2+}, albeit with lower affinities. The concentrations of EDTA and EGTA that were employed in this context are commonly used for the removal of divalent cations (James et al., 2000). Even if all of the MIP-1α molecules present in the Sephadex G25 eluate were in a tetrameric state or higher with 2 calcium ions associated with every tetramer, EDTA and EGTA would still be present in a molar excess of at least 100 and 200 fold, respectively, which should result in a complete removal of calcium ions. Although crystallisation studies have suggested that no other ions are involved, the presence of both EDTA and EGTA also ensured the removal of other divalent cations which may replace calcium as a mediator of aggregation.

Figure 3.3: Position of the calcium ions in the MIP-1α tetramer

Ribbon diagram of the MIP-1α tetramer with the position of the calcium ions shown in red. The one on the left is coordinated by the two backbone carbonyl groups on either side of Cys34, while the one on the right is coordinated by Asp64 and Leu67.

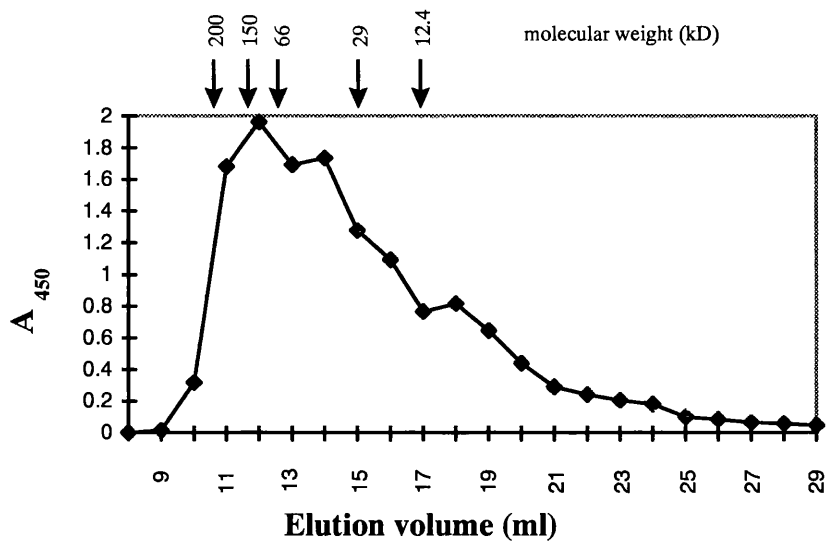


Figure 3.4: Effect of Calcium Removal on MIP-1α aggregation

0.7 ml of murine MIP-1α (~13 mg/ml) was run on an HR 10/30 Sephacryl S200 sizing column in the presence of 1 mM EDTA, 2 mM EGTA and 0.1% BSA (in PBS) at a flow rate of 0.5 ml/min. 1 ml fractions were collected and analysed for their MIP-1α content using a murine MIP-1α-specific ELISA (A_{450}). Sizes were determined using a range of molecular weight standards (see top of figure).

The protein was eluted from the desalt column in a volume of 750 µl, 0.7 ml of which was loaded onto a Sephacryl S-200 sizing column, pre-equilibrated with PBS again containing 1 mM EDTA and 2 mM EGTA (to maintain a calcium-free environment) and 0.1% BSA, which was included in order to minimise non-specific interactions of MIP-1α with the gel matrix, and the column run at a flow rate of 0.5 ml/min. 1 ml fractions of the eluate were collected and analysed for the presence of MIP-1α using an ELISA as above. As shown in Fig. 3.4, by comparison to the elution profiles of molecular weight standards, it was determined that the cation-depleted MIP-1α was present in a range of aggregation states, from monomers up to 16mers (128 kD) and higher, with the bulk of the protein eluting as oligomers of more than four monomeric units. Since the concentration of MIP-1α that was loaded onto the sizing column was between 10-13 µg/ml, the elution profile is as expected for this protein which is largely aggregated at that concentration. It compares with the elution profiles obtained previously for murine MIP-1α at 10 µg/ml (data not shown) which suggests that the presence of calcium (or possibly other divalent cations) is not required for the formation of higher order aggregates. These results also demonstrate that aggregation and disaggregation are reversible processes since completely dissociated MIP-1α (in the presence of 100 µM acetic acid) will re-aggregated upon removal of the acid.

3.4. Summary

Above is given an account of what is currently known about chemokine aggregation, in particular the aggregation of MIP-1α. It was previously known that the ionic and hydrophobic strength and the pH of the buffer as well as a number of negative (Graham et al., 1994) and positive (Graham et al., 1996) charges in the molecule, some of which form important salt bridges in the oligomers, have a profound effect on the ability of MIP-1α to form self-aggregates. In addition, serial dilutions of human MIP-1α not only demonstrated the effect of concentration on aggregation, but also made it possible to isolate differentially aggregated human MIP-1α (Graham et al., 1994). The above findings show that the same is possible for murine MIP-1α, where different dilutions of this protein and their subsequent analysis by gel filtration chromatography demonstrated that at 5 µg/ml, murine MIP-1α is predominantly in a tetrameric state which disaggregates further to dimers at 1 µg/ml, while at concentrations below 0.5 µg/ml, the protein is almost entirely monomeric. Since human MIP-1α needs further dilution to below 0.1 µg/ml in order to be completely disaggregated, it seems that forces stabilising the aggregates in human MIP-1α are slightly stronger than in murine MIP-1α. The above findings also show that divalent cations, in particular

calcium ions, are not essential for the aggregation of murine MIP-1α, as their removal by the chelators EDTA and EGTA has no effect on the ability of MIP-1α to form higher order aggregates as expected from the concentrations of murine MIP-1α used in this experiment. Finer dissociation constant measurements, however, are required in order to demonstrate whether these ions have any influence on the aggregation process. The function of the two calcium ions associated with the murine MIP-1α tetramer therefore remains unknown and may just represent a phenomenon caused by the conditions under which the tetramer was crystallised. The fact that the ions could not be removed without disrupting the crystal indicates that their presence may be required for the crystallisation of the tetramer, however not for its formation under physiological conditions.

The physiological relevance of chemokine aggregation *in vivo* is not entirely clear (see Introduction), especially since the dissociation constants (K_d) for the oligomers lie in the μM range while the K_d's for binding of chemokines to their receptors lie in the nM range. However, local concentrations of chemokines during or following secretion may be high enough to induce a certain amount of aggregation. It has also been suggested that aggregation of chemokines may be relevant for their interaction with proteoglycans (Hoogewerf et al., 1997) as will be analysed for murine MIP-1α in the following chapter.

Chapter 4. RESULTS - Effect of Aggregation on the Heparin Binding Affinity of MIP-1α

4.1. Introduction

Chemokines bind to heparin with varying affinities, ranging from a relatively strong interaction found between RANTES and a heparin matrix, needing salt concentrations of 0.9 M NaCl to be disrupted, to relatively weak interactions as are characteristic for MIP-1α - heparin binding which can be disrupted by 0.39 M NaCl (Kuschert et al., 1999). Heparin binding sites have been defined in a number of chemokines and are characterised by clusters of basic residues in the proteins that are thought to interact with the negatively charged sulphate groups of the glycosaminoglycan chains. In most cases, the number of positive charges and therefore the extent of the heparin binding surface have been shown to positively correlate with the strength of the chemokine - heparin interaction. Furthermore, in some cases, such as PF4 (Mikhailov et al., 1999; Stringer and Gallagher, 1997) and IL-8 (Spillmann et al., 1998), positive cooperativity was observed between the binding sites on different monomers which resulted in a higher affinity for heparin in the oligomers as the heparin binding area was increased. For example, in PF4, individual binding sites come together in the tetramer to form a ring of positive charges around the molecule that runs perpendicular to the α helices. It has even been suggested that the interaction between chemokines and glycosaminoglycans can mediate the oligomerisation of these proteins (Hoogewerf et al., 1997). The following sections describe experiments that were carried out in order to establish whether the aggregation of murine MIP-1α is also accompanied by an increase in heparin binding affinity, as this may represent another mechanism by which MIP-1α aggregation is controlled and may also be relevant for the way MIP-1α is presented to its target cells in the context of the bone marrow microenvironment.

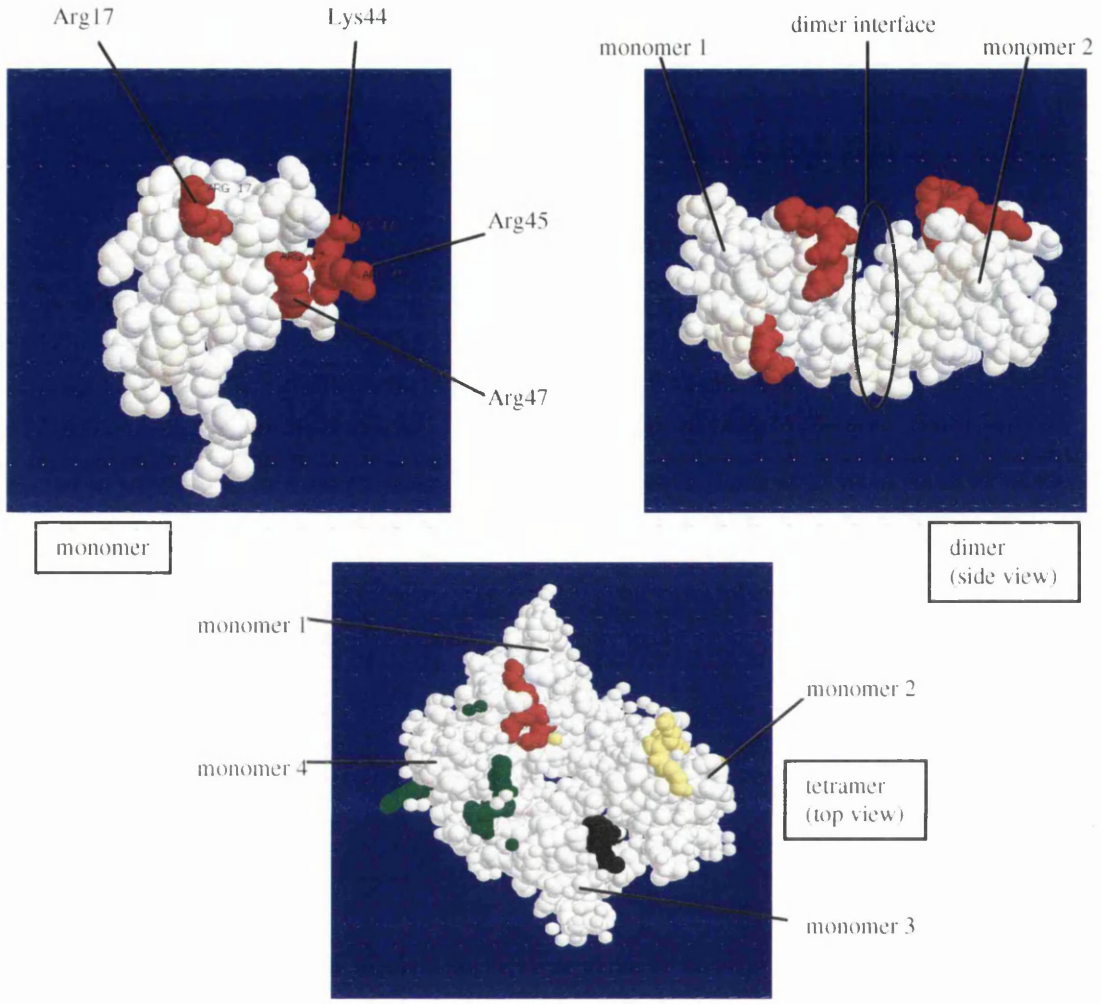


Figure 4.1: Position of the Heparin Binding Sites

Above are shown space-fill models of the murine MIP-1α monomer, dimer and tetramer. The residues implicated in heparin binding (Arg17, Lys44, Arg45, Arg47) are coloured in order to demonstrate their relative positions in the molecules. The heparin binding sites in the individual monomeric units in the tetramer are represented in different colours.

4.2. The Heparin Binding Site in murine MIP-1α

To see whether a similar cooperativity of individual binding sites takes place in MIP-1α upon self-association, the orientation of these sites relative to each other was first of all examined in the crystal structure of the monomer, dimer and tetramer. As can be seen in Fig. 4.1, the four residues that have been implicated in the interaction of MIP-1α with heparin, Lys44, Arg45, Arg47 and, to a lesser extent, Arg17 (Graham et al., 1996; Koopmann and Krangel, 1997), form a positive cluster at one end of the monomeric molecule. Although these sites are slightly twisted away from each other in the dimer, a glycosaminoglycan chain may still be accommodated within the cleft between the two monomers, simultaneously interacting with the two different binding sites. In the tetramer, the binding sites form a ring on one face of the molecule. A glycosaminoglycan chain that runs across this circle (top left to bottom right in Fig. 4.1) may be able to make extensive contacts with more than one binding site in the tetramer. The following experiments were therefore intended to formally check whether there is a relationship between aggregation and heparin binding.

4.3. Heparin Binding Affinities of mMIP-1α Aggregation Mutants

In a first approach to determining whether aggregation has any impact on heparin binding affinity, three aggregation mutants (see Introduction and previous section) of MIP-1α were tested for their binding to a 1 ml HiTrap heparin affinity column. These three differentially aggregating mutants had been previously derived by the sequential neutralisation of negative charges in the C terminus of murine MIP-1α (Graham et al., 1994). A mutant that could not aggregate past the tetrameric state (PM1) was obtained by the neutralisation of one acidic residue, E66Q, while the neutralisation of additional negative charges, E66Q + D64N and E66Q + D64N + E60Q, generated mutants that could not aggregate past the dimeric (PM2) and monomeric state (PM3), respectively. 500 µl of each mutant at a concentration of 0.1 mg/ml in PBS, a concentration at which they should have reached their maximal aggregation (see Fig. 3.2), was applied to the heparin column that had been washed with 0.1 M NaCl/0.02 M Tris (pH 7.6) and a subsequent step to 2 M NaCl/0.02 M Tris (pH 7.6) and then, prior to the run, been equilibrated with the starting buffer (0.1 M NaCl/0.02 M Tris pH 7.6). The proteins were eluted using a flow rate of 1 ml/min and by developing a continuous gradient from 0.1 M NaCl to 0.5 M NaCl with a subsequent step

to 2 M NaCl. This particular continuous gradient from 0.1-0.5 M NaCl was chosen, as it was known that wild type MIP-1α can be dissociated from a heparin matrix using 0.39 M NaCl (Kuschert et al., 1999). The subsequent step to 2 M NaCl ensured that any protein still attached to the matrix would be eluted. The elution of the mutants was followed by absorbance measurements at 280 nm, as the protein concentrations were sufficiently high to be detected by this method and as there was no BSA included in the running buffer that would otherwise complicate the A_{280} readings. The elution profiles are shown in Fig. 4.2 and demonstrate that the different mutants do indeed bind heparin with differing affinities. All three of them eluted completely over the continuous gradient (between 0.1-0.5 M NaCl) with no further elution detected following the step to 2 M NaCl. The symmetrical nature of the peaks indicates that the preparations applied to the column are indeed homogeneous populations of aggregates that have not dissociated any further. Interestingly, and contrary to expectations, the tetramer (PM1) displayed the lowest affinity for heparin, eluting at 0.34 M NaCl, followed by the dimer (PM2) at 0.4 M NaCl and the monomer at 0.45 M NaCl with the strongest binding. It is unlikely that these differences in elution represent molecular sizing effects as the matrix chosen for affinity chromatography is of an inert and very porous nature and will not resolve proteins that are greater than 5kD.

Therefore, one possible interpretation of these results is that, in contrast to PF4, aggregation of MIP-1α interferes progressively with heparin binding, perhaps by partially restricting access to the binding sites. An alternative interpretation is provided by the fact that the interaction of chemokines with proteoglycans is mainly of an electrostatic nature, with positive residues in the proteins interacting with the negatively charged sulphate groups of the glycosaminoglycan chains. Thus, the neutralisation of repelling negative charges may alter the overall charge of MIP-1α in a way that favours the interaction with heparin which would explain the higher affinity of the monomeric mutant in which three negative charges have been neutralised. This latter possibility may also explain why MIP-1α, which has a net negative charge unlike other chemokines, such as RANTES, which are basic proteins (see Fig. 3.1), is a weak heparin-binding protein. This would also mean that clusters of positive charges are not the only determinants for heparin binding affinities, but that the overall charge distribution of a protein is also important. In order to be able to discriminate between an aggregation or a charge effect on the binding affinities of the three mutants, further experiments were carried out, as described below.

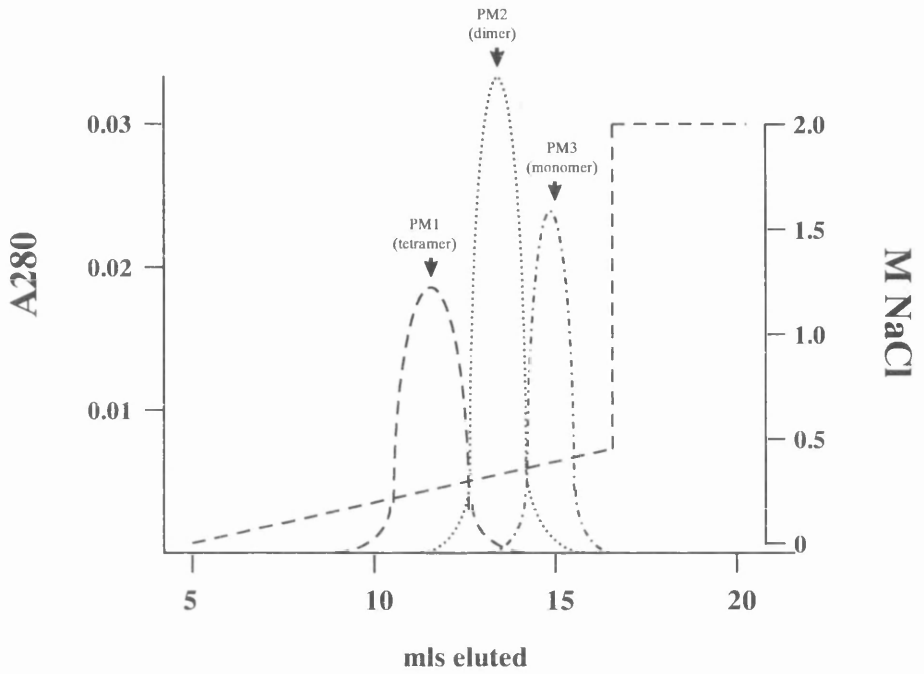


Figure 4.2: Binding of aggregation mutants to heparin

Differentially aggregated MIP-1α mutants were applied (0.5 ml at 0.1 mg/ml) to a HiTrap heparin column and eluted with a 0.1-0.5 M NaCl gradient at a flow rate of 1 ml/min. Protein elution was monitored by absorbance measurements at 280 nm.

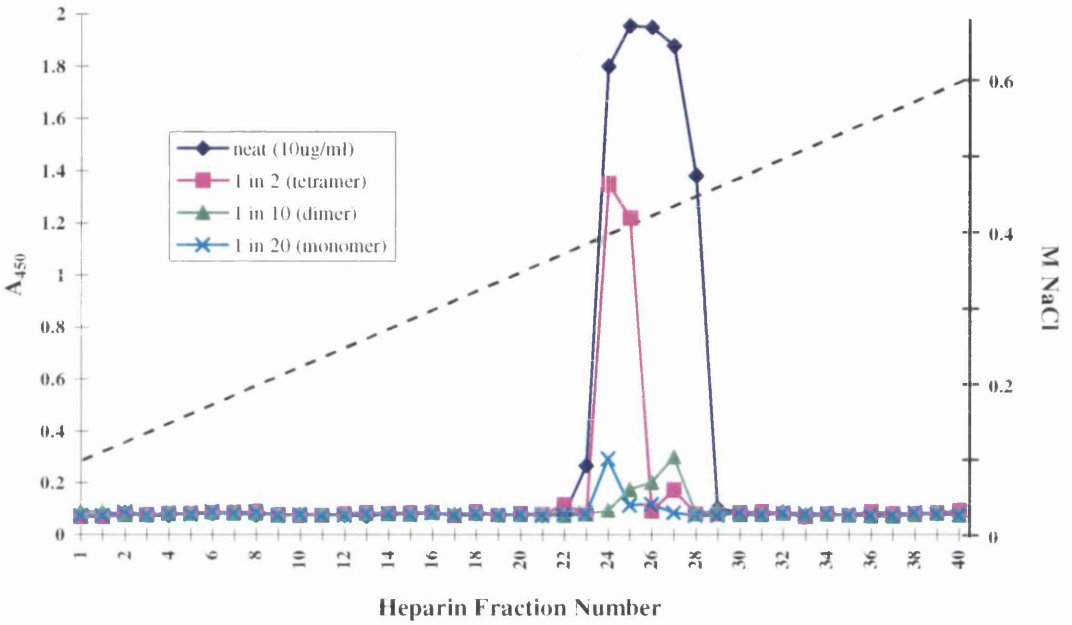


Figure 4.3: Effect of dilution on heparin binding of MIP-1α

Differentially aggregated MIP-1α was applied (0.5 ml) to a HiTrap heparin column and eluted using a 0.1-0.6 M NaCl gradient and a flow rate of 1 ml/min. 0.25 ml fractions were collected over the gradient and analysed for their MIP-1α content using a murine MIP-1α-specific ELISA. Note, the differences in peak sizes was caused through the necessity of extensively diluting the samples prior to their analysis in the ELISA.

4.4. Heparin Affinities of Differentially Aggregated MIP-1α

The first step in trying to resolve the above question involved finding an alternative method for obtaining differentially aggregated preparations of murine MIP-1α that circumvented the problem of altering the overall charge of the molecule. As shown in Fig. 3.2 in the previous chapter, progressively diluting MIP-1α produces preparations that contain predominantly monomers, dimers or tetramers as analysed on a Sephacryl S200 sizing column and subsequently in a murine MIP-1α ELISA. Thus, at concentrations of 5 μg/ml, MIP-1α is predominantly tetrameric with some evidence of higher order aggregates, whilst at 1 μg/ml it is dimeric and at concentrations of 500 ng/ml or less it is almost entirely monomeric. As explained in section 3.2., further dilution of the samples may occur on their way through the column and the elution profile of the eluate may not be a completely accurate representation of the original sample composition. Therefore, gel filtration fractions that were known to contain the different aggregation states (as illustrated in the ELISA measurements) were directly applied in a volume of 0.5 ml to a 1 ml HiTrap heparin affinity column in order to analyse the heparin binding affinities of the different oligomers. However, when the fractions collected from the heparin column were analysed in an ELISA, it became apparent that a further dilution of the samples had occurred which made it increasingly difficult to detect the protein in the ELISA and no clear picture of the elution profiles of the different oligomers could be obtained (data not shown).

To circumvent this problem, dilutions that had produced monomers, dimers, tetramers and higher order aggregates on the gel filtration column (i.e. 0.5 μg/ml, 1 μg/ml, 5 μg/ml and 10 μg/ml, respectively) were directly applied to the HiTrap heparin column. As mentioned above, dilution of the samples and possibly a further disaggregation may have occurred during their path through the sizing column, which would mean that the preparations before gel filtration may also contain oligomers of slightly higher molecular weights, however, a similar dilution effect also occurs on the heparin column, which makes the approach of taking the original dilution a valid one. The samples were applied to the heparin column in a volume of 500 μl and the protein eluted over a gradient from 0.1 M NaCl to 0.6 M NaCl with a subsequent step to 2 M NaCl. 250 μl fractions were collected over the gradient and diluted aliquots again analysed in an ELISA for their MIP-1α content, as the protein concentrations were too small to be detected by absorbance measurements. As shown in Fig. 4.3, no effect of aggregation was seen on the affinity of binding to heparin with all of the differentially aggregated dilutions eluting on average at a

concentration of 0.36 M NaCl which is similar to the elution point of PM1 (Fig. 4.2) and wild type MIP-1α (Kuschert et al., 1999). The different sizes of the protein peaks were brought about by the necessary and extensive dilutions of the fractions prior to the ELISA. It also appears from Fig. 4.3 that the elution peak of the dimer is shifted slightly with respect to the peaks of the monomer and tetramer. However, that shift is unlikely to be relevant, as all three peaks display an extensive overlap with each other and all coincide with the peak of the 10 μg/ml preparation. It is more likely due to a pipetting error that occurred during the dilution of the fractions prior to the ELISA which would also explain the lack of symmetry in the peaks.

The fact that all the different wild type MIP-1α aggregates tested bound to heparin with similar affinities suggests that it is indeed the differences in overall charge rather than the different aggregation status of the three mutants PM1, PM2 and PM3 that cause these proteins to elute at different times from the heparin column. However, one slight concern with the findings described above is the possibility that the salt concentrations used to elute the proteins off the column may in fact cause a partial disaggregation of the oligomers which has been observed in the past at NaCl concentrations of 1-2 M (Graham et al., 1992). Therefore, in order to verify the above observations, stable, covalently linked MIP-1α oligomers were generated by a novel chemical cross-linking method (Fancy and Kodadek, 1999) as described below.

4.5. Heparin Affinities of Cross-linked MIP-1α

Most commercially available cross-linkers are unsuitable for the cross-linking of MIP-1α due to the fact that their side chain specificity and the size of their linker arms requires the presence of specific residues at a defined distance from each other within the three-dimensional structure of the protein which are not available in MIP-1α. The method by Fancy and Kodadek (Fancy and Kodadek, 1999) circumvents these problems by using a completely different cross-linking mechanism. It relies on the absorption of visible light (452 nm) by the photoactivatable compound ruthenium(II) tris-bipyridyl cation ($Ru(II)bpy_3^{2+}$) which converts it into an excited state that allows it to donate an electron to an acceptor such as persulphate, thus resulting in the generation of $Ru(II)bpy_3^{3+}$ (a potent oxidant) and a sulphate radical. These two very reactive species have been proposed to induce the cross-linking of protein side chains by various mechanisms involving a number of radical intermediates and nucleophilic attacks. Thus, this process of oxidative coupling

results in the direct coupling of nearby residues without an intervening linker arm which makes this a very local, short distance effect that is unlikely to perturb the overall structure of the protein. There is some evidence to suggest that the amino acid side chains that can be cross-linked in this fashion include tyrosine, lysine, cysteine, tryptophan and possibly others as well (Kodadek, personal communication).

Due to limitations in the available equipment and because of the fact that the details of the cross-linking reaction are still largely unknown which makes it impossible to predict the efficiency with which a given protein is cross-linked by this method, the best conditions for the cross-linking of murine MIP-1α had first of all to be determined empirically. In a first trial, the cross-linking of murine MIP-1α was attempted using different light sources and irradiation times in order to establish the best conditions. To 16 µl of murine MIP-1α at 10 µg/ml in PBS was added 2 µl of 1.25 mM $Ru(bpy)_3Cl_2$ and 2 µl of 25 mM Ammonium Persulphate. Light was then shone for various time lengths directly into the top of the open Eppendorf screw cap tube. Two different light sources were tested, a mercury lamp with irradiation times of 1, 5 and 30 sec, and a weaker 2.5V-0.5A krypton lamp, with irradiation times of 5 sec, 30 sec and 5 min. The reactions were quenched immediately by adding 5 µl of SDS and DTT-containing SDS PAGE loading buffer. DTT is easily oxidised and therefore serves as a good quenching reagent. 5 µl of bromophenol blue/glycerol were then also added to each reaction and the samples analysed on a 17.5% SDS PAGE gel and subsequently visualised by Western Blotting using a polyclonal anti-murine MIP-1α antibody. As shown in Fig. 4.4, murine MIP-1α can indeed be cross-linked by this method, as evident from the presence of monomers, dimers, tetramers, octamers and even higher order aggregates. It seems to be a highly specific interaction since no trimers could be detected. Nevertheless, in subsequent experiments, Histidine, an electron-donating amino acid that has been shown to be able to "tone down" the degree of cross-linking and sort out tight contacts from weak or transient associations (Fancy and Kodadek, 1999), was included at 0.1 mM in the reaction in order to keep the amount of nonspecific cross-linking at a minimum level. The best cross-linking results were obtained using a mercury lamp as a light source and an irradiation time of approximately one second. Longer irradiation times resulted in a disappearance of the protein band on the Western Blot, as shown in Fig. 4.4, lanes 8 and 9. A possible explanation for this may be that a very high degree of cross-linking has altered the overall structure of the protein to such a degree that it was no longer recognised by the specific antibodies.

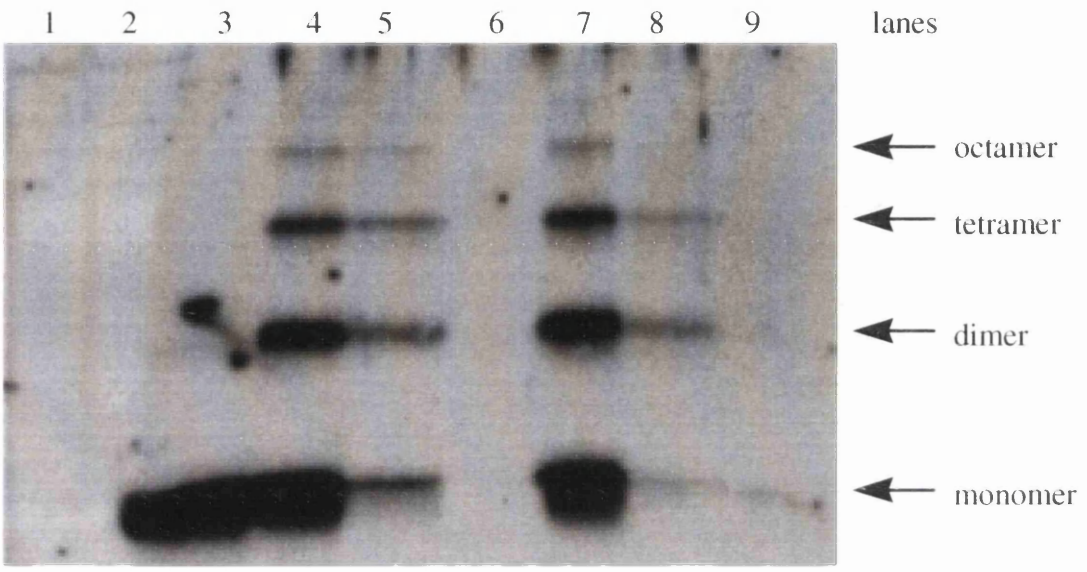


Figure 4.4: Chemical cross-linking of MIP-1α

lanes 1,2: molecular weight markers; **lane 3**:uncross-linked MIP-1α; **lanes 4-6**:MIP-1α cross-linked with a 2.5V-0.5A krypton lamp for 5 sec, 30 sec, 5 min; **lanes 7-9**:MIP-1α cross-linked with a mercury lamp for 1, 5, 30 sec

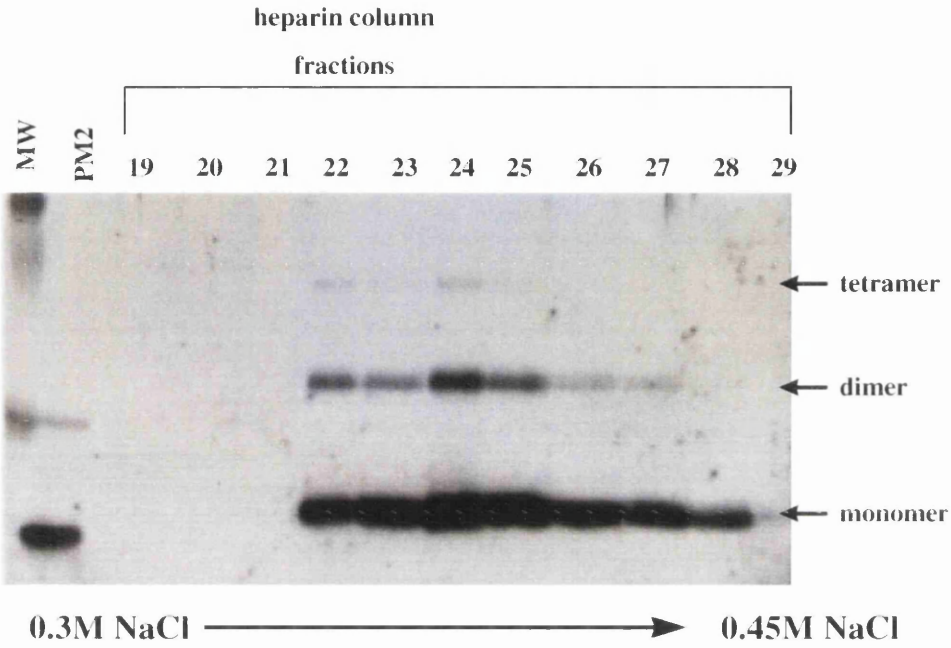


Figure 4.5: Binding of cross-linked MIP-1α to immobilised heparin

MIP-1α was cross-linked as described in the text, and the cross-linked material applied to a HiTrap heparin column. Protein was eluted by a salt gradient (0.1-0.6 M NaCl) and fractions analysed by Western Blotting using a MIP-1α-specific polyclonal antibody.

Once the optimal conditions had been established, this technique was used to cross-link wild type murine MIP-1α at a concentration of 100 μg/ml in a volume of 100 μl in PBS with 10 μl of 1.25 mM $Ru(bpy)_3Cl_2$, 10 μl of 1 mM Histidine and 10 μl of 25 mM Ammonium Persulphate. As the cross-linked material was then to be analysed for heparin binding, it was not possible to quench the reaction through the addition of DTT since that would have disrupted the disulphide bridges which may have an impact on the protein's tertiary structure. The reaction was therefore immediately diluted through the addition of 970 μl of PBS and 1 ml of the reaction instantly applied to a HiTrap heparin column (same chromatography method as above). The material was eluted with increasing salt concentrations from 0.1 M NaCl to 0.6 M NaCl and the elution profile analysed by SDS gel electrophoresis and Western Blotting of the 250 μl column fractions. Western Blotting was employed for the detection of the protein since the concentrations were high enough for detection and the different covalently-linked oligomers could be visualised by this method. Fig. 4.5 shows that aggregation again has no effect on the heparin binding affinity of MIP-1α, with the stable monomers, dimers and tetramers eluting at identical points from the column, peaking at fraction 24 which corresponds to a salt concentration of approximately 0.36 M NaCl which is again very similar to PM1 and wild type MIP-1α. It can thus also be assumed that the process of cross-linking MIP-1α did not cause any major structural disturbances, as the proteins are still able to bind to heparin with the affinity of the wild type protein.

4.6. Heparin Elution of Differentially Aggregated MIP-1α

Immobilised heparin can act as an ion exchange matrix as well as an affinity matrix. It is therefore possible that in the experiments described above the heparin chains, which are attached to the beads of the matrix at several points along their chains in the HiTrap column and may therefore not be free to interact in a more specific and physiologically relevant manner with MIP-1α, acted predominantly as ion exchangers, especially under those buffer conditions (using increasing ion concentrations for protein elution). One way of addressing this possibility is by using increasing concentrations of soluble heparin (a competing ligand) rather than salt to elute the protein from the column, thus giving a more accurate estimate of the heparin binding affinity. Thus, murine MIP-1α dilutions were prepared and applied to the HiTrap column as before (see section 4.4.), but eluted with a gradient of 0-5 mg/ml (sufficient for the elution of the majority of proteins) of soluble heparin in 0.1 M NaCl/0.02 M Tris pH 7.6, over which 250 μl fractions were collected.

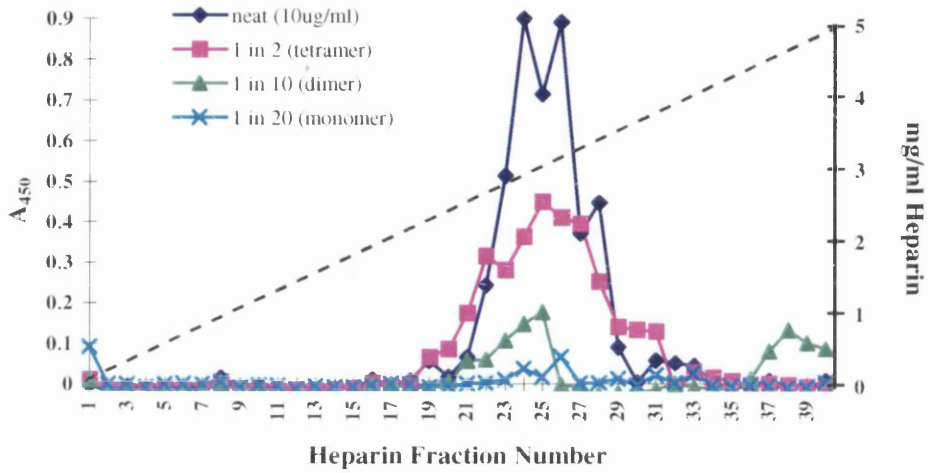


Figure 4.6: Binding of differentially aggregated MIP-1α to heparin

Differentially aggregated MIP-1α was loaded (0.5 ml) onto a HiTrap heparin column and eluted with increasing concentrations of soluble heparin (0-5 mg/ml) at a flow rate of 1 mg/ml. 0.25 ml fractions were collected over the gradient and analysed for their MIP-1α content using a MIP-1α-specific ELISA (A_{450}).

Analysing the fractions in an ELISA showed that again there was no difference in the avidity with which the different MIP-1α aggregates interacted with heparin (Fig. 4.6), as they all eluted at approximately 3 mg/ml soluble heparin. Some further protein elution seems to occur at 5 mg/ml heparin. However, that was not seen consistently, and repeating the experiment with a gradient of 0-10 mg/ml soluble again showed that the aggregation state has no impact on the heparin binding affinity (data not shown).

4.7. Interaction of murine MIP-1α with a Heparin Matrix Produced by Reductive Amination

Another method for testing the affinity of differentially aggregated MIP-1α for heparin in a way that more closely represents an affinity-based interaction was employed and involved the use of a matrix to which heparin chains had been attached by reductive amination. This is an end-coupling method that attaches heparin to the agarose matrix by its reducing end, leaving the remainder of the molecule unmodified and free to interact with its ligand in a more physiological manner, thus reducing the amount of non-specific binding (Funahashi et al., 1982). A 1 ml HR 5/5 column was packed with this type of heparin-agarose resin and equilibrated with 0.1 M NaCl/0.02 M Tris pH 7.6 at a flow rate of 0.5 ml/min. The experiments described above were repeated using this new matrix

Murine MIP-1α dilutions (10 μg/ml, 5 μg/ml, 1 μg/ml, 0.5 μg/ml) were prepared in PBS as above, 0.5 ml of each loaded onto the new column and eluted with a salt gradient (0.1-0.6 M NaCl) as before, but at a flow rate of 0.5 ml/min. 250 μl fractions were collected over the gradient and analysed for the MIP-1α content in an ELISA. The results of the ELISA, as shown in Fig. 4.7, demonstrate that again there was no difference in the heparin binding affinities of the different MIP-1α aggregates even on this matrix. They did, however, elute on average at a slightly later point, needing a salt concentration of approximately 0.4 M NaCl. The peaks were also somewhat broader, indicating a slightly different mode of interaction.

The behaviour of stable oligomers was also analysed on the new matrix. Cross-linked murine MIP-1α was therefore generated as above and also applied to and eluted from the new column as before. The Western Blot analysis of the fractions, as shown in Fig. 4.8, provides further evidence that the aggregation status of MIP-1α does not have any effect on heparin binding, with the monomer, dimer and tetramer eluting at identical points, at

approximately 0.36 M NaCl. The reason why the cross-linked material in Fig. 4.8 elutes at a slightly earlier point (identical to the elution points of the stable oligomers on the HiTrap column - Fig. 4.5) from the column than the native protein in Fig. 4.7 is not clear.

The observation that, even with a matrix that mimics more closely the interaction between MIP-1α and heparin *in vivo*, different aggregates of murine MIP-1α display identical binding affinities was also confirmed by the elution of different dilutions of murine MIP-1α with increasing concentrations (0-5 mg/ml) of soluble heparin (Fig. 4.9). However, as it had been observed for the salt elution of different MIP-1α dilutions (Fig. 4.7), binding to the matrix prepared by reductive amination was stronger than to the HiTrap column with broader elution profiles. In this case the shift in the elution peaks was even more pronounced with 4 mg/ml of soluble heparin, instead of 3 mg/ml (Fig. 4.6), needed for competing MIP-1α off the matrix-bound ligand which explains why elution had not been completed by the end of the run (at 5 mg/ml heparin). Thus, native murine MIP-1α seems to interact more strongly with this matrix as compared to the HiTrap matrix, and the actual point of elution differs with different ways of eluting the protein. However, these differences are conserved among the different aggregates, thus lending further support to the assumption that aggregation has no impact on the heparin binding affinity of murine MIP-1α.

4.8. Summary

Using several different approaches, it was demonstrated that MIP-1α's affinity for heparin does not change upon aggregation and that it rather seems to depend on the overall charge of the protein. Even the use of different heparin matrices (HiTrap and reductive amination-prepared), different methods of protein elution (salt and heparin) and different ways of producing differentially aggregated murine MIP-1α (dilution and cross-linking) still always produced results that all led to the same conclusion that, unlike in the PF4 tetramer, in the MIP-1α oligomers the individual heparin binding sites on the different monomeric units do not display positive cooperativity, but rather act as independent units, resulting in a heparin affinity that does not change upon oligomerisation. For that reason, it can be assumed that the different elution profiles of the three aggregation mutants, PM1, PM2, PM3, are not caused by an obstruction of the heparin binding site in the oligomers, but are related to changes in the overall charge of the proteins that were induced when the acidic residues were replaced by neutral ones. This demonstrates for the first time that the affinity of

chemokines for heparin is not only determined by the presence of clusters of positive charges that can interact with the sulphate groups of the glycosaminoglycan chain, but is also influenced by the overall charge of the protein, with the number of negative charges being negatively correlated with the strength of the interaction.

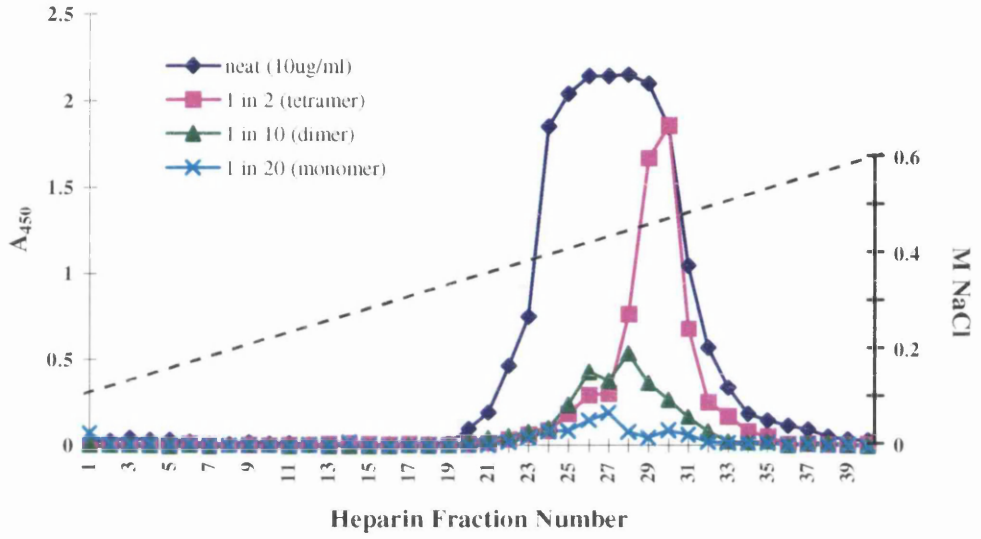


Figure 4.7: Binding of differentially aggregated MIP-1α to a heparin matrix prepared by reductive amination

0.5 ml samples of differentially aggregated murine MIP-1α (see figure legend) were loaded onto a heparin column (with a matrix prepared by reductive amination) and eluted using increasing salt concentrations (0.1-0.6 M NaCl) and a flow rate of 0.5 ml/min. 250 µl fractions were collected over the gradient and analysed in a murine MIP-1α-specific ELISA (A_{450}).

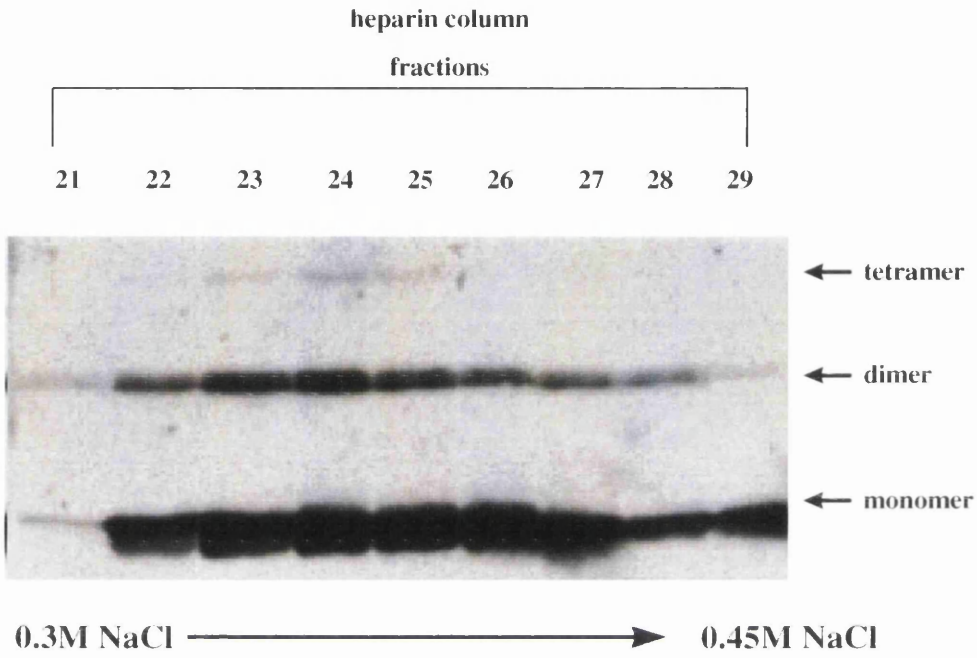


Figure 4.8: Binding of cross-linked MIP-1α to a heparin matrix prepared by reductive amination

MIP-1α was cross-linked as described in the text and the cross-linked material (1 ml) run on a heparin column at a flow rate of 0.5 ml/min. The protein was eluted by developing a gradient between 0.1 and 0.6 M NaCl. 250 µl fractions were collected over the gradient and analysed for the presence of MIP-1α by Western Blotting.

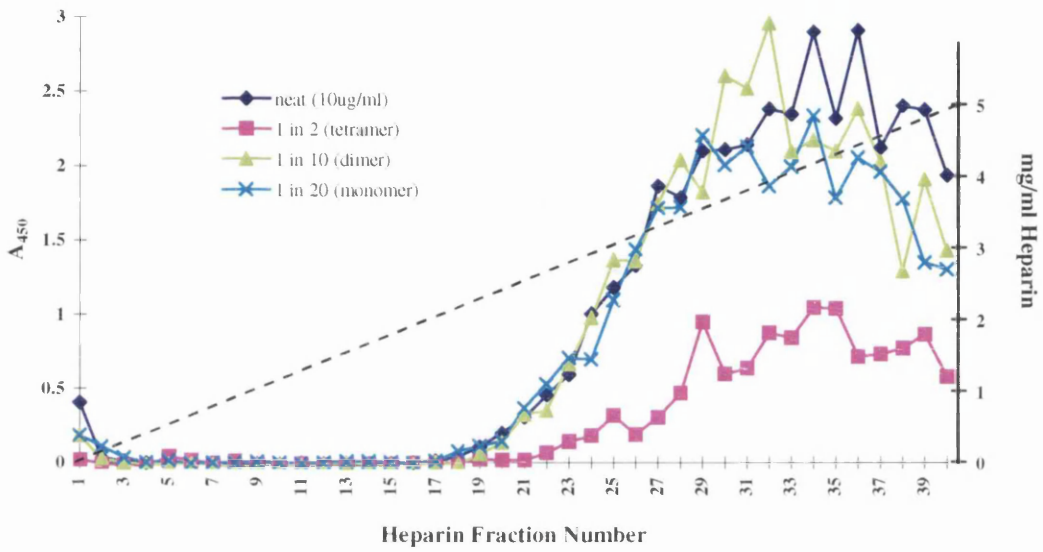


Figure 4.9: Heparin elution of differentially aggregated murine MIP-1α from a heparin matrix prepared by reductive amination

Differentially aggregated murine MIP-1α (see figure legend for concentrations in PBS) was loaded in 0.5 ml samples onto a heparin affinity column containing a matrix prepared by reductive amination. The protein was eluted at a flow rate of 0.5 ml/min with increasing concentrations of soluble heparin (0-5 mg/ml). 0.25 ml fractions were collected over the gradient and analysed for their MIP-1α content in an ELISA (A_{450}).

Chapter 5. RESULTS - The MIP-1α Inhibitory Receptor

5.1. Introduction

The last two chapters dealt with two properties of MIP-1α, its ability to aggregate and to interact with proteoglycans, which can potentially influence MIP-1α's inhibitory activity *in vivo*. However, the key players in this process, apart from MIP-1α itself, are the receptor that conveys MIP-1α's inhibitory signal as well as the downstream signalling events that follow receptor activation. As described in the Introduction, MIP-1α was the first chemokine to be shown to affect the proliferation of haemopoietic stem cells (Graham et al., 1990). Since then, it has been demonstrated to have even more wide-spread effects on primitive as well as more mature cells. The inhibitory function of MIP-1α is conveniently studied in the CFU-A assay which is an *in vitro* assay for the detection of transiently engrafting haemopoietic stem cells, thus termed CFU-A cells. This assay involves the plating of total murine bone marrow in the presence of the growth factors murine SCF, human M-CSF and murine GM-CSF which support the development of colonies from CFU-A cells that become visible as large colonies of 2 mm or more in diameter after 11 days of incubation. Very little is known about the molecular mechanism underlying MIP-1α-mediated CFU-A cell inhibition, and the receptor responsible for conveying this activity has not been studied. The following sections present data that add to the current knowledge of MIP-1α's mode of action and may prove to be instrumental in the identification of the inhibitory receptor. The knowledge of the receptor and signalling mechanisms involved in stem cell inhibition is not only important for the understanding of the regulation of stem cell proliferation and their possible clinical applications in bone marrow transplants and gene therapy, it may also shed some light on how these processes have been altered in diseases such as leukaemia, as stem cells from Chronic Myeloid Leukaemia patients have been shown to be refractory to the inhibitory effect of MIP-1α (Eaves et al., 1993; Holyoake et al., 1993).

5.2. Effects of MIP-1α and other chemokines on the proliferation of CFU-A cells

In order to study the possible involvement of any of the known chemokine receptors in stem cell inhibition, the following chemokines/ligands were included in CFU-A assays, by adding 200 µl of each at a concentration of 1 µg/ml in PBS to the bottom of the plates. This was intended to establish whether any of them, at the final concentration of 100 ng/ml, are capable of influencing the proliferation of these transiently engrafting haemopoietic stem cells.

CC chemokines: two of the mMIP-1α aggregation mutants (PM1 and PM2; see section 4.3.), hMIP-1αP (LD78β), hMIP-1β, hMCP-1, hMCP-2, hHCC-1, hHCC-2, hHCC-4, hLARC, hMPIF-1, hMPIF-2, mC10, hI-309, hDCCK-1, mEotaxin, mESkine, mMDC, hRANTES, mSLC, mTECK;

CXC chemokines: hIP-10, mSDF-1, hIL-8 (72 and 77 amino acid isoforms);

CX_3C chemokines: hFractalkine;

viral chemokine homologues: vMIP-II.

As can be seen in Fig. 5.1, only the addition of MIP-1α (the two murine aggregation mutants or the human homologue) results in a marked and reproducible inhibition of the proliferation of CFU-A cells, as reflected in the reduced number of large CFU-A colonies while none of the other chemokines display any effects. From Fig. 5.1 and data published previously (Graham et al., 1994), it is also apparent that mutations in MIP-1α that interfere with its ability to aggregate do not influence its inhibitory function. Interestingly, MIP-1β, which is highly related to MIP-1α, and DCCK-1, the sequence of which following the CC motif is highly homologous to the corresponding sequence in MIP-1α, do not, however, share its inhibitory effect on CFU-A cells. Similarly, chemokines that have previously been shown to inhibit the proliferation of certain haemopoietic stem cell subtypes, such as MPIF-1 and MPIF-2 (Patel et al., 1997), have, nevertheless, no effect on the proliferation of CFU-A cells.

In a separate experiment, ligands for all the currently known CC chemokine receptors were tested in a CFU-A assay in order to establish whether any of them showed stem cell inhibitory activity, thus implicating one of the known CCRs in stem cell inhibition. However, Table 5.1 shows that none of them displays any consistent activity that was comparable to MIP-1α, thus suggesting that a novel, currently uncharacterised, receptor is involved. It seems, therefore, that MIP-1α (human as well as murine) is unique among chemokines in its ability to inhibit CFU-A cell proliferation which suggests that these cells may carry a MIP-1α-specific receptor through which this effect is mediated. This also makes the CFU-A assay suitable for specifically studying MIP-1α-mediated inhibition since no other chemokines display any activity in this assay.

5.3. MIP-1α does not utilise CCR5 or D6 for Stem Cell Inhibition

Murine MIP-1α has been shown to bind to four murine receptors, to CCR5 and D6 with high affinity and to CCR1 and CCR3 with low affinity (Nibbs et al., 1997), however a number of observations suggest that none of these receptors mediate MIP-1α's inhibition of CFU-A cells. One of the first indications came from studies on a mutant of MIP-1α which has lost its ability to bind to heparin (Graham et al., 1996). The mutation that destroyed the heparin binding site, also completely abolished binding of this mutant to CCR1 and resulted in lower affinities for CCR3, CCR5 and D6. Yet this mutant was just as potent as wild type MIP-1α in the inhibition of CFU-A cells, suggesting that the inhibitory signal is not conveyed by CCR1. Furthermore, the fact that all four MIP-1α receptors also bind other ligands (see Table 1.1 and Table 5.1), none of which have any effect on CFU-A cell proliferation, further supports the suggestion that stem cell inhibition is mediated through a novel, possibly MIP-1α-specific receptor. For the same reason, none of the other known CC chemokine receptors are likely to be involved since none of the ligands for CCR1-CCR11 display CFU-A cell inhibition activity (see above and Table 5.1).

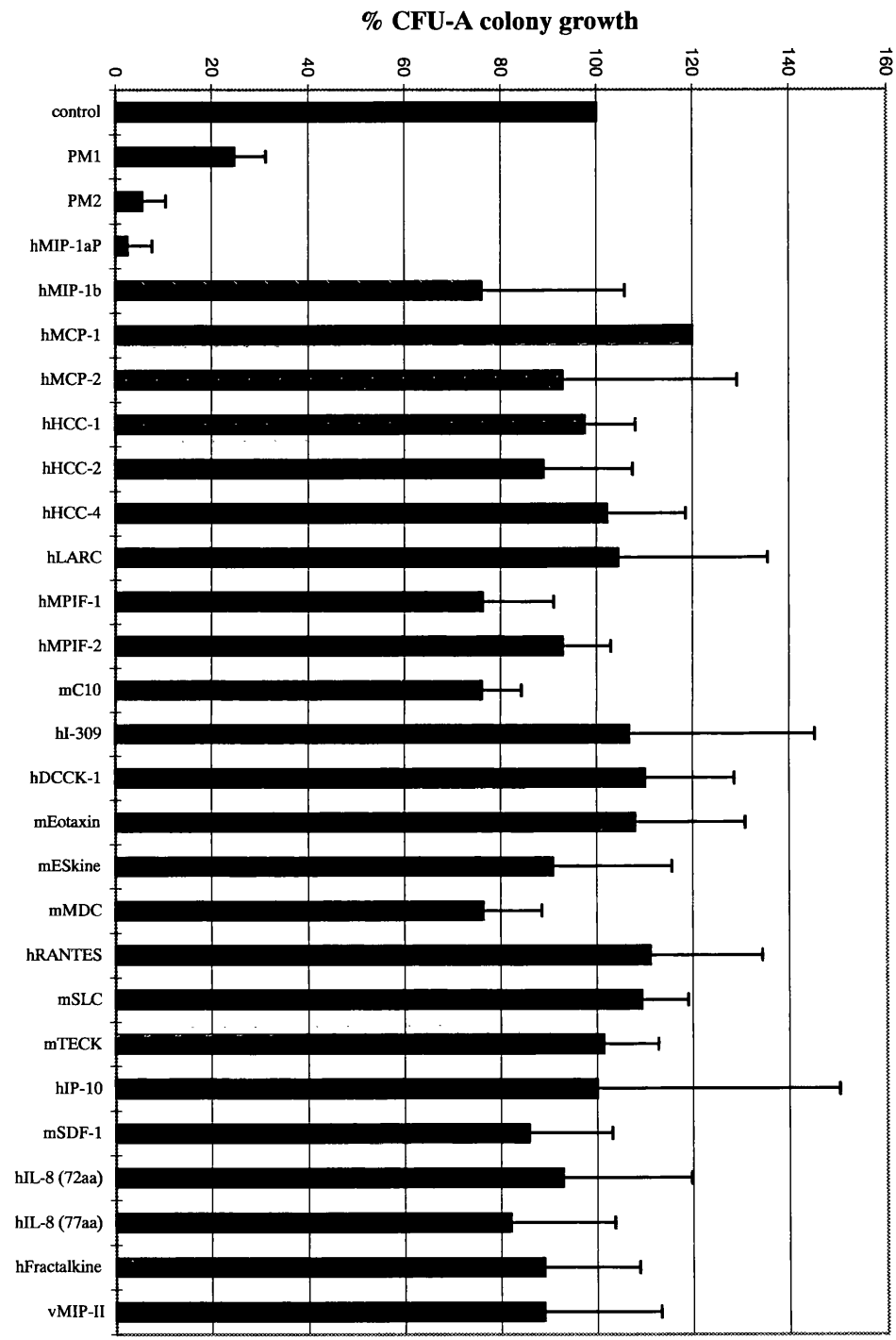


Fig. 5.1: Effect of different chemokines on CFU-A cell proliferation

A CFU-A assay was performed as described in the Methods section. All chemokines were added to the plates at a final concentration of 100 ng/ml in PBS and a volume of 0.2 ml.

Chemokine (100ng/ml)	Systematic name	% colony growth	Receptors used
hMIP-1β	CCL4	75.0±18.8	CCR5, D6
hRANTES	CCL5	111.4±26.3	CCR1, 3, 5, D6
hMCP1	CCL2	112.6±18.8	CCR2, D6
hMCP2	CCL8	93.4±9.4	CCR1, 2, 3, 5, D6
hHCC1	CCL14	93.4±9.4	CCR1 (D6)
hHCC2	CCL15	88.9±20.8	CCR1, 3
hHCC4	CCL16	102.2±18.2	CCR1
hMPIF1	CCL23	76.3±16.5	CCR1
mEotaxin	CCL11	107.9±25.7	CCR3
mMDC	CCL22	83.3±17.1	CCR4
hLARC	CCL20	104.5±34.8	CCR6
mSLC	CCL21	109.2±11.0	CCR7, 11
hI-309	CCL1	106.5±43.1	CCR8,
mTECK	CCL25	101.3±12.8	CCR9, 11
mESkine	CCL27	90.8±27.7	CCR10

Table 5.1: Effect of ligands for all of the CC chemokine receptors on CFU-A cell proliferation

Above are listed the results (in % colony growth) of a CFU-A assay in which the listed CC chemokines (common names and systematic names; h=human, m=murine) were tested for stem cell inhibition at a final concentration of 100 ng/ml. Listed are also the receptors these chemokines bind to which include all of the currently known CC chemokine receptors.

Recently, two human isoforms of MIP-1α have been characterised (Menten et al., 1999; Nibbs et al., 1999), known as LD78α and LD78β. These two proteins differ in only three amino acids, at position 2 (Ser/Pro), 39 (Gly/Ser) and 47 (Ser/Gly), in the mature protein (see figure below).

LD78α/MIP-1αS

ASLAADTPTACCFSYTSRQIPQNFIADYFETSSQCSKPGVIFLTKRSRQVCADPSEEWVQKYVSDLELSA

APLAADTPTACCFSYTSRQIPQNFIADYFETSSQCSKPSVIFLTKRGRQVCADPSEEWVQKYVSDLELSA

LD78β/MIP-1αP

The two studies have established that the functional differences between these two isoforms and their different affinities for CCR1, CCR5 and D6 can be almost exclusively attributed to the amino acid found at position 2, which in LD78α is a serine residue, whereas LD78β has a proline at that position, which makes the latter isoform more similar to murine MIP-1α. For that reason, these proteins will be referred to as MIP-1αS and MIP-1αP, respectively, for the remainder of this thesis. Sequence analysis of MIP-1αS has predicted two alternative signal sequence cleavage sites (Nibbs et al., 1999), producing a protein with the putative full-length amino terminus (with serine at position 2) as well as a protein in which the four amino terminal amino acids are missing (-4 variant). However, so far, only the -4 variant has been isolated from natural sources (Menten et al., 1999). Despite sequence analysis predicting only the full-length MIP-1αP protein, both full-length and a -4 variant were purified from natural sources (Menten et al., 1999), however in our lab only the full-length MIP-1αP has been detected. Functional differences observed between the full-length and -4 variants further support the findings regarding the importance of the penultimate amino acid. When the two full-length isoforms were analysed for their binding to CHO cells stably transfected with murine CCR1, CCR5 or D6 (Nibbs et al., 1999), it was found that MIP-1αS was a slightly better ligand for CCR1 than MIP-1αP, while the latter had a much higher affinity for CCR5, for which it is the highest affinity ligand yet identified, and for D6 than MIP-1αS. Thus, MIP-1αP resembles murine MIP-1α more closely in its binding to CCR5 and D6, while MIP-1αS has a CCR1 affinity more similar to murine MIP-1α. The MIP-1αS-4 and MIP-1αP-4 variants display indistinguishable binding activities for all three receptors. They have a slightly increased affinity for mCCR1 (higher than full-length MIP-1αS and comparable to murine MIP-1α), while their affinities for mCCR5 and mD6 were greatly reduced, thus resembling full-length MIP-1αS.

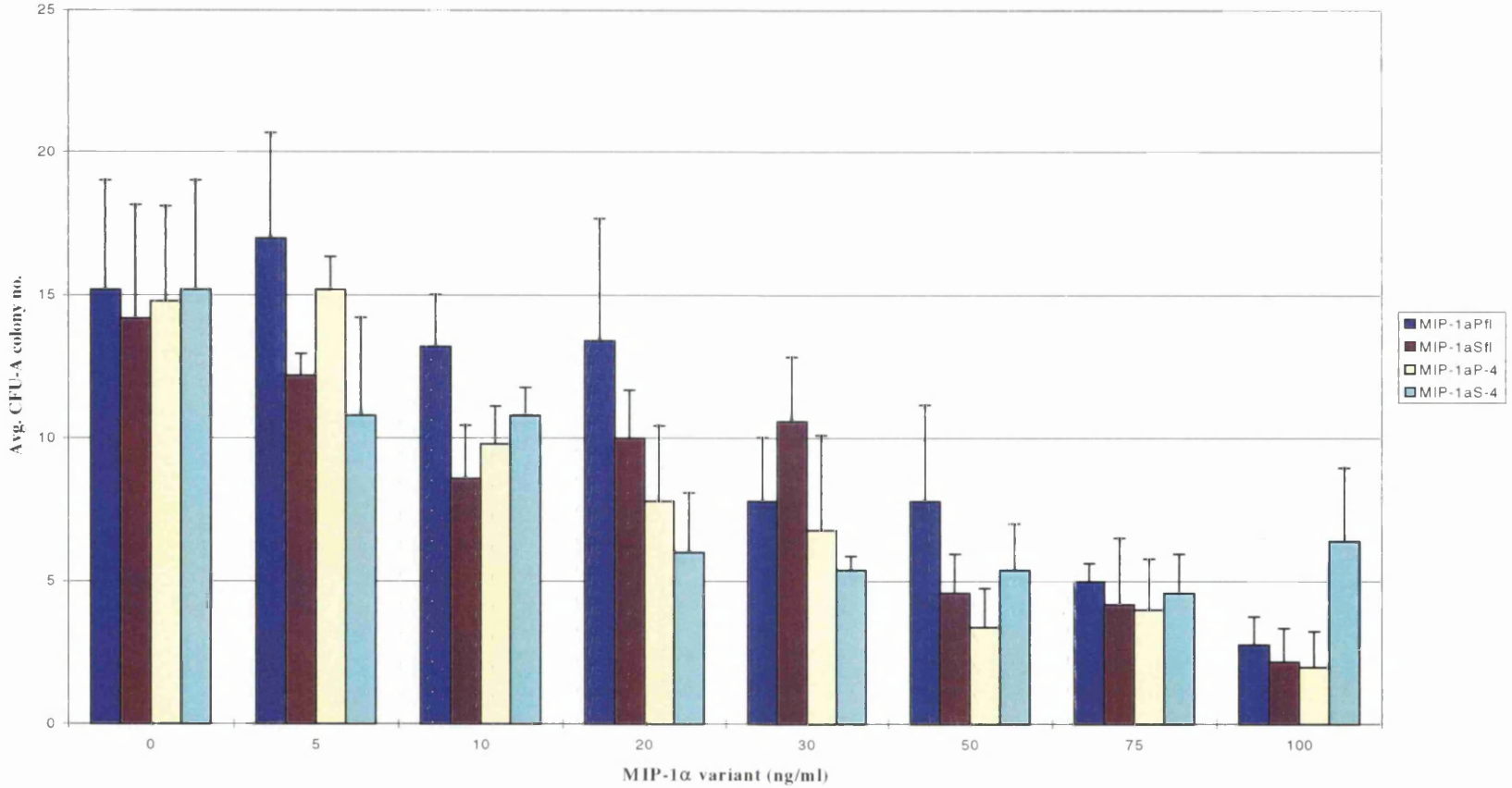


Fig. 5.2: Effect of different human MIP-1α isoforms on CFU-A cell proliferation

A CFU-A assay was set up as described in the Methods section and the MIP-1α variants added at the following concentrations (see figure legend): 0, 5, 10, 20, 30, 50, 75, 100 ng/ml in PBS. Bars: blue=MIP-1αP full-length; red=MIP-1αS full-length; yellow=MIP-1αP-4; cyan=MIP-1αS-4

Because of the differences with which the four isoforms interact with the three murine receptors for MIP-1α, all four human isoforms were tested for stem cell inhibition in the CFU-A assay in order to shed some light on the possible identity of the inhibitory receptor. As shown in Fig. 5.2, all four isoforms are potent inhibitors of CFU-A cell proliferation, with no significant differences observed between them. This suggests that CCR5 and D6 are unlikely to be involved in stem cell inhibition as the marked differences in binding affinities for the four ligands would be expected to be reflected in their potencies as stem cell inhibitors. It also demonstrates a rather limited role for the extreme N terminus of MIP-1α in stem cell inhibition, as a truncation of up to four amino acid residues does not have a negative influence on the potencies of the four proteins. This is very unusual since the integrity of the N terminus of CC chemokines has been shown to be essential for receptor activation in several cases. It suggests that the inhibitory receptor may be quite divergent from the other known murine MIP-1α receptors.

5.4. MIP-1α does not employ any of its known Receptors for Stem Cell Inhibition

Although the data in sections 5.2. and 5.3. suggest that none of the known murine MIP-1α receptors are involved in stem cell inhibition, as none of the other ligands for these receptors show any activity in the CFU-A assay and the human MIP-1α isoforms are all equally potent as inhibitors, different ligands have been shown to elicit different responses through the same receptor (Zhang et al., 1999; Zimmermann et al., 1999) and one ligand has been demonstrated to elicit two different responses via the same receptor (Poznansky et al., 2000). This raises the possibility that MIP-1α uses one of its known receptors in a different way for stem cell inhibition. Therefore, the most reliable way of testing whether any of the known MIP-1α receptors are involved in haemopoietic stem cell inhibition is to obtain bone marrow from mice in which the genes for the receptors have been deleted and test whether their CFU-A cells are still responsive to MIP-1α when analysed in the CFU-A assay. Mice in which the gene for CCR1 was deleted show protection against pancreatitis-associated lung injury (Gerard et al., 1997), they have defects in neutrophil trafficking and in steady state and induced trafficking and proliferation of myeloid progenitors (Gao et al., 1997), and their more mature myeloid progenitors do not undergo MIP-1α-induced stimulation and mobilisation, while the inhibition of more immature stem cells appeared normal (Broxmeyer et al., 1999). CCR5 null mice develop normally, but display reduced efficiency in the clearance of *Listeria* infections (Zhou et al., 1998) and defects in innate

immunity and organ-specific leukocyte trafficking (Huffnagle et al., 1999). $CCR3^{-/-}$ (Humbles *et al*, manuscript in preparation) and $D6^{-/-}$ (Cook *et al*, manuscript in preparation) mice have only just been generated but appear to develop normally. Bone marrow was isolated from these mice, frozen for transport and then thawed for the CFU-A assays. CFU-A cells tolerate the freezing and thawing process generally well, but 5-10 fold higher bone marrow cell numbers had to be used in order to obtain the same number of colonies per plate as with freshly isolated bone marrow. As shown in Fig. 5.3, the CFU-A cells from all four receptor-null mice are inhibited by MIP-1α, suggesting that none of these receptors are involved in stem cell inhibition. It appears as if CFU-A cells from $CCR3^{-/-}$ mice are slightly refractory to inhibition, needing up to 200 ng/ml of MIP-1α for complete inhibition. However, this seems to be due to these cells forming unusually large colonies which complicates the scoring of these colonies despite the fact that they show clear signs of inhibition.

Although the data presented above strongly indicates that a novel receptor is involved in stem cell inhibition, it remains possible that MIP-1α is able to utilise more than one receptor for the inhibition of CFU-A cells. In that case, the absence of one receptor in the knock-out mice may be compensated for by another MIP-1α receptor. In order to test for this possibility, the chemokine variant AOP-RANTES which can bind to CCR1, CCR3, CCR5 and D6 (Elsner et al., 2000 and data not shown) was included in the CFU-A assays in order to compete with MIP-1α for its receptors since it has been shown to act as a dominant negative chemokine. As shown in Fig. 5.4, AOP-RANTES is inactive as a stem cell inhibitor even at concentrations as high as 500 ng/ml. It was therefore included in the presence of MIP-1α at a 5-10 fold excess in order to compete with MIP-1α for its four receptors. However, the presence of AOP-RANTES did not in any way influence MIP-1α's ability to inhibit CFU-A cell proliferation (Fig. 5.4), thus providing further evidence for the proposed existence of a novel receptor through which MIP-1α mediates its inhibitory effect.

5.5. Summary

Data presented above have established that MIP-1α inhibits the proliferation of transiently engrafting haemopoietic stem cells (CFU-A cells) via a novel, as yet uncharacterised receptor. It was shown that it is unlikely to be any of the known CC chemokine receptors, as their ligands are unable to produce the same response in CFU-A assays as MIP-1α. This

also demonstrates that MIP-1α (both murine as well as human) is unique in its ability to inhibit CFU-A cells since this function is not shared by any of the CC, CXC, CX_3C and viral chemokines tested. This suggests that the novel receptor on CFU-A cells, through which MIP-1α mediates its inhibition, may be MIP-1α-specific and also quite divergent from the other known MIP-1α receptors, since the integrity of the MIP-1α N terminus is not required for its activation, as it has been demonstrated to be the case for most other CC chemokine receptors. The assumption that the inhibitory receptor is a novel receptor is further supported by the fact that CFU-A cells obtained from bone marrow of mice which had each individual known MIP-1α receptor (CCR1, CCR3, CCR5 and D6) deleted, were still inhibited by MIP-1α, even in the presence of AOP-RANTES, a chemokine homologue that can compete with MIP-1α for its four receptors.

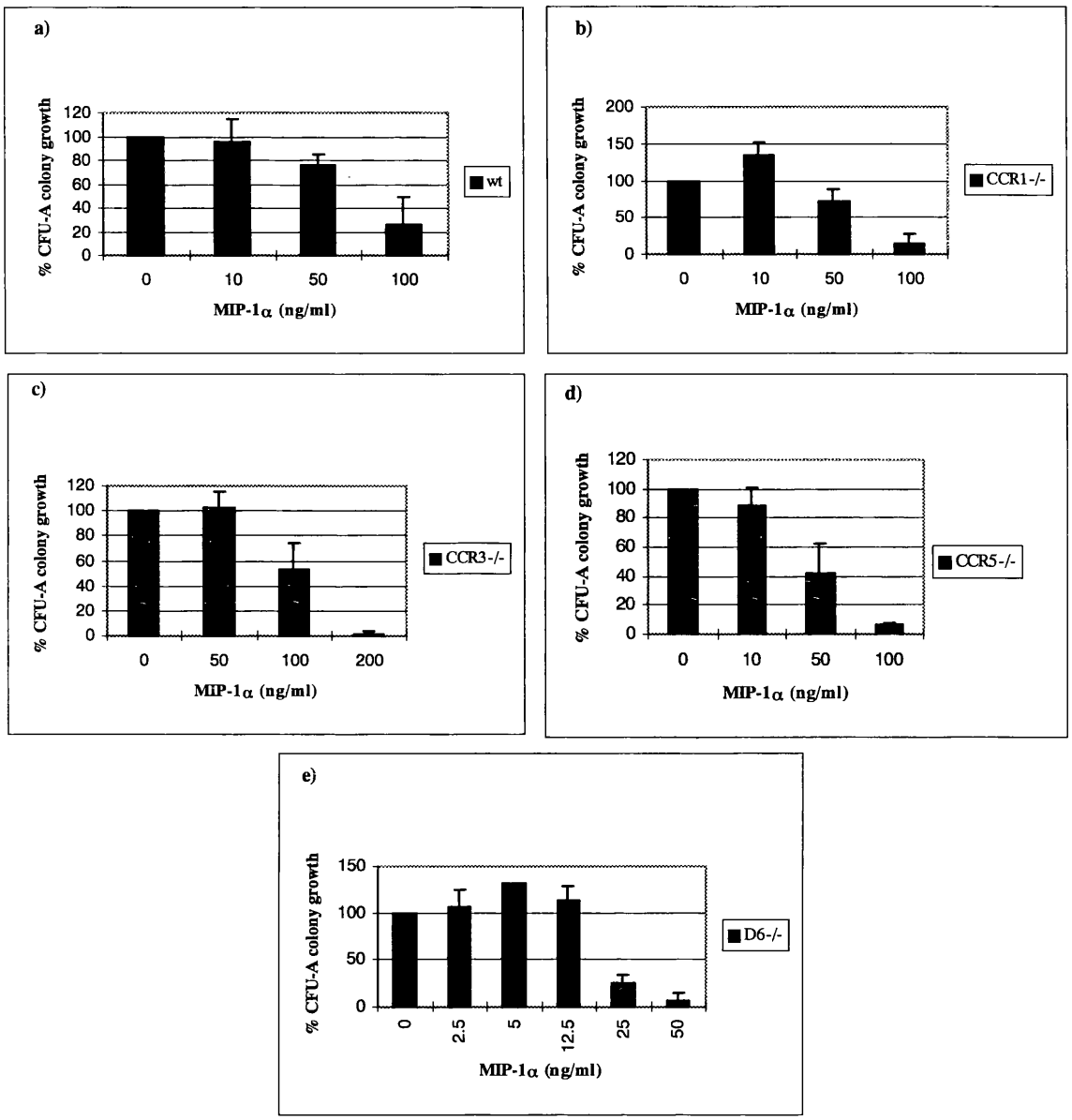


Figure 5.3: CFU-A assay with bone marrow cells from MIP-1α receptor null mice

The ability of murine MIP-1α (concentrations in PBS provided in figure legends) to inhibit CFU-A cell proliferation in the absence of each one of its known receptors was tested in CFU-A assays (see Methods section) using a) wild type, b) $CCR1^{-/-}$, c) $CCR3^{-/-}$, d) $CCR5^{-/-}$, e) $D6^{-/-}$ bone marrow cells.

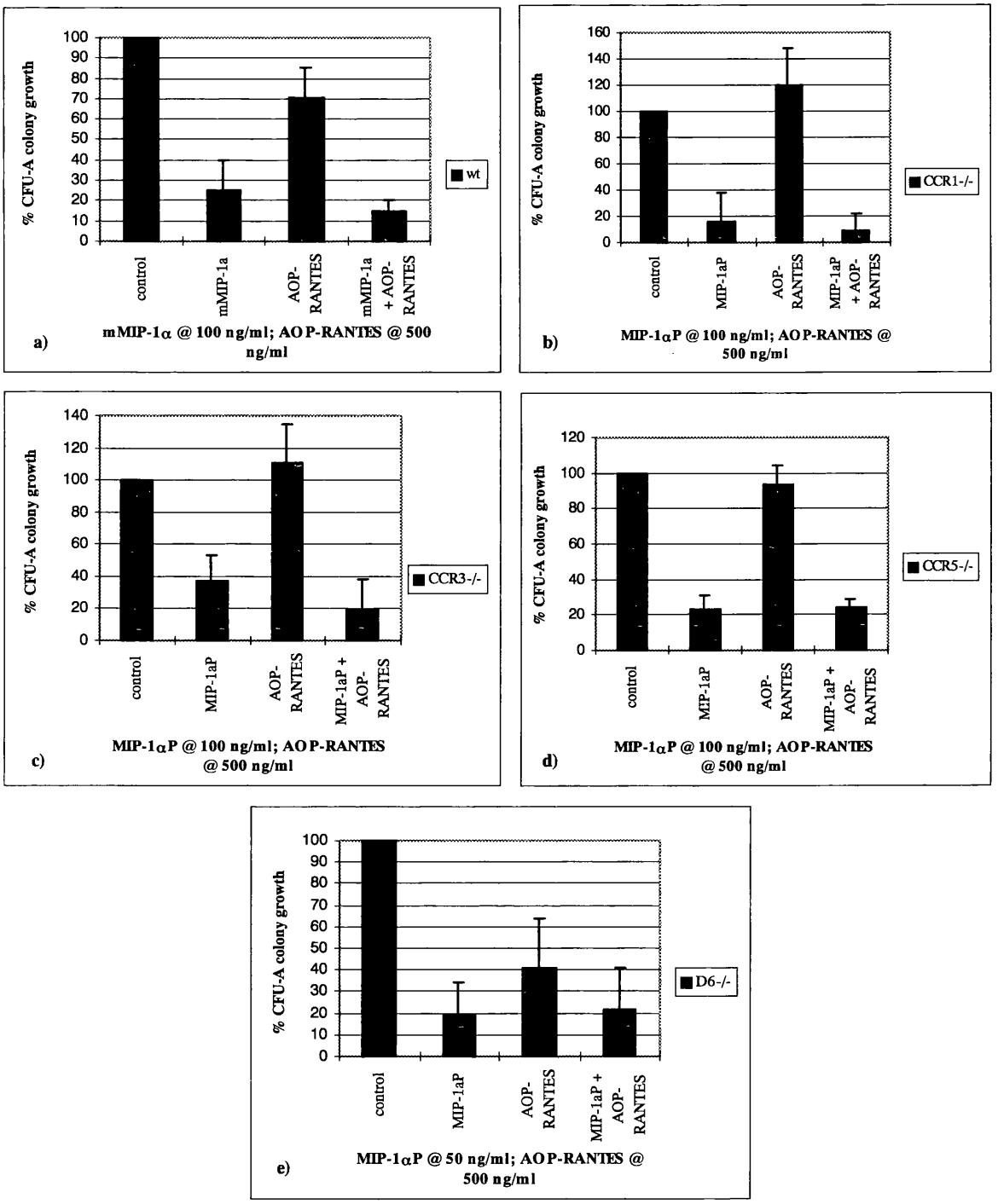


Figure 5.4: Ability of MIP-1α to inhibit CFU-A cell proliferation in the presence of an antagonist

CFU-A assays were carried out as described in the Methods section, using a) wild type bone marrow (BM), b) CCR1$^{-/-}$BM, c) CCR3$^{-/-}$BM, d) CCR5$^{-/-}$BM, and e) D6$^{-/-}$BM. Chemokine concentrations (in PBS) are given in the figure legends.

Chapter 6. RESULTS - Molecular Dissection of the MIP-1α Inhibitory Motif

6.1. Introduction

Since the identity of the inhibitory receptor still remains elusive, an alternative approach to gathering some knowledge of the molecular mechanism underlying MIP-1α-mediated stem cell inhibition was initiated and involved experiments aimed at identifying the responsible region within MIP-1α itself and possibly characterising an inhibitory motif. It is hoped that the identification of a more or less contiguous inhibitory motif may lead to the design and generation of a peptide which can interact with the inhibitory receptor and may thus have agonistic or antagonistic properties in CFU-A cell inhibition. This peptide could be further useful for raising "inhibition-specific" antibodies and for cloning out the inhibitory receptor.

To this aim, chimaeras were designed in which structural domains were exchanged between murine MIP-1α and the highly related CC chemokine human RANTES. As the crystal structure of murine MIP-1α has recently been solved (MacLean, J unpublished results) and shown to be virtually superimposable on the NMR structure of human RANTES (Skelton et al., 1995) with almost identical domain borders, a rational basis for the construction of the chimaeras was provided and domains exchanged in a way that tried to minimise structural disturbances. MIP-1α and RANTES were divided into three basic units (see Fig. 6.1 for an alignment of the two sequences): (1) the N terminal region up to the CC motif, (2) the main body of the chemokine, including the loop following the CC motif and the triple-stranded β sheet, and (3) the C terminal α helix.

6.2. Generation of MIP-1α/RANTES Chimaeras

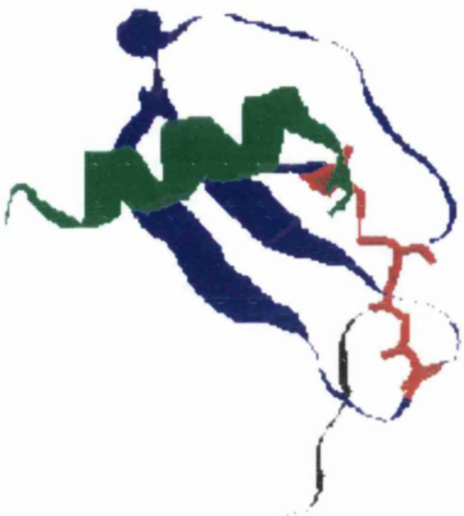
The three basic units were exchanged between murine MIP-1α and human RANTES using a technique known as Overlap PCR (see Fig. 6.2 for schematic diagram). Briefly, in a first round of PCR, a chimaeric primer spanning the region of exchange and a primer to the extreme amino or carboxy terminus were used to amplify either the N or C terminus, resulting in a double-stranded chimaeric product. This product from the first round was subsequently used in a second round of PCR, together with primers against the N and C

termini, resulting in the full-length chimaeric cDNA. These chimaeric cDNAs were also used as templates for the generation of double domain swaps. The PCR reactions were carried out using *Pfu* polymerase as outlined in the Materials and Methods section. Fig. 6.3 lists the final constructs which included the two wild type cDNAs (full length murine MIP-1α and human RANTES), the four single domain swaps (two amino terminal swaps and two carboxy terminal swaps) and the two double swaps, all of which were named according to the domains they contained, e.g. RRR for wild type RANTES and RMR for the main body of MIP-1α attached to the RANTES N and C termini. The final double-stranded, blunt-ended PCR products were subcloned into pCR-Script, which is specifically designed for blunt-ended ligations and also contains T3 and T7 promoter sites for sequencing, which was carried out by the Beatson Sequencing Service.

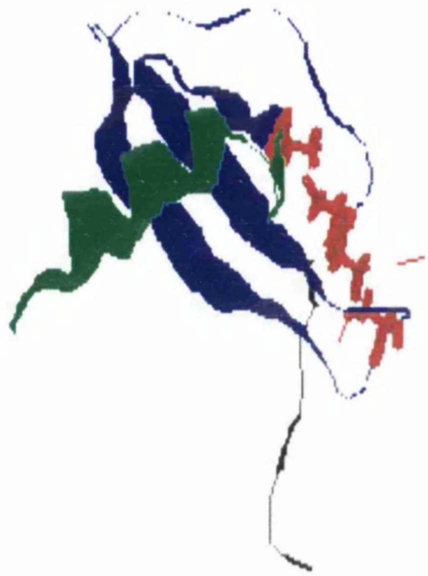
Once their correct sequences had been confirmed, they were transferred into the pVL1392/1393 Baculovirus Transfer Vector (using NotI and BamHI restriction), sequenced again for correct orientation and the vectors prepared for transfection into Sf9 insect cells.

6.3. Production of Recombinant Proteins

A brief outline of the basic principles of the Baculovirus System is provided in Fig. 6.4. The large size of the baculovirus genome excludes the possibility of cloning cDNAs directly into it. For that reason, the transfer vector containing the gene of interest is co-transfected with the linearised baculovirus genome, both of which are incapable of replicating on their own. They have to undergo homologous recombination within the nucleus of Sf9 cells which generates a complete, functional baculovirus genome containing the gene of interest (under the high-expressing promoter of the polyhedrin gene which normally produces a structural protein) and capable of producing recombinant protein. Simultaneously, recombinant viral particles are produced which can bud from the cell and infect neighbouring cells, thereby spreading the virus and amplifying protein production. As Sf9 cells are eukaryotic cells, they are capable of carrying out posttranslational modifications and, most importantly in the case of chemokines, they can process and secrete proteins containing a signal sequence. The cell culture medium can then be tested for the expression of recombinant protein by Western Blotting.



mMIP-1α



hRANTES

```
                         loop     turn   β1      loop     β2  turn   β3   turn    α-helix

                  A         B        C     D        E       F     G     H     I        J
                       <-------->       <--->             <->      <--->        <-------->
MIP-1a    APYGADTPTA CC FSY SRKIPRQFIVDYFE TSSL  C SQPGVIFLTKRNRQI C ADSKETWVQEYITDLELNA

RANTES    ASPYSSDTTP CC FAYIARPLPRAHIKEYFY TSGK  C SNPAVVFVTRKNRQV C ANPEKKWRVEYINSLEMS
              *** *      *          * *   *   **                       *** **     *   *
```

Figure 6.1: Comparison of the murine MIP-1α and human RANTES primary and tertiary structures

Tertiary structure (upper part): Ribbon diagrams of the monomeric units of murine MIP-1α and human RANTES. In red are shown the four cysteine residues that form the two disulphide bonds. In black, blue and green are shown the three domains that were exchanged in the MIP-1α/RANTES chimaeras; the N terminus, the main body (loop and triple-stranded β sheet) and the C terminus (α helix), respectively.

Primary sequence (lower part): The bold and underlined amino acids represent residues implicated in heparin binding, while the asterisks denote non-conservative changes between MIP-1α, MIP-1β and RANTES. The borders of the individual domains (denoted by bold capital letters) are represented by the arrows above the sequences.

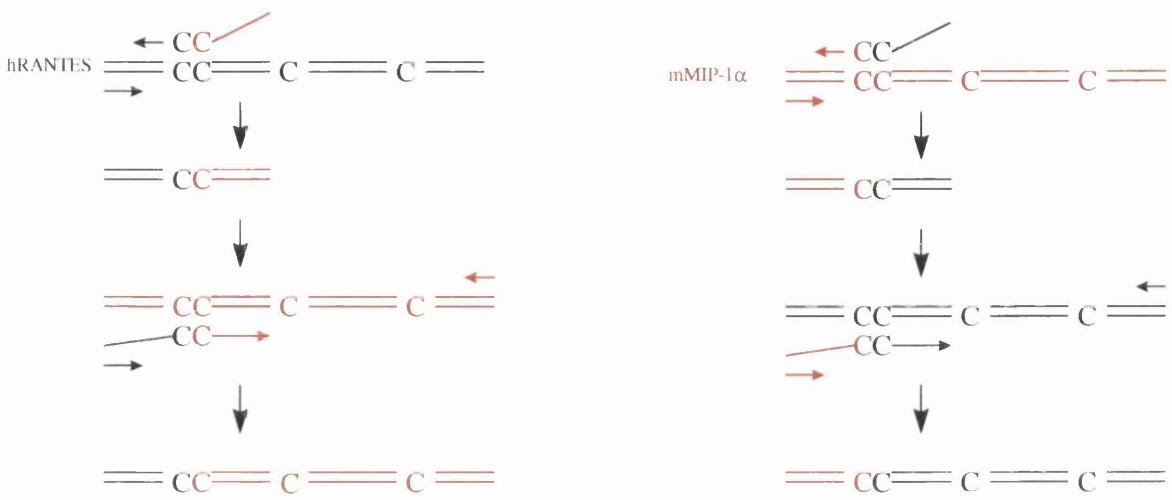


Figure 6.2: Principles of generation of chimaeras by Overlap PCR

Schematic diagram of the generation of single, N terminal domain swaps by Overlap PCR (as described in the text). A similar chimaeric primer spanning the region around the final cysteine residue and running in the opposite direction was employed for single C terminal swaps. Single domain chimaeras were used as templates in the generation of double swaps.

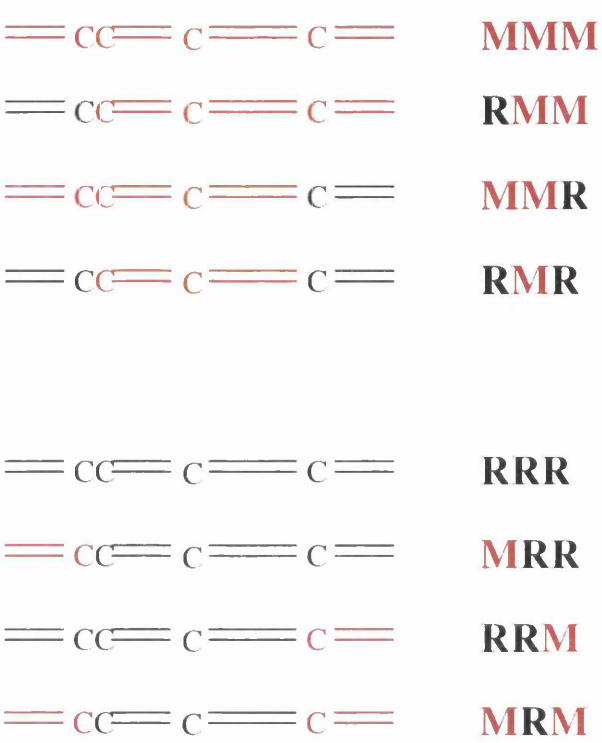


Figure 6.3: Summary of final constructs

List of the final PCR products that were prepared for transfection into Sf9 cells. The letters M and R stand for MIP-1α and RANTES, respectively and describe the domain composition of the chimaeras, with RRR and MMM being the wild type sequences.

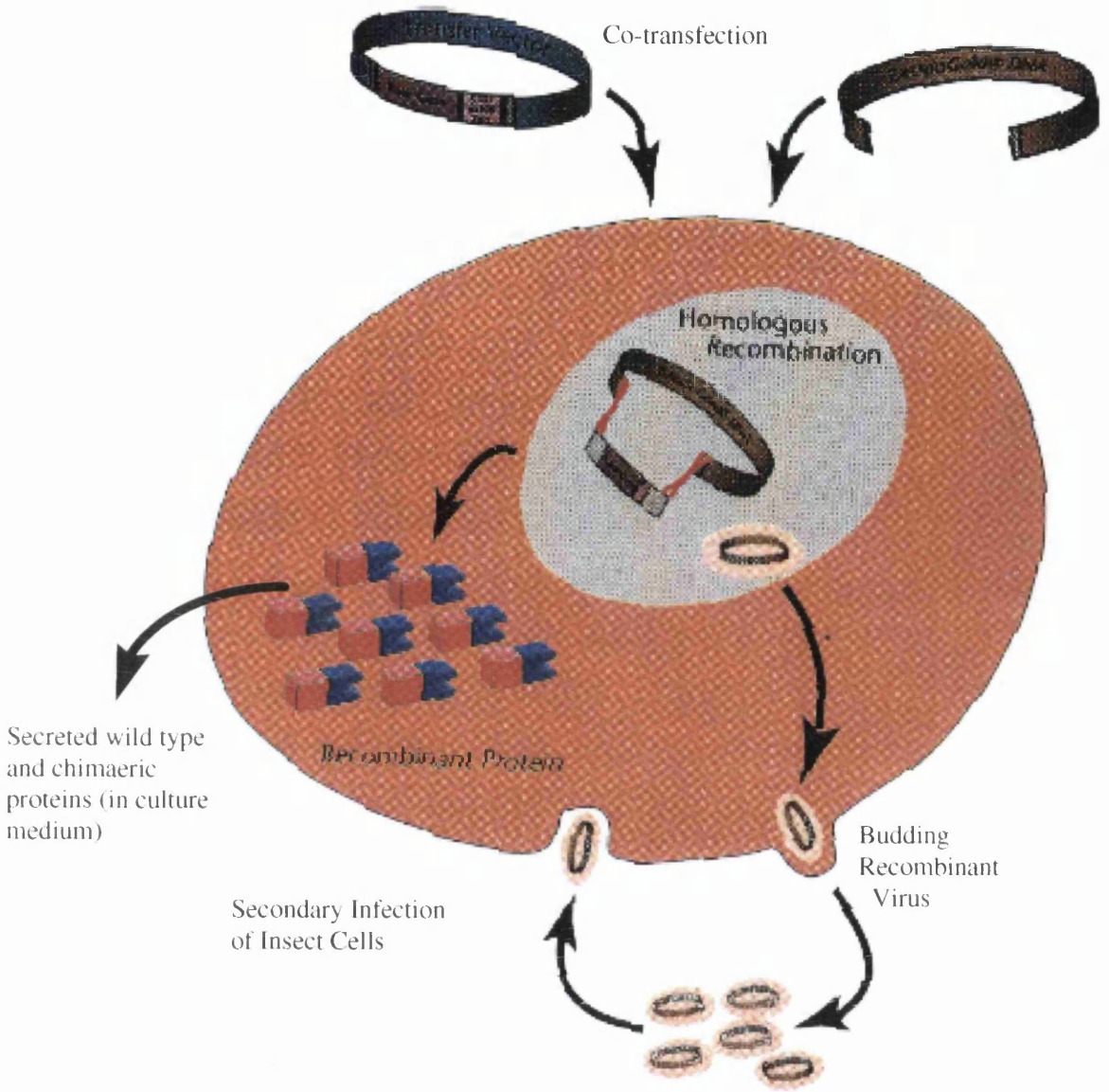


Figure 6.4: Overview of the Baculovirus System

Diagram adapted from the PharMingen Baculovirus laboratory manual.

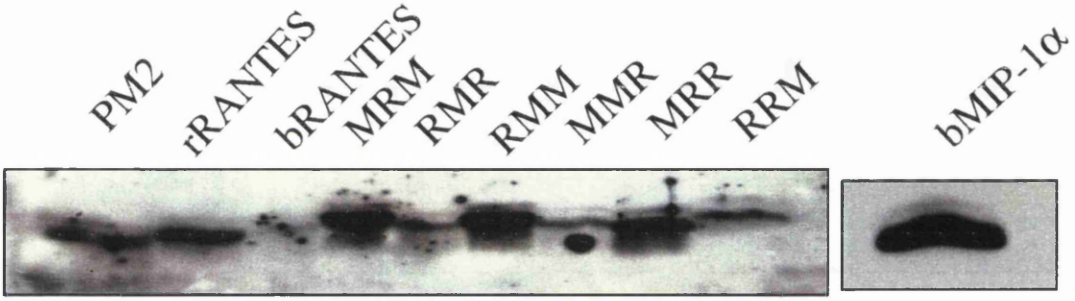


Figure 6.5: Protein production by the final clones

20 µl samples of murine MIP-1α (PM2) and recombinant human RANTES (rRANTES) (both at 5 µg/ml in PBS) as controls and 20 µl samples of the undiluted supernatant from cells infected with the final recombinant viral clones were run on a 17.5% SDS gel and Western blotted with a mixture of MIP-1α- and RANTES-specific antibodies (bRANTES/bMIP-1α = RANTES/MIP-1α produced in the baculovirus system).

For the expression of the recombinant proteins in the baculovirus system, 2-5 μg of the Baculovirus Transfer Vectors containing the wild type and chimaeric cDNAs were contransfected with 0.5 μg of the linearised baculovirus DNA into $2x10^6$ Sf9 cells. The cells were incubated at 27°C for 5 days and the cell supernatant then tested for the expression of recombinant protein by Western Blotting. To obtain high expressing viral clones, 2-3 rounds of plaque assays and plaque 'pick-ups' were carried out and the expression followed by Western Blotting.

Fig. 6.5 shows a Western Blot of the expression of recombinant proteins by the selected final clones, using a mixture of polyclonal anti-murine MIP-1α and anti-human RANTES antibodies in a 1:1 ratio in order to assure the detection of all of the chimaeric proteins. The secondary antibody was coupled to Horseradish Peroxidase in order to allow for the detection of the antibody complexes with the Amersham Pharmacia Biotech Enhanced Chemiluminescence (ECL) System and their visualisation on x ray films. It is immediately noticeable that there are marked differences in the level of expression, with wild type MIP-1α produced in the baculovirus system (bMIP-1α) showing the highest expression, while RANTES is expressed at the lowest level. There is no obvious pattern behind the levels of expression, and the reason for these differences is unknown.

Aliquots of different sizes (in order to compensate for the discrepancies in expression) of these recombinant protein-containing supernatants were directly tested in a CFU-A assay for stem cell inhibition. However, apart from the observation that baculovirus-produced murine MIP-1α was a competent stem cell inhibitor, no obvious activity was detected in any of the other preparations. It was therefore decided that the preparations were too crude to allow for the perception of subtle differences in activity and that large preparations of relatively pure protein were needed, in which active protein had been separated from inactive protein. For that purpose, large working stocks of high titre recombinant virus were first prepared in suspension culture by infecting cells at a density of $5x10^5$ cells/ml with 0.1-0.2 plaque-forming units (pfu)/ml and the virus-containing supernatants harvested after 7-9 days. Aliquots of those were used to infect 300-500 ml suspension cultures of Sf9 cells (at $1x10^6$ cells/ml) and the recombinant protein-containing supernatant harvested after 6 days. Viral particles were removed by centrifugation.

6.4. Purification of Recombinant Proteins

After a first attempt, the production and purification of recombinant wild type human RANTES was discontinued because of the difficulties associated with its low expression. The recombinant human RANTES used in subsequent experiments has therefore been obtained from commercial sources. For all of the other recombinant proteins, the purification protocols had to be derived from first principles as they all behaved quite differently and unpredictably during the chromatography process (see below and Fig. 6.6). In an initial purification protocol, the first step involved running 300-500 ml of the cell supernatant through a 200 ml heparin affinity column at a flow rate of 5 ml/min and eluting the proteins by a stepwise increase of the salt concentration in the running buffer. The size of the steps was varied according to the requirements of the individual proteins (see Fig. 6.6 for the conditions used for each individual protein). The elution of proteins was followed by absorbance measurements at 280 nm and the eluate collected over protein peaks. As shown in Fig. 6.6, there were marked differences in the strength with which individual chimaeras bound to the heparin column as reflected in the concentration of salt needed to elute the protein. Salt concentrations for elution ranged from 0.2 M for MMM and MRM to 0.8 M for MMR, with stronger binding generally being related to the presence of the RANTES C terminal domain which contains two additional positive charges and two fewer negative charges when compared to the MIP-1α C terminus (see Fig. 3.1). Fractions containing the protein of interest (as determined by Western Blotting) were further purified on a MonoQ anion exchange column, and the proteins eluted by a continuous increase in salt (NaCl) concentration. Again there were marked differences in the behaviour of the individual chimaeras on this column, with RRM and MRM not even being able to bind to the column and therefore eluting in the flow through and with RMR eluting over the entire gradient, including the flow through. In the latter case, the interaction with the cationic matrix may have caused some degree of denaturation. The remaining proteins displayed moderate binding, needing 0.2-0.3 M NaCl for elution.

Positive fractions were then subjected to further purification on a reversed phase chromatography column and proteins eluted by a continuous increase in acetonitrile. The concentration of acetonitrile needed to elute the proteins from the column were generally very similar, ranging from 30-40%. Fractions were analysed for the presence of the protein of interest by Western Blotting and positive fractions analysed for purity by silver staining of the protein gel. In a few cases, such as RRM (RANTES with a MIP-1α carboxy

terminus) and MRM (RANTES with a MIP-1α C and N terminus), the protein was sufficiently pure and the fractions could therefore be pooled, freeze-dried and resuspended in PBS. In all of the other cases, the proteins needed further purification and were therefore run on a 1 ml HiTrap heparin affinity column, eluted in a continuous gradient and positive fractions cleaned up in a final reversed phase chromatography step. Wild type MIP-1α and RMM were then sufficiently pure to be dried down, while so little protein remained of MMR, RMR and MRR that further production and the use of an alternative purification protocol were required.

Therefore, instead of eluting the protein from the initial 200 ml heparin column by a stepwise increase of the salt concentration, a continuous gradient was used which has the advantage of producing a higher resolution of the protein peaks resulting in a higher purity, while having the disadvantage of potentially increasing the volume over which a specific protein is recovered. The positive fractions were then further purified on a reversed phase chromatography column as before and the recombinant protein-containing fractions freeze-dried and resuspended in PBS. Fig. 6.6 provides a summary of the purification steps applied to each recombinant protein and shows that the conditions were different for each one of them and therefore had to be tailored to the individual needs. Fig. 6.7 contains images of the silver-stained 17.5% SDS PAGE gels on which the final preparations in PBS were run in order to assess their purity and to estimate their concentrations in the presence of known amounts of murine MIP-1α (PM2). While the preparations of wild type MIP-1α and MRM were completely pure, the preparations of the other chimaeras contained various amounts of contaminating proteins. However, a concentration of the recombinant proteins into a smaller volume was achieved by the purification and the final concentration for each one of them was 14 μg/ml of wild type MIP-1α, 15 μg/ml of MRM, 20 μg/ml of RMR, 10 μg/ml of MMR, 15 μg/ml of RMM, 15 μg/ml of RRM and 40 μg/ml of MRR, as assessed by comparison to known amounts of murine MIP-1α (PM2). An aliquot of each protein was sent to Alta Bioscience (Birmingham, UK) for N terminal sequencing (the first 5 amino acid residues). It showed that all N termini were correct and intact (data not shown).

MMM - 0.2M NaCl step heparin, 0.25M NaCl gradient MonoQ, 30-35% Acetonitrile rpc gradient, heparin gradient, rpc gradient

RMM - 0.3M NaCl step heparin, <0.13M NaCl gradient MonoQ,, 30-35% Acetonitrile gradient rpc, heparin gradient, rpc gradient

MMR - 0.8M NaCl step heparin, 0.3M NaCl gradient MonoQ, 40% Acetinitrile rpc gradient; big heparin gradient, rpc gradient

RMR - 0.5M NaCl step heparin, smear over MonoQ, smear over rpc; big gradient heparin, gradient rpc

RRR - discontinued

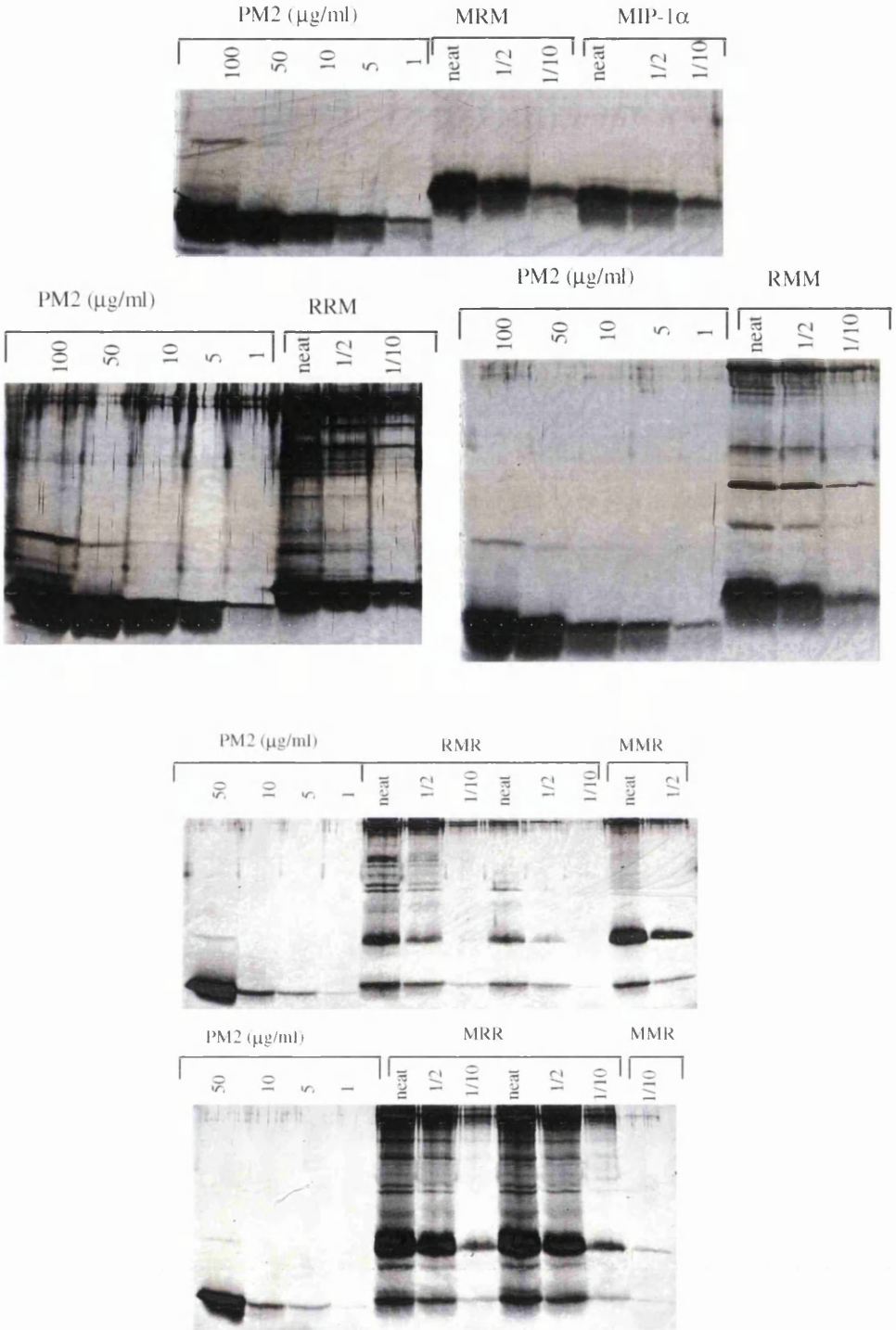
MRR - 0.6M NaCl step heparin, 0.2-0.3M NaCl gradient MonoQ, gradient rpc; big heparin gradient, rpc gradient

RRM - 0.4M NaCl step heparin, FT MonoQ, 30-35% Acetonitrile rpc gradient

MRM - ~0.2M NaCl step heparin, FT MonoQ, 30-33% Acetonitrile rpc gradient

Figure 6.6: Purification Protocol

Summary of the purification strategy applied to each individual recombinant protein. (FT=flow through)



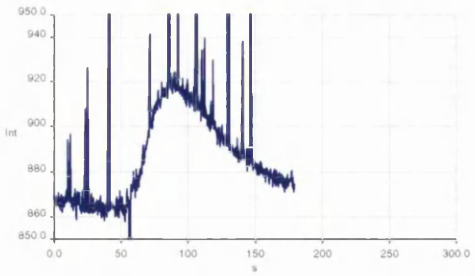
Estimated concentrations (µg/ml):			
MIP-1α	14	RMM	15
MRM	15	RRM	15
RMR	20	MRR	40
MMR	10		

Figure 6.7: Silver-stained gel showing the purified chimaeras

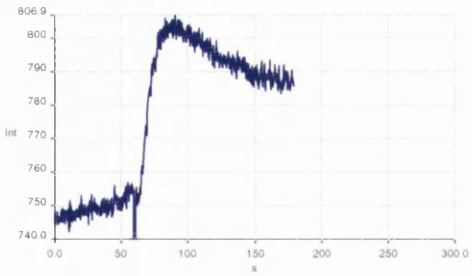
20 µl samples of the purified recombinant proteins (undiluted, 1in2, 1in10 in PBS) were analysed on a silver-stained 17.5% SDS gel as to their purities. Estimates of their concentrations were made by comparing them to known amounts of murine MIP-1α (PM2). The concentration of each undiluted preparation is given in the table.

6.5. Functional Test in Calcium Flux Assay

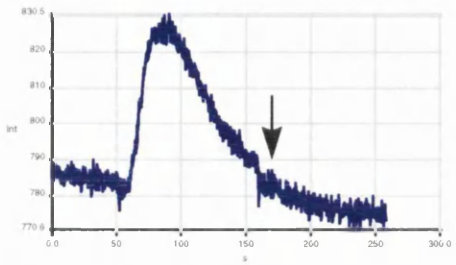
In order to determine whether the purified chimaeras were properly folded, functional proteins, they were tested for the ability to induce the release of intracellular calcium in HEK293 cells that were stably transfected with human CCR5. In this so-called calcium flux assay, cells are loaded with the calcium-sensitive fluorescent dye Fura-2-AM, the fluorescence of which changes upon binding of calcium. Bursts of intracellular calcium release, induced by stimuli such as chemokine binding, can thus be measured as transient increases in fluorescence. Murine MIP-1α as well as human RANTES are capable of binding to (with similar affinities) and eliciting a calcium flux through human CCR5 at a final concentration of 50 ng/ml (Fig. 6.8 and see below) which makes this assay ideally suitable for testing the functionality of the chimaeric proteins. Another feature of the calcium flux assay which is important in this context is that it also provides a test for specificity in a process known as desensitisation. When a chemokine binds to its receptor and elicits a transient calcium signal, then a second stimulus provided either by the same chemokine or another ligand for the same receptor will not result in another calcium burst when added immediately after the first. This occurs because the receptor has been desensitised by the first stimulus and also typically indicates that both stimuli utilise the same receptor. As shown in Fig. 6.8, both human RANTES and PM2 (murine MIP-1α) induce a calcium flux of similar magnitude through hCCR5. The same was found for baculovirus-produced MIP-1α, MRM, MMR and MRR, all of which did so through CCR5 since subsequently administered PM2 did not induce a second flux. With RRM and RMR which also fluxed through CCR5, the desensitisation was not complete, as a second flux induced by PM2 was observed, albeit of lower magnitude than PM2 on its own. RMM only gave a signal when 100 µl were added to the reaction. It did, however, desensitise the receptor against subsequent stimulation by RANTES. It therefore seems that RMM may be at least ten fold less active than the other chimaeras and it was therefore decided to use this protein at a higher concentration in the experiments that followed.



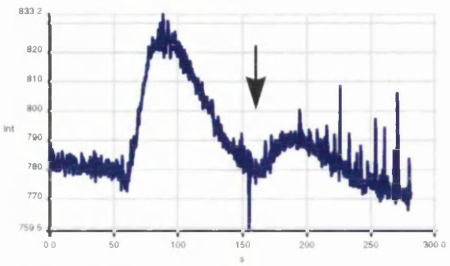
10 µl rhRANTES (10µg/ml)



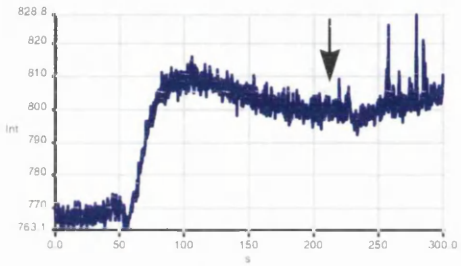
10 µl PM2 (10µg/ml)



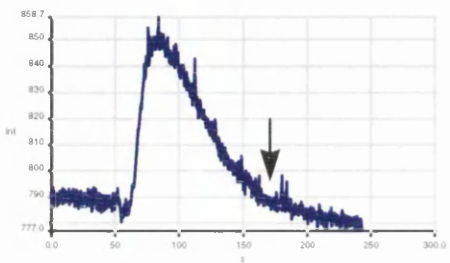
10µl MRM + 10µl PM2 (10µg/ml)



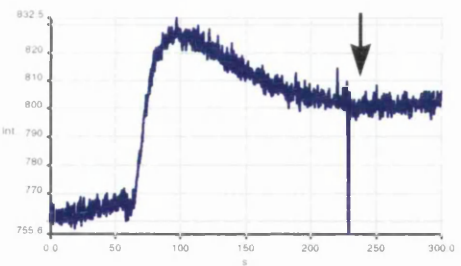
10µl RRM + 10µl PM2 (10µg/ml)



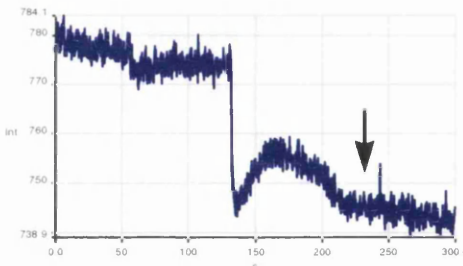
10µl MMR + 10µl PM2 (10µg/ml)



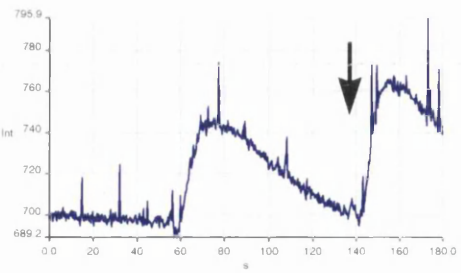
10µl bMIP-1α +10µl PM2 (10µg/ml)



10µl MRR + 10µl PM2 (10µg/ml)



10µl RMM + 100µl RMM + 10µl RANTES (10µg/ml)



20 µl RMR + 10 µl bMIP-1a

Figure 6.8: Calcium Flux Assays

Calcium flux assays with human CCR5-transfected, Fura-2-AM-loaded HEK293 cells were performed as described in the Methods section. All proteins were tested undiluted (unless stated otherwise). For desensitisation tests, a second stimulus ("+ chemokine") was added at a specific time point as indicated by the arrow.

6.6. CFU-A Cell Inhibition by MIP-1α/RANTES Chimaeras

In order to examine whether any of the recombinant proteins are capable of inhibiting stem cells, they were tested by direct addition to CFU-A assay plates. Fig. 6.9 gives the results obtained with titrations of all the recombinant proteins as compared to titrations of PM2 and commercially obtained RANTES. The preparation of MIP-1α that was produced in the Baculovirus System (bMIP-1α) was a very powerful inhibitor with complete inhibition observed at 100 ng/ml and half maximal activity at about 25 ng/ml. It even had a higher activity than the control preparation of murine MIP-1α (PM2). The only one that showed consistent inhibition of CFU-A colony formation similar to MIP-1α itself, albeit not as potently, was RMR. Despite the fact that it also showed half maximal inhibition at 25 ng/ml, inhibition was not complete at 100 ng/ml. MRM, MRR, RRM and MMR which had all produced a robust flux in CCR5-transfected cells, were nevertheless inactive as stem cell inhibitors. The poor performance of RMM in the calcium flux assays suggested that only a portion of the protein was active, therefore making it necessary to use it at a higher concentration in the CFU-A assay. In order to be able to use it at a higher concentration, it was first concentrated four fold by desalting it, drying it down and resuspending it in a volume that was four times smaller than the original. It was then tested in a one-layer CFU-A assay that was set up in a 24-well plate in order to reduce the amount of protein needed. Fig. 6.10 shows the result of this assay which indicates that even at concentrations 30-60 fold higher than used normally with chemokines, RMM does not inhibit CFU-A cells to any significant level, although there may be some residual activity within the bulk of mostly inactive protein.

The observation that RMR was the only chimaeric protein with inhibitory activity suggests that the main determinants for CFU-A cell inhibition are located within the main body of MIP-1α between the CC motif and the C terminal α helix which seems to be supported by the fact that MRM, which lacks the MIP-1α main body but retains the rest of the MIP-1α framework, was a potent inducer of intercellular calcium release, but showed no activity in the CFU-A assay. A dominant role for the MIP-1α N and C termini was also ruled out by the fact that MRR and RRM, both of which induced a robust calcium flux, were inactive in the CFU-A assay. However, the fact that RMR is not as potent as native MIP-1α in stem cell inhibition may have several causes. The conformation of the main body of MIP-1α may be slightly altered when attached to the RANTES N and C termini, therefore resulting in a suboptimal interaction with the receptor. It could also be that a proportion of the

protein in the RMR preparation is inactive as suggested by its aberrant behaviour on the chromatography columns and the relatively low calcium flux induced through CCR5 (see Fig. 6.6 & 6.8), which would then suggest that the active portion of the preparation is in fact a very potent stem cell inhibitor. Alternatively, the fact that RMR, which only contains the MIP-1α main body, is not quite as potent an inhibitor as wild type MIP-1α, may also suggest that other important residues either in the MIP-1α N or C terminus are required in combination with residues in the main body for full inhibitor activity. In this context, it is surprising to note that neither RMM nor MMR display any significant inhibitory activity, despite the presence of the MIP-1α main body in these proteins. This may mean, that these particular combinations of RANTES and MIP-1α domains produce proteins with slightly altered conformations which may explain why the expression of MMR was relatively low (Fig. 6.5) and the activity of RMM in the calcium flux assay reduced (Fig. 6.8).

In order to determine whether RMR inhibits stem cells by a mechanism similar to the one employed by MIP-1α, i.e. by inhibiting cell proliferation, a CFU-A assay was set up in which the top agar layer was replaced by methylcellulose. This allowed CFU-A colonies to be picked and analysed further for cell numbers. This could not be carried out for RMM or MMR as no more purified material was available, and these two had not shown any significant inhibitory activity. 5-10 colonies were picked from each plate containing the different chimaeras, pooled separately, single cell suspensions obtained in ice-cold PBS and the cells counted in a Schärfe System CASY 1 Cell Counter. Table 6.1 presents the results of the cell counts which indicate that the number of cells per colony is significantly lower in MIP-1α- and RMR-inhibited colonies and slightly higher in RMR-inhibited than in MIP-1α-inhibited colonies which corresponds well with the data presented in Fig. 6.9. The cell numbers per colony counted on the control plates were similar to numbers published previously, albeit a bit lower ($2x10^4$ as compared to $4x10^4$ cells/colony) which may be due to the fact that the colonies were a bit smaller than the one in the previous report (Pragnell et al., 1988).

Furthermore, colonies were also picked from the methylcellulose assays for the analysis of the cell content in colonies in order to determine whether the different proteins affected the differentiation potential of CFU-A cells. Cells were cytospun and stained with GIEMSA which facilitates the morphological identification of different cell types. Inspection of the stained cells, obtained from colonies grown in the presence of the different chimaeras, under a light microscope established that the cell composition is the same in the colonies

from each plate (control as well as in the presence of the different recombinant proteins). All slides contained mainly macrophages with a few blast-like cells (<5%) and about 1 neutrophil per slide which compares to previously reported cell content analyses of CFU-A colonies (Pragnell et al., 1988). This suggests that MIP-1α (and RMR) is a true inhibitor of proliferation (indicated by the lower cell counts per colony) and does not influence the differentiation potential of CFU-A cells.

6.7. Summary

Once it had been established that the identity of the MIP-1α inhibitory receptor is unknown, attention was turned to MIP-1α itself in an attempt to identify a region within the protein that confers this activity and that may even contain an identifiable inhibitory motif. This task was approached by constructing chimaeras that had three domains (the N terminus up to the CC motif, the main body, and the C terminal α helix) exchanged between murine MIP-1α and the highly related, yet non-inhibitory chemokine human RANTES. The aim was to take away from MIP-1α the inhibitory activity and transfer it onto RANTES. The chimaeric cDNAs were generated by Overlap PCR, the chimaeric proteins produced using the Baculovirus System and purified in several chromatography steps. The functionality of the individual proteins was ascertained in calcium flux assays through CCR5, with only RMM showing markedly reduced activity. When the individual proteins were tested for stem cell inhibition in CFU-A assays, it was established that only the chimaera that contained the MIP-1α main body (flanked by the RANTES C and N termini) displayed any inhibitory activity similar, yet not as potent, as wild type MIP-1α. From that it was concluded that the main determinants for CFU-A cell inhibition reside within the main body of MIP-1α, with possibly other residues in the N and C termini required for full activity. It is very unusual that the activity of a chemokine is predominantly determined by a region other than the N terminus and may suggest that the receptor for this activity is quite different from the other CC chemokine receptors that have been identified to date. Additional chimaeras that have smaller units within the MIP-1α main body exchanged with the corresponding regions of RANTES have already been constructed at the DNA level and will be expressed and analysed for stem cell inhibitory activity in due course. It is hoped that this will narrow down the inhibitory region even further and lead to the generation of a peptide that has antagonistic or agonistic activities and is specific for the inhibitory receptor as this would be a valuable tool in the search for the identity of this receptor.

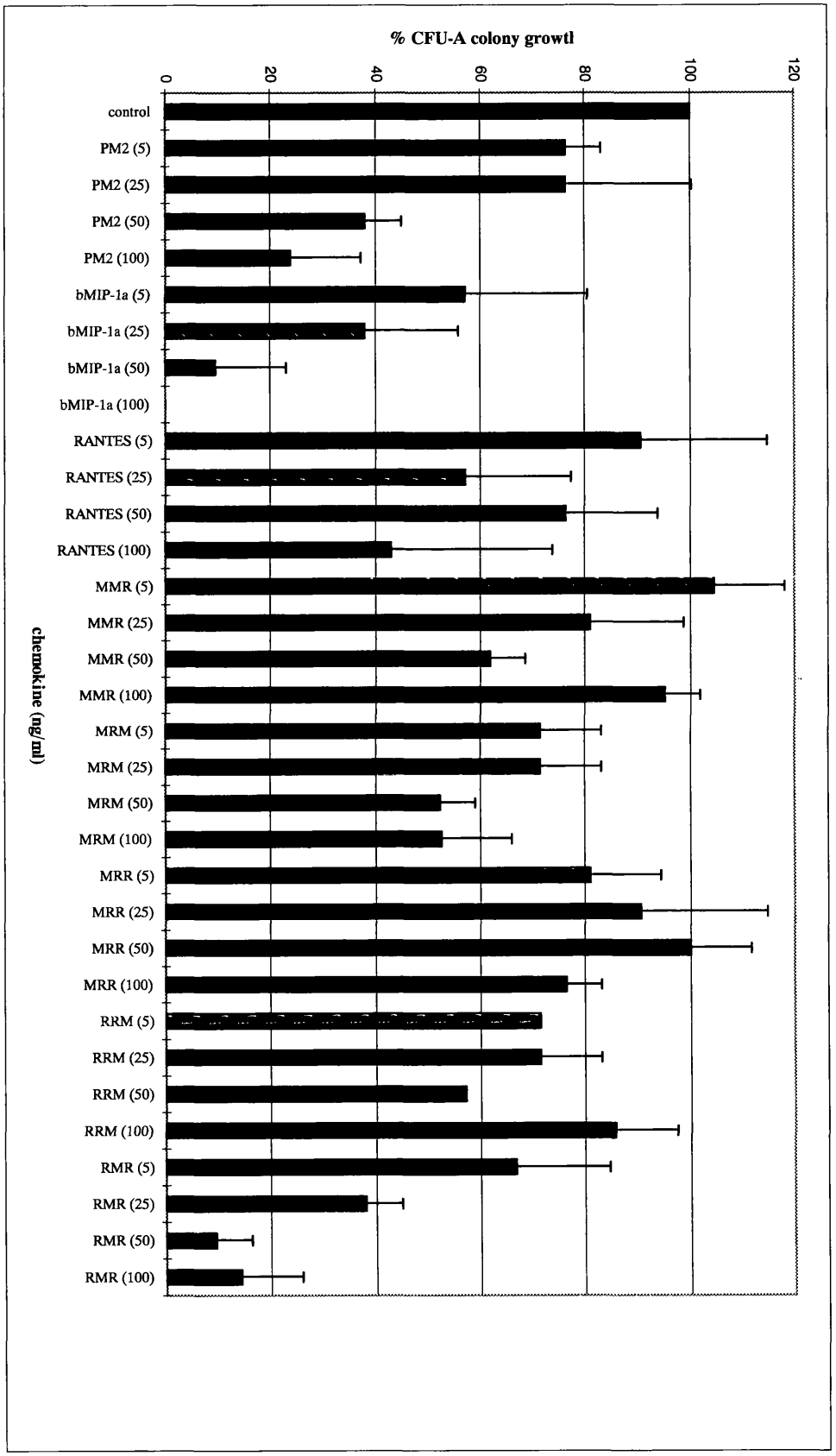


Fig. 6.9: Effect of chimearas on CFU-A cell proliferation

A CFU-A assay was performed as described in the Methods section and the purified proteins added at the concentrations given in brackets (in PBS). bMIP-1a = murine MIP-1α produced in the baculovirus system

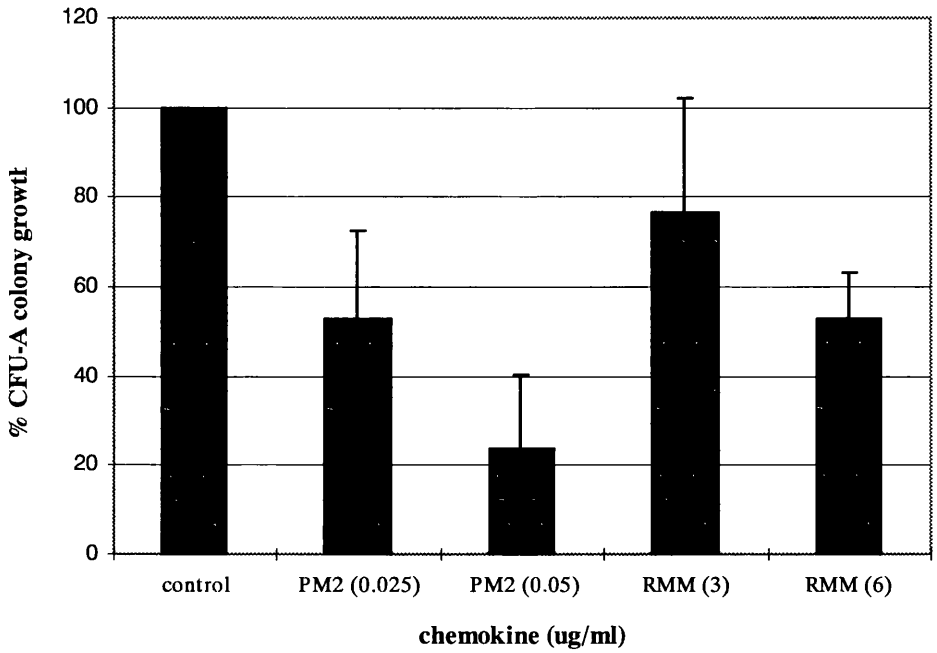


Figure 6.10: Effect of RMM on CFU-A cell proliferation

A one-layer 24-well plate CFU-A assay was performed as described in the Methods section. The chemokines were added to the wells at the concentration given in brackets (in μg/ml in PBS).

Proteins @ 100 ng/ml	Number of Cells per Colony ($x10^4$)
control	2.055
bMIP-1α	0.8025
RMR	1.0435
MRR	1.785
RRM	2.172
MRM	2.892

Table 6.1: Cell numbers in the chimaera-treated CFU-A colonies

A methylcellulose CFU-A assay was set up as described in the Methods section. At the end of the 11 day incubation period, 5-10 colonies were picked per chimaera, pooled in PBS and the number of cells determined using a cell counter.

Chapter 7: DISCUSSION

7.1. Introduction

Since their first description as proinflammatory mediators, chemokines have been implicated in many other areas including regulation of leukocyte trafficking during normal immune surveillance, angiogenesis and vascularisation, and regulation of haemopoietic stem/progenitor cell proliferation, mobilisation and homing (Zlotnik et al., 1999). MIP-1α is one such chemokine that has been shown to be involved in a number of different biological processes, including inflammatory responses, the regulation of haemopoietic stem and progenitor cells and the inhibition of keratinocyte proliferation.

Chemokines mediate their effects through members of the heptahelical G protein-coupled cell surface receptor family which are found on a wide range of different cell types. However, two other properties of chemokines can potentially influence their biological activities. The first one of these is their tendency to self-associate to form aggregates of varying sizes depending on their concentration and the ionic strength, pH and hydrophobicity of their environment (Clark-Lewis et al., 1995). Not all chemokines, however, seem to share this ability, with experimental evidence suggesting that HCC-2 (Sticht et al., 1999), I-309 (Keizer et al., 2000), SDF-1 (Crump et al., 1997) and Fractalkine (Mizoue et al., 1999) only exist as monomers. Others, such as IL-8 (Clore et al., 1990) and PF4 (St. Charles et al., 1989) cannot aggregate past the dimeric and tetrameric state, respectively, while MIP-1α, MIP-1β and RANTES are able to form large and heterogeneous aggregates (Lodi et al., 1994; Patel et al., 1993; Skelton et al., 1995). Despite the fact that aggregation-incompetent chemokine mutants are still fully functional in bioassays *in vitro* and that the affinity for self-association is lower than the affinity for chemokine receptor binding, a number of functions have been suggested for chemokine aggregation, including increased resistance to proteolysis (Paolini et al., 1994), a mechanism for limiting the active amount of circulating chemokine in the blood (Skelton et al., 1995) and the activation of specific signalling pathways at high chemokine concentration (Czaplewski et al., 1999).

The other property of chemokines that has the potential to influence their biological activities *in vivo* is their ability to interact with glycosaminoglycans. The study of this interaction and the determination of its biological relevance are complicated by the

diversity of glycosaminoglycans, both in their location as well as in their type and composition, and by the fact that different chemokines have very different affinities for these molecules. A further degree of complexity is introduced by the observation that the aggregation state of chemokines may influence their glycosaminoglycan-binding affinities. In the case of PF4, for example, the formation of the tetramer brings the individual binding sites on the monomers together in such a way that the overall glycosaminoglycan-binding area is increased which therefore results in a higher affinity of the tetramer for glycosaminoglycans than the individual monomers (Mikhailov et al., 1999; Stringer and Gallagher, 1997). Binding sites for heparin (a model glycosaminoglycan) have been identified in a number of chemokines and, as a general rule, seem to involve clusters of positive amino acids that interact with the negatively charged sulphate groups of glycosaminoglycans. As with the aggregation of chemokines, the relevance of chemokine-glycosaminoglycan interaction *in vivo* is not entirely clear. Mutants of MIP-1α (Graham et al., 1996; Koopmann and Krangel, 1997), SDF-1 (Amara et al., 1999), MIP-1β (Koopmann et al., 1999) and MCP-1 (Chakravarty et al., 1998) that have lost their ability to interact with the glycosaminoglycans, are nevertheless still fully functional in bioassays *in vitro*. It has, however, been suggested that this interaction is relevant for a number of situations *in vivo*, including immobilisation of chemokines on vessel walls, their efficient packaging into secretory vesicles, enhancement of their anti-viral activity and sequestration of chemokines.

The aim of the work presented in this thesis was to characterise the relationship between the structure of murine MIP-1α and its function as an inhibitor of the proliferation of transiently engrafting haemopoietic stem cells. In this context, the two properties of chemokines described above, aggregation and the interaction with heparin, were first of all considered. In chapter 3, potential mechanisms for the regulation of murine MIP-1α aggregation, the possible influence of divalent cations and the effect of concentration of MIP-1α on its aggregation, were investigated and a method for the isolation of different MIP-1α aggregation states developed. In the following chapter, the control of the interaction of murine MIP-1α with heparin was analysed and how this may be influenced by its aggregation state. Finally, the actual mechanism underlying MIP-1α-mediated haemopoietic stem cell inhibtion was examined. It was confirmed that none of the known murine MIP-1α receptors are involved in this particular function of MIP-1α. Furthermore, a region within murine MIP-1α was identified that confers stem cell inhibitory properties. Thus, the results presented in this thesis and discussed in the following sections have made

a further contribution towards the understanding of the way in which murine MIP-1α aggregation and proteoglycan binding is regulated. In addition, data was obtained on how MIP-1α controls the proliferation of haemopoietic stem cells and that this control is mediated through a novel receptor.

7.2. The Aggregation of MIP-1α

A number of observations regarding the forces that control the aggregation of MIP-1α had already been made. For example, it had been established that human MIP-1α at 0.5 mg/ml exists predominantly as a range of large self-associated multimers that are held together by hydrophobic as well as electrostatic interactions (Patel et al., 1993). More specifically, it was shown that dimerisation and tetramerisation is driven through the interaction of hydrophobic residues, whereas the formation of larger aggregates (past the tetramer) is controlled by electrostatic forces. However, partial and complete disaggregation of MIP-1α was also achieved by the neutralisation of specific negative charges. When the aspartate residue at position 26 of human MIP-1α was changed to an alanine residue, a mutant was generated that existed in an equilibrium between monomers, dimers and tetramers, but could not aggregate past the tetrameric state (Hunter et al., 1995). In the crystal structure of the murine MIP-1α tetramer, the Asp26 residue of one dimer is seen to form a salt bridge with the Arg45 residue of the other dimer, which, in addition to extensive hydrophobic interactions, helps to stabilise the tetramer. In another mutagenesis study (Graham et al., 1994), the neutralisation of a glutamate residue at position 66 of murine MIP-1α was also seen to result in a mutant that could not aggregate past the tetrameric state. This residue is, according to the crystal structure of the murine MIP-1α tetramer, also in a position that allows it to form a salt bridge with Arg45/Arg47, and has therefore been implicated in the stabilisation of tetramer-tetramer interactions. The neutralisation of a further acidic residue in murine MIP-1α (E66Q + D64N) resulted in a mutant that could not aggregate past the dimeric state (Graham et al., 1994). However, Asp64 is located on the external surface of the α helix where the distance to the nearest residue in the other dimer is about 10 Å which is too far for a direct interaction. The fact that a mutation at this position nevertheless interferes with aggregation, suggests that the overall charge distribution is also critical for the aggregation process. When, in addition to Glu66 and Asp64, a third acidic residue in the α helix is neutralised (E60Q), a complete disaggregation of murine MIP-1α to monomers is achieved (Graham et al., 1994). Since this residue is seen to point away from the tetramer and is thus not involved in monomer-monomer or dimer-dimer interactions

but may form a critical salt bridge with Arg45/Arg47 in tetramer-tetramer contacts, the dramatic effect this mutation nevertheless has on MIP-1α aggregation again supports the assumption that changes in the overall charge may disturb the monomer-dimer-tetramer equilibrium found in the native protein.

In addition to these mutagenesis studies, other investigations have characterised additional factors that have the potential to influence MIP-1α aggregation. It was shown that partial disaggregation was achieved in the presence of 1-2 M NaCl, while complete disaggregation occurs in the presence of 10 μM acetic acid (Graham et al., 1992). Serial dilutions of human MIP-1α were also demonstrated to have a profound effect on aggregation, with a predominantly monomeric MIP-1α solution found at concentrations below 0.1 μg/ml (Graham et al., 1994).

The work presented in chapter 3 represents further investigations into the factors controlling murine MIP-1α aggregation. It was first of all demonstrated that, analogous to the behaviour of human MIP-1α, dilutions of the murine homologue also produced differentially aggregated protein (Fig. 3.2). At concentrations of 10 μg/ml or higher, murine MIP-1α formed a range of oligomers, with most of the protein being present in higher order aggregates (tetramer and higher, data not shown). At 5 μg/ml it was mainly tetrameric (with some evidence of higher order aggregates), at 1 μg/ml predominantly dimeric and at concentrations below 0.5 μg/ml MIP-1α was completely disaggregated. As mentioned above, human MIP-1α requires further dilution to below 0.1 μg/ml for complete disaggregation which suggests that the forces that stabilise the human MIP-1α oligomers are slightly stronger than for the murine counterpart. This may be explained by the fact that human MIP-1α is more negatively charged than its murine homologue. Although the broadness and asymmetry of some of the peaks in Fig. 3.2 suggests that the preparations do not contain a completely homogeneous population of oligomers, the results nevertheless demonstrate that differentially aggregated MIP-1α can be prepared in which the separate aggregation states are isolated.

Another potential method of regulating MIP-1α aggregation emerged when the crystal structure of the murine MIP-1α tetramer was solved. Two different tetrameric structures of murine MIP-1α were detected, sometimes even within the same droplet. Both of these, however, were in association with calcium ions in almost identical positions (see Fig. 3.3). Because of the fact that 2/3 of the tetramer-tetramer contacts for one of the two types of

tetramers were mediated by these calcium ions, it was suggested that calcium may be involved in the aggregation of murine MIP-1α, which received further support from the observation that the MIP-1α tetramer could only crystallise when calcium was present. Despite there being evidence for the involvement of calcium in the self-association of other proteins (Ruano et al., 2000), this type of aggregation has so far not been described for chemokines. Ca^{2+} is a very ubiquitous intracellular mediator that is involved in many different processes, including neural signalling, muscle contraction, secretion and cell proliferation. In fact, chemokines are also known to induce intracellular calcium fluxes upon binding and activating their receptors. It may therefore not come as such a surprise that this universal messenger may be involved in the self-association of chemokines which would endow the cell with another means of regulating chemokine aggregation by varying the local Ca^{2+} concentration. This would also allow a fine tuning of chemokine aggregation. However, data presented in section 3.3. suggests that the presence of calcium ions is not required for the formation of higher order aggregates of murine MIP-1α. Murine MIP-1α was first completely disaggregated in 100 μM acetic acid in order to make the calcium ions accessible which were then chelated with 1 mM EDTA and 2 mM EGTA. This Ca^{2+}-depleted preparation of murine MIP-1α at ~13 μg/ml was then analysed for its aggregation state on a sizing column. Fig. 3.4 shows that even in the absence of calcium ions murine MIP-1α is still capable of forming higher order aggregates well beyond the tetrameric state as would be expected at that concentration. It is unlikely that other ions present in the solution substituted for the removal of calcium and mediated the aggregation process in its absence for two reasons. First, during the crystallisation of the MIP-1α tetramer it was noticed that it was not possible to replace the calcium ions with a number of other ions tested, thus suggesting that it was specifically calcium ions that were required for the crystallisation of the tetramer. Second, although EDTA and EGTA bind calcium ions with the highest affinity, they also complex other divalent cations, such as magnesium. It can therefore be assumed that the presence of EDTA and EGTA at these concentrations also resulted in a depletion of other divalent cations. The above results also demonstrate that the aggregation of MIP-1α is a dynamic and reversible process since the complete disaggregation in the presence of acetic acid was reversed upon its removal, thus allowing the re-formation of oligomers.

Thus, the aggregation of murine MIP-1α, like the aggregation of other chemokines, is controlled by the ionic strength and the pH of its environment and not by the presence of specific divalent cations. This may nevertheless represent a physiologically relevant control

mechanism since salt concentrations similar to the salt concentrations found in the blood were shown to favour the formation of the MIP-1β dimer at 10 μM (Laurence et al., 1998) - a concentration at which some chemokines have been shown to be secreted from stimulated cells (Sotsios et al., 2000). The fact that the aggregation of MIP-1α is concentration-dependent may also be of relevance *in vivo*. Although the concentration of free, circulating chemokines is likely to fall below the threshold for aggregation as do the concentrations required for the mediation of most of their biological effects, high local chemokine concentrations may nevertheless be achieved near sites of secretion. In addition, changes in the local environment, as described above, as well as immobilisation of chemokines on proteoglycans may create conditions under which chemokine aggregation can be achieved. Aggregation mutants of a number of chemokines have been generated (see Introduction) which generally display full activity in all bioassays. For example, all three aggregation mutants of murine MIP-1α that were described above retain full stem cell inhibitory capacity in an *in vitro* assay (Graham et al., 1994). However, bioassays for chemokine function do not reflect the environment under which these functions are carried out *in vivo*. It may therefore be that aggregation is essential for certain processes for which there are currently no *in vitro* assays available. Indeed, it was only recently demonstrated that RANTES is able to trigger two different signalling pathways depending on its concentration. At nanomolar concentrations, chemotaxis is initiated via the activation of G protein-coupled receptors. At higher concentrations, micromolar and above, a different signalling pathway is activated which involves a Protein Tyrosine Kinase-mediated signal, leading to cell activation, and which requires RANTES aggregation, since an aggregation mutant which cannot aggregate past the dimeric state is unable to initiate the latter signalling pathway even at high concentrations (Appay et al., 1999; Bacon et al., 1995). Other aggregation-dependent activities may therefore still be discovered for other chemokines.

7.3. MIP-1α - Heparin Interactions

As mentioned above, there is evidence to suggest that the presence of proteoglycans may enhance chemokine aggregation (Hoogewerf et al., 1997). Equally, there is evidence that shows that aggregation enhances proteoglycan binding. A first indication for this phenomenon came from studies on PF4 when it was shown that the individual heparin binding sites on each separate monomer are arranged in the tetramer in a way that increases the overall heparin binding surface which results in a higher affinity of the tetramer for

heparin than each of the monomers alone (Mikhailov et al., 1999; Stringer and Gallagher, 1997). Indeed, it is believed that PF4 is released from the α-granules of platelets as a macromolecular complex in which the tetramer is bound to a proteoglycan carrier. Other studies have also since demonstrated the existence of positive cooperativity between the individual glycosaminoglycan binding sites in chemokine oligomers which results in an induction of chemokine oligomerisation, when bound to proteoglycans, and thus in an increase in the local chemokine concentration (Hoogewerf et al., 1997).

It was therefore decided to undertake similar studies on murine MIP-1α in order to determine whether its affinity for self-association can be increased in the presence of proteoglycans. If that were the case, one could assume that MIP-1α aggregation may occur more easily *in vivo* which would result in high local concentrations of immobilised chemokine.

The following basic residues in MIP-1α have been implicated in heparin binding, Arg17, Lys44, Arg45 and Arg47, which are seen to form a cationic cluster on one side of the monomer (Graham et al., 1994; Koopmann and Krangel, 1997; and see Fig. 4.1). Upon dimerisation, these two clusters are slightly twisted away from each other, however, the three closely spaced basic residues, Lys44, Arg45 and Arg47, of both monomeric units are still found on top of the dimer and may therefore be able to interact with the same glycosaminoglycan chain. This may in fact be comparable to findings that were made with regards to the interaction of the IL-8 dimer with a single glycosaminoglycan chain (Spillmann et al., 1998). The heparin binding sites of IL-8 are located on the solvent-exposed side of the α helices and are thus positioned at opposite ends on the top of the dimer. The authors, however, isolated a glycosaminoglycan chain that displayed high affinity binding to the IL-8 dimer and which was shown to contain two highly sulphated regions that were separated by a distance just long enough to bridge the gap between the heparin binding sites on top of the two helices of the IL-8 dimer. Fig. 4.1 also shows the arrangement of the individual heparin binding sites in the MIP-1α tetramer where they are found to be organised in a circle on top of the oligomer. Again, it could be envisaged that a glycosaminoglycan chain is aligned on top of the tetramer in such a way that allows it to interact with more than one of the four heparin binding sites.

The interaction of the MIP-1α monomer, dimer and tetramer with glycosaminoglycans was analysed using heparin affinity columns as sources for immobilised glycosaminoglycan chains since these allowed the measurement of the relative affinities of the different

oligomers as expressed by the concentration of NaCl or soluble heparin required to interrupt the protein - heparin interactions and elute the protein from the columns. As a first approach, the relative heparin binding affinities of the three murine MIP-1α aggregation mutants (described in section 4.3.) PM1 (tetramer), PM2 (dimer) and PM3 (monomer) were determined as molarity of salt needed for their displacement. Fig. 4.2 shows that there is indeed a difference in their affinities. However, it came as a surprise to see the tetramer elute first (0.34 M NaCl), followed by the dimer (0.4 M NaCl) and the monomer (0.45 M NaCl), the latter of which had the highest affinity for heparin. This suggested that there was no positive cooperativity between the individual heparin binding sites in the oligomers and that, in fact, the opposite was observed. There are two alternative explanations for this observation. First, a progressive obstruction of the heparin binding site may occur upon aggregation which reduces the access of the sulphate groups of the glycosaminoglycan chains to the basic residues in MIP-1α. Indeed, it was suggested that the dimerisation surface of murine MIP-1α may slightly overlap with its heparin interaction site (MacLean, personal communication). The other possible explanation is that the progressive neutralisation of negative charges, the process by which these aggregation mutants were generated, results in an increasing affinity for heparin. The interaction of proteins with heparin is primarily of an electrostatic nature which could explain why a change in the overall charge of a protein may cause a change in its affinity for heparin. Indeed, it was shown in another study that chemokines in which negatively charged amino acids are interspersed between the positive heparin binding determinants exhibit more selective binding to heparin with a higher affinity for glycosaminoglycan chains in which the sulphate groups are spaced in a way that avoids a clash with the negative residues in the protein (Witt and Lander, 1994). The neutralisation of negative charges in the aggregation mutants may therefore result in higher affinities as repelling charges are removed.

In order to be able to distinguish between these two possibilities, differentially aggregated murine MIP-1α was prepared by progressive dilution as described in the previous section, with 10 μg/ml containing a range of higher order aggregates, 5 μg/ml mainly tetramers, 1 μg/ml predominantly dimers and 0.5 μg/ml monomers (see Fig. 3.2). However, when these were applied to the heparin column, no significant difference was observed between the individual elution profiles (Fig. 4.3) with the protein from all four preparations eluting on average at 0.36 M NaCl which is identical to the elution point of the tetrameric variant. This suggests that aggregation has no impact on murine MIP-1α's affinity for heparin and that the differences observed between the elution profiles of the aggregation mutants were

in fact due to their charge differences. As there may be a slight chance that the salt concentrations used for the elution of differentially aggregated MIP-1α may lead to a partial disintegration of the complexes, the same preparations of differentially aggregated MIP-1α were prepared, applied to the same column, but this time eluted with increasing concentrations of soluble heparin. The results, which are shown in Fig. 4.6, seem to support the previous findings that the aggregation state of MIP-1α is irrelevant for its heparin binding affinity. However, it may still be possible that the samples become further diluted on the heparin column, resulting in a complete disaggregation, or that the interaction with heparin causes the complexes to fall apart, especially if there is indeed a partial occlusion of the heparin binding sites in the aggregates. The latter possibility may be less likely as that would imply that the forces holding the heparin - MIP-1α complex together are stronger than the forces stabilising the oligomers. This is probably not the case since the interactions between the MIP-1α molecules are more extensive than between MIP-1α and heparin as reflected in the fact that a NaCl concentration of as little as 0.36 M is enough to disrupt MIP-1α - heparin interactions, whereas 1-2 M NaCl are required to achieve at least a partial dissociation of MIP-1α aggregates. However, in order to rule out these possibilities completely, covalently cross-linked and therefore stable MIP-1α oligomers were also analysed for their binding to the heparin column and found to elute at exactly the same point (0.36 M NaCl) as the oligomers prepared by dilution, regardless of their aggregation state (Fig. 4.5). These stable oligomers were derived from a direct cross-linking of side chains from different monomeric units, as described in detail in section 4.5. Since there is no linker arm involved, this is a very local method for cross-linking and therefore expected to take place only where there is a true subunit interface. The specificity of this method seems to be confirmed in the fact that no trimers were observed. It is therefore likely that the cross-linked oligomers are a true representation of the native MIP-1α oligomers and that the tertiary structure of MIP-1α was not grossly perturbed as suggested by the fact that the cross-linked species bind to heparin with wild type affinity.

HiTrap heparin matrices such as the one employed in the experiments described above may occasionally act more like an ion-exchange matrix rather than a specific affinity matrix since the individual heparin chains are immobilised via multiple interactions with the agarose beads and therefore not completely free to interact with their ligands in a more physiological manner. For that reason, a different heparin matrix was obtained in which the glycosaminoglycan chains had been attached to the agarose beads by a process called reductive amination which immobilises the chains by their reducing ends, thus leaving the

remainder of the molecule free to interact with the ligands in a way that is more likely to reflect interactions that occur *in vivo*. The experiments described above were repeated using this new heparin affinity column. The salt elution of differentially aggregated murine MIP-1α (Fig. 4.7), the salt elution of cross-linked murine MIP-1α (Fig. 4.8) and the heparin elution of differentially aggregated MIP-1α (Fig. 4.9) all demonstrate that there really is no difference in the affinities of the different murine MIP-1α oligomers for heparin. The position and shape of the elution peaks of the differentially aggregated MIP-1α were slightly altered on the heparin matrix prepared by reductive amination which indicates that MIP-1α may interact somewhat differently with this matrix as compared to the HiTrap matrix. However, the results are consistent in that self-association does not affect murine MIP-1α's affinity for heparin, suggesting that there is no positive cooperativity between the individual binding sites which seem to be positioned in a way that only allows one site at a time to interact with a given glycosaminoglycan chain. Aggregation, on the other hand, also does not interfere with heparin binding as first suggested by the results obtained with the aggregation mutants (Fig. 4.2). The heparin binding site does not become partially buried upon oligomerisation since the covalently linked oligomers retain wild type affinity. It can therefore be assumed that the difference observed between the aggregation mutants can be exclusively attributed to their charge differences. In summary, the affinity of murine MIP-1α for heparin is not determined by its aggregation state, but rather by its overall charge which may explain why, in comparison to other chemokines, murine MIP-1α is a fairly weak heparin binder since it has a net negative charge, while most other chemokines have a net positive charge.

Despite the fact that the same results were obtained using two different heparin matrices, the possibility nevertheless remains that an effect of aggregation on heparin binding may have been observed with heparan sulphate chains that have a specific distribution and spacing of sulphate groups that mirrors the position of the heparin binding sites in the MIP-1α oligomers more closely. Heparin chains are generally very highly sulphated, whereas heparan sulphate chains often have longer stretches of undersulphated disaccharide units between highly sulphated regions. The importance of undersulphated regions in bridging the space between individual heparin binding sites, especially when this intervening space contains negatively charged amino acids that would otherwise clash with sulphate groups of the glycosaminoglycan chain, has already been demonstrated (Spillmann et al., 1998; Witt and Lander, 1994). It may therefore be the case that *in vivo*, where there exists a wide range of different glycosaminoglycan chains with different patterns of sulphation, specific

chains may be found that display increased affinities for the different MIP-1α oligomers. However, as far as the binding to heparin is concerned, different aggregation states of MIP-1α have no selective advantages over other aggregation states as all the oligomers tested showed identical binding to heparin.

It may still be the case that binding of MIP-1α to heparin induces oligomerisation and that the affinities measured above may in fact represent the affinity of MIP-1α oligomers that form while bound to the heparin matrix. Such an oligomerisation-inducing effect of heparin has previously been demonstrated where immobilised heparin was shown to induce MIP-1α dimerisation and, in a separate experiment, the apparent molecular weight of MIP-1α, as determined by gel filtration chromatography, was seen to increase from 19.9 kD (dimer) to 52 kD in the presence of soluble heparin (Hoogewerf et al., 1997). However, if immobilised heparin indeed induces MIP-1α dimerisation, it would not explain the indistinguishable affinity of the stable tetramer (see Fig. 4.5 and 4.8), again arguing that the aggregation state does not influence MIP-1α's heparin binding affinity. The MIP-1α concentration used in the study by Hoogewerf *et al.* was also up to 50 times higher than the highest concentration employed in this thesis. At concentrations as high as that, MIP-1α oligomers are expected to form and, if the soluble heparin chains employed by Hoogewerf *et al.* were long enough, each glycosaminoglycan chain may have been able to bind to more than one MIP-1α molecule. However, in the work presented in this thesis, MIP-1α's heparin binding affinity was tested at concentrations of as low as 10 ng/ml, when MIP-1α is completely disaggregated, and still found to be the same as for the MIP-1α oligomers (data not shown). It was therefore concluded that the affinity of murine MIP-1α for heparin does not change upon oligomerisation of this chemokine.

7.4. Molecular Mechanism of MIP-1α-mediated Haemopoietic Stem Cell Inhibition

MIP-1α's activity as a haemopoietic stem cell inhibitor was first demonstrated in 1990, when it was shown to inhibit the proliferation of transiently engrafting stem cells in the CFU-A assay, an *in vitro* assay that detects a cell that has properties indistinguishable from day 12 CFU-S cells (Graham et al., 1990). This property of MIP-1α has since been confirmed *in vivo*, and, in addition, a stimulatory effect on the proliferation and the mobilisation of more mature progenitor cells described. However, the molecular

mechanism underlying MIP-1α-mediated stem cell inhibition is still largely unknown. It has been illustrated that aggregation is not required for the direct inhibition of CFU-A cells, since aggregation-incompetent MIP-1α mutants are fully active as stem cell inhibitors. A further MIP-1α mutant in which the heparin binding site had been destroyed also retained full stem cell inhibitory capacity, suggesting that MIP-1α does not need to be immobilised on proteoglycans in order to affect the proliferation of CFU-A cells.

A number of different chemokines have now been shown to be involved in the regulation of haemopoietic stem cells and, more specifically, in the inhibition of their proliferation. However, data presented in this thesis demonstrates that none of the chemokines tested have any effect on the proliferation of CFU-A cells (Fig. 5.1). Not even chemokines that are very closely related to MIP-1α, such as MIP-1β, RANTES and DCCK-1, or chemokines that have been shown to inhibit the proliferation of other haemopoietic stem cells, such as MPIF-1 and MPIF-2 (Patel et al., 1997), had any noticeable effect on the formation of CFU-A colonies. Among the chemokines tested for CFU-A cell inhibition were ligands for all of the currently known CC chemokine receptors (Table 5.1). Furthermore, included also were other ligands for each one of the four murine MIP-1α receptors, CCR1, CCR3, CCR5 and D6. Since none of these ligands (neither for the MIP-1α receptors, nor for any of the other known CC chemokine receptors) showed any significant activity as a CFU-A cell inhibitor, it is unlikely that any of the known CC chemokine receptors are involved in CFU-A cell inhibition. It therefore seems that this effect is mediated by a novel, as yet uncharacterised receptor, which is expressed on CFU-A cells and likely to be specific for MIP-1α.

Further support for the assumption that none of the known MIP-1α receptors are involved in MIP-1α-mediated stem cell inhibition was obtained from the analysis of the inhibitory potential of a number of human MIP-1α variants (see also section 5.3.). The two human MIP-1α isoforms, LD78α and LD78β, which have recently been characterised (Menten et al., 1999; Nibbs et al., 1999), are almost identical in sequence apart from single amino acid substitutions at positions 2, 39 and 47. In addition, sequence analysis predicts two alternative signal sequence cleavage sites for LD78α (Nibbs et al., 1999) producing the full-length protein and a -4 variant, in which the first four amino acids are missing. However, so far only the -4 variant has been isolated from natural sources (Menten et al., 1999). For LD78β, on the other hand, the full length as well as a -4 variant have been detected *in vivo* (Menten et al., 1999), however, studies from our laboratory suggest that

only the full length LD78β protein is produced in cells. Receptor binding studies have highlighted differences in which these four proteins bind to murine CCR1, CCR5 and D6 which can be attributed to the two different amino acids found at position 2 in the two full length proteins, which are absent in the two -4 variants. LD78α has a serine residue at that position, thus it has been alternatively named MIP-1αS and will be referred to as such from now on, while LD78β has a proline in that position and is thus now referred to as MIP-1αP. MIP-1αS is a slightly better ligand than MIP-1αP for CCR1, while MIP-1αP has a much higher affinity for CCR5 and D6 than MIP-1αS (Nibbs et al., 1999). The binding affinities for the two -4 variants are indistinguishable from each other, but are high as far as binding to CCR1 is concerned, whereas their affinity for CCR5 and D6 is very poor. Because of the differences with which these four human MIP-1α variants bind to these three murine receptors, they were ideally suited for the investigation of the possible identity of the inhibitory receptor. If indeed murine CCR1, CCR5 or D6 were involved in this process, then it could be expected that the differences in the receptor affinities of the four variants will be reflected in their potencies as haemopoietic stem cell inhibitors, as measured in the CFU-A assay. However, results presented in Fig. 5.2 clearly indicate that all four human MIP-1α isoforms are potent inhibitors of CFU-A cells with no significant difference observed between them. This again points to a novel receptor as being the mediator of MIP-1α's inhibitory signal - a receptor, which binds to all four human MIP-1α variants with indistinguishable affinities and which does not require the first four amino acids for full activation.

However, the most unequivocal way of determining whether any one of the four murine MIP-1α receptors is involved in stem cell inhibition is by testing bone marrow from mice, in which the genes for each individual MIP-1α receptor are deleted, in CFU-A assays for inhibition. If their CFU-A cell proliferation is still inhibited in the presence of MIP-1α, it would then rule out the participation of the knocked-out receptors in stem cell inhibition. Bone marrow was therefore obtained from $CCR1^{-/-}$, $CCR3^{-/-}$, $CCR5^{-/-}$ and $D6^{-/-}$ mice and their stem cells analysed for their sensitivity to MIP-1α. None of these null mice displayed any defects that might be explained by a lack of haemopoietic stem cell control (Broxmeyer et al., 1999; Gao et al., 1997; Gerard et al., 1997; Huffnagle et al., 1999; Zhou et al., 1998; A. Humbles, unpublished results; D. Cook, unpublished results). The $CCR1^{-/-}$ mice exhibited a phenotype that suggested that this receptor mediates MIP-1α's stimulation of more mature progenitors. However, MIP-1α-mediated stem cell inhibition appeared to be normal (Broxmeyer et al., 1999). Indeed, Fig. 5.3 shows that CFU-A cells from all four

knock-out mice are inhibited by MIP-1α, again demonstrating that none of these receptors are required for stem cell inhibition. It may appear from Fig. 5.3 c) that CFU-A cells from $CCR3^{-/-}$ mice are slightly refractory to the effects of MIP-1α as a concentration of 200 ng/ml, 2 fold higher than required for the cells from the other knock-out mice, is necessary for full inhibition. The CFU-A colonies formed by these cells are also on average a lot larger than the colonies formed by CFU-A cells from the other null mice. In fact, it is the unusually large size of these colonies that interferes with proper assay scoring, since uninhibited CFU-A colonies are identified on the basis of having a diameter of larger than 2 mm. Therefore, despite the fact that the colonies formed by $CCR3^{-/-}$ cells showed the typical signs of inhibition at 100 ng/ml (denser/more darkly stained and very well defined colony edges), their diameter nevertheless remained above 2 mm. It is unknown whether and how the absence of CCR3 can result in this altered colony morphology. It is not even clear whether it is a cell migratory or a cell proliferation effect, however evidence points to the former possibility since these colonies are paler and more diffuse looking which suggests that they contain the same number of cells spread out over a larger surface. It is unlikely that this effect is mouse strain specific, as the cells from the $CCR1^{-/-}$ mice, which are of the same strain, do not form such large colonies. However, since the $CCR3^{-/-}$ colonies inhibited with 200 ng/ml were indistinguishable from the other inhibited colonies, it was assumed that MIP-1α-mediated stem cell inhibition was not impaired by the absence of CCR3.

One might still be able to argue that MIP-1α, in the absence of one of its receptors, may revert to the use of one of its other receptors for stem cell inhibition. In order to be able to rule out this possibility, the chemokine analogue AOP-RANTES which can bind to CCR1, CCR3, CCR5 and D6 (Elsner et al., 2000 and data not shown) was included in the CFU-A assays at a concentration 5-10 fold higher than MIP-1α and thus competed with MIP-1α for binding to these receptors (Elsner et al., 2000; Townson et al., 2000). Fig. 5.4 shows that AOP-RANTES on its own does not have any effect on stem cell proliferation. Furthermore, it shows that its presence does not abrogate or even impair MIP-1α's ability to inhibit CFU-A cell proliferation, even when the CFU-A assays were carried out using bone marrow from the four receptor knock out mice. As AOP-RANTES at these concentrations will displace MIP-1α from its known receptors (Elsner et al., 2000; Townson et al., 2000), one must assume, the MIP-1α conveys its inhibitory signal via another, as yet uncharacterised receptor.

As all of the data presented above make a very strong case for the existence of a novel inhibitory receptor and thus ruled out the involvement of any of the known MIP-1α receptors in stem cell inhibition, attention was then turned to MIP-1α itself and attempts made at identifying an inhibitory motif within the structure of this chemokine which can then be of assistance in the search for the inhibitory receptor.

The best way of proving that a particular region of MIP-1α is involved in stem cell inhibition is by separating it from the rest of the molecule and by testing whether it can effect inhibition in isolation. However, since the conformation of an effector domain is likely to be as crucial for activity as its primary sequence, it is necessary to present the detached domain in a framework that will conserve the domain's native secondary structure, but that will not in itself participate in the reaction. Such an inert framework is found in the chemokine RANTES which has never been demonstrated to possess any activity on haemopoietic stem cells and which, as shown in Fig. 5.1, does not inhibit CFU-A cells. However, despite this functional difference between MIP-1α and RANTES, their structures are very highly homologous (Fig. 6.1), thus making it possible to exchange domains between them in a way that will preserve the secondary structure of the exchanged domain and that will not alter the overall tertiary structure of the protein. A rational basis for the design of these chimaeric molecules was provided by the availability of the NMR structure of human RANTES (Skelton et al., 1995) and the crystal structure of murine MIP-1α (J MacLean, unpublished results). Initially, it was decided to divide the chemokines into three major domains; (1) the flexible amino terminus up to the CC motif, (2) the main body of the chemokine between the second and the fourth cysteine residue which includes the loop, that leads into the first β strand, and the triple-stranded β sheet, and (3) the C terminal α helix. These domains were swapped singly or doubly in all possible combinations between MIP-1α and RANTES by a PCR-based method, and the chimaeric proteins expressed in the baculovirus system and purified from the cell culture medium. All of the recombinant proteins were functional, as determined by their ability to induce a calcium flux through CCR5 (Fig. 6.8) with the exception of RMM (MIP-1α + N terminus of RANTES) which was only partially functional. When all of the recombinant proteins were tested in the CFU-A assay for stem cell inhibition, only the plates that contained wild type MIP-1α and RMR (MIP-1α body + RANTES N/C terminus) showed clear signs of inhibition (Fig. 6.9 and 6.10), as reflected in the reduced number of CFU-A colonies found on the plates, in the morphology of the inhibited colonies which in both cases were smaller and denser, and in the reduced number of cells per colony (Table 6.1).

Despite the fact that MRR, RRM and MRM induced a robust calcium flux in CCR5-transfected cells, they showed no activity in the CFU-A assay, thus indicating that the MIP-1α N and C terminus are not sufficient for stem cell inhibition and that the main determinants for stem cell inhibition reside within the main body of the chemokine. The fact that RMR is not quite as potent as wild type MIP-1α in CFU-A cell inhibition suggests that further residues either in the amino or the carboxy terminus of MIP-1α may nevertheless be required for full activity, or that the structure of the MIP-1α main body becomes slightly altered when inserted between the RANTES N and C termini. It was surprising in that context that the other two chimaeric proteins that contained the main body of MIP-1α, MMR and RMM, did not inhibit the proliferation of CFU-A cells. This suggests that the conformation of the main body of MIP-1α is better maintained when the adjacent N and C terminus both originate from RANTES. This is supported by the poor performance of RMM in the calcium flux assays. Experiments are currently underway that will further subdissect the MIP-1α main body in an attempt to narrow down the inhibitory region even more.

What do these results suggest about the way in which MIP-1α interacts with its inhibitory receptor and how does this compare to what is already known about the interaction of chemokines with their receptors? Although it has been suggested that chemokines may exert their inhibitory effects by displacing growth factors required for the proliferation of stem cells from proteoglycans on which they are presented to the growth promoting receptors on the stem cells, it is more likely that such a specific effect as seen with MIP-1α is mediated through a specific receptor. It is also unlikely that this is a receptor other than a member of the family of G protein-coupled receptors since no chemokine has ever been shown to be able to interact with other types of protein receptors. Most activated chemokine receptors couple to the $G_{\alpha i}$ type of G protein subunits which is inhibited by Pertussis Toxin, thus making this a commonly employed test for the involvement of this type of G protein. This approach was also applied to this case in order to establish whether $G_{\alpha i}$ subunits are part of the signalling pathway that leads to MIP-1α-mediated stem cell inhibition. Pertussis Toxin was added to the CFU-A assay plates at concentrations of 1, 10, 100 and 1000 ng/ml, as commonly used (data not shown). However, even in the absence of MIP-1α, Pertussis Toxin has an inhibitory effect on the formation of CFU-A colonies which makes it unsuitable for this approach.

The current dogma of how chemokines interact with their receptors (see Introduction) states that this is a 2-step process in which the first step involves the initial docking of the chemokine, possibly via its N loop (the region between the cysteine motif and the first β strand), to the receptor. This step is determined by how specific the ligand is for this particular receptor and is also the main determinant for receptor-ligand affinities. This is followed by the activation of the receptor by the flexible N terminus of chemokines. The crucial role that the N terminus of chemokines plays in receptor activation has been confirmed by a great number of mutagenesis studies. In a subgroup of CXC chemokines, a three amino acid motif - ELR - which directly precedes the CXC motif has been shown to be essential for receptor activation, while in most CC chemokines the extreme N terminal residues are the most important ones for receptor interactions, as reflected in the differences in the binding of the four human MIP-1α variants to CCR1, CCR5 and D6 (Nibbs et al., 1999). It was therefore surprising to see that an integrity of the N terminus of MIP-1α is not required for the binding to and activation of the inhibitory receptor, as all four human MIP-1α variants which had shown such marked differences in binding to CCR1, CCR5 and D6 displayed the same potencies as stem cell inhibitors. This also suggests that the inhibitory receptor found on CFU-A cells is quite different from the other known MIP-1α receptors which receives further support from the observation that AOP-RANTES, which can interact with all four known MIP-1α receptors, does not seem to be able to bind to the inhibitory receptor. The assumption that the N terminus may not be of such major importance for the interaction with the inhibitory receptor also receives further support from the studies with the chimaeric mutants which suggest that the region between the second and the fourth cysteine residue contains the main determinants for inhibition. It can, however, not be ruled out that there are also crucial residues in the N terminus which are conserved between MIP-1α and RANTES. One could thus imagine the possibility that the presence of the MIP-1α main body in RMR allowed this protein to dock to the inhibitory receptor (something that RANTES cannot do because it lacks the docking site) and that, once it was tethered to the receptor, residues that are conserved between the RANTES and the MIP-1α N termini may result in almost full activation. A possible role for the MIP-1α N terminus is also suggested by the fact that DCCK-1, a CC chemokine whose N terminal region is very different from MIP-1α's sequence while the rest of the molecule shows a high degree of homology, is nevertheless nonfunctional as a stem cell inhibitor.

When one compares the tertiary structure of MIP-1α to those of MIP-1β and RANTES, they are found to be most divergent in the N terminal region as well as in the loop connecting strand 1 and strand 2 of the β sheet. On comparison of the primary sequence of human RANTES and murine MIP-1α, the greatest number of nonconservative changes is found just in front of the CC motif, between cysteines 2 and 3 and around the α helix. As most of the evidence listed above, including the data from the chimaera studies, point to the main body of MIP-1α as the site for the main determinants for inhibition, future mutagenesis studies will focus on that region, especially the stretch between cysteines 2 and 3 which contains a lot of nonconservative changes between MIP-1α and RANTES.

7.5. Conclusions and Aspects for Further Investigations

The aim of this thesis was to undertake extensive structural studies on the murine chemokine MIP-1α that may shed some light on the mechanism by which this protein inhibits the proliferation of transiently engrafting haemopoietic stem cells. Attention was first turned to the two well known properties of chemokines, their ability to self-associate and to interact with proteoglycans. In addition to what was already known about the factors controlling murine MIP-1α aggregation, it was shown that this process is in large part concentration-dependent, however, the concentrations required for its aggregation lie beyond the concentrations required for receptor activation, thus making it unlikely to be important for the direct inhibition of stem cells. It has previously been demonstrated that aggregation-incompetent murine MIP-1α mutants are potent stem cell inhibitors, thus lending further support to the assumption that MIP-1α interacts with its receptor as a monomer. Nevertheless, it remains possible that aggregation is important for other, as yet undescribed, activities of MIP-1α and that it is of relevance for the production and secretion of MIP-1α.

It was further demonstrated that the aggregation process was not influenced by the presence of calcium ions, nor did there seem to be a connection between the aggregation state of MIP-1α and its affinity for heparin. It is still possible that MIP-1α, at high local concentrations, interacts with proteoglycans as an oligomer, but as far as the binding to heparin is concerned, aggregation was not of any advantage. Despite the fact that heparin is often used in the study of chemokine - proteoglycan interactions, it does not accommodate for the diversity of proteglycans, especially in the sulphation patterns of the glycosaminoglycan moieties. It has been demonstrated that specific sulphation patterns of

heparan sulphate chains in the stem cell niche can have differential effects on haemopoietic stem cells (Drzeniek et al., 1999; Gupta et al., 1998), and chemokines were shown to have different affinities for different heparin/heparan sulphate chains depending on the frequency and distribution of the sulphate groups along the chain (Witt and Lander, 1994). It may therefore be possible that there are specific proteoglycans present in the bone marrow microenvironment that display differential binding of MIP-1α oligomers which may play a role in the immobilisation and sequestration of this chemokine. Since a mutant of MIP-1α that has lost its ability to interact with heparin is nevertheless a potent stem cell inhibitor as demonstrated in the *in vitro* CFU-A assay, it is unlikely that the interaction with proteoglycans, in a process of ligand presentation, is directly required for the interaction of MIP-1α with its inhibitory receptor. For the same reason, it is unlikely that MIP-1α exerts its suppressive effect by displacing growth factors, that are required for CFU-A cell proliferation, from proteoglycans. It was therefore assumed that stem cell inhibition by MIP-1α is mediated via a specific, probably G protein-coupled, receptor.

It was further shown in this thesis that the inhibitory receptor is not one of the four known murine MIP-1α receptors, but a novel, as yet unidentified, receptor. The analysis of a number of murine MIP-1α chimaeras and human MIP-1α variants established that the main body of MIP-1α contains the major determinants for interacting with the inhibitory receptor and suppressing the proliferation of CFU-A cells and that the extreme N terminus plays a rather negligible role. This distinguishes this receptor from the other known MIP-1α receptors and therefore makes it an interesting target for future studies. The identification of the inhibitory receptor is also of particular interest in the context of leukaemia, as stem cells from patients suffering from Chronic Myeloid Leukaemia are resistant to MIP-1α–mediated inhibition, suggesting a defect in the signalling pathway following the binding of MIP-1α to these cells (Chasty et al., 1995; Eaves et al., 1993; Holyoake et al., 1993). For that same reason, it is also important to identify the downstream signals following the activation of the inhibitory receptor by MIP-1α. As it is currently impossible to obtain a pure population of MIP-1α-sensitive haemopoietic stem cells for direct analysis, other, more indirect ways of investigation have to be employed. These include a continuation of the chimaera approach that may eventually lead to the identification of an inhibitory motif and to the derivation of an inhibitory peptide that spans the identified domain. Furthermore, it is possible to obtain bone marrow from mice in which the genes for individual components of signalling pathways have been deleted and which can then be tested in CFU-A assays for the integrity of the signalling pathway that

leads to inhibition. Protocols for the enrichment for MIP-1α-sensitive stem cells by fluorescence-activated cell sorting also exist, which may make it possible to obtain a population of cells that is sufficiently pure to allow their mRNA to be used for the construction of an expression library that can then be screened for the expression of receptors that bind to either the full length MIP-1α protein or, if available, an inhibitory peptide.

Although the phenotype of the MIP-1α knock-out mice suggests that MIP-1α is not one of the key players in stem cell inhibition and that back-up systems exist that can compensate for its absence, it is nevertheless important to continue the research in this particular field for several reasons. The dissection of the mechanism by which MIP-1α inhibits haemopoietic stem cells will no doubt provide valuable insights into the way transiently engrafting stem cells, the more active members of the stem cell compartment, are controlled. Furthermore, as mentioned above, these findings may have a therapeutic value as they may shed light on the possible defects underlying stem cell proliferative disorders, such as leukaemia.

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