Molecular Mechanisms of IgA Nephropathy

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Publication and abstracts arising from this work

Publication

McDonald KJ, Cameron AJ, Allen JM & Jardine AG. Expression of Fc alpha/mu receptor by human mesangial cells: a candidate receptor for immune complex deposition in IgA nephropathy. *Biochem Biophys Res Commun* 2002; 290: 438-442.

Abstracts presented

McDonald KJ, Allen JM, and Jardine AG. Expression of Fc receptors on human mesangial cells. *Scottish Renal Association, Kilmarnock, May 2001.* (Oral presentation)

McDonald KJ, Cameron AJM, Allen JM, and Jardine AG. Expression of a novel IgA receptor by human mesangial cells. *Renal Association, London, September 2001.* (Oral presentation)

McDonald KJ, Allen JM, and Jardine AG. Human mesangial cells express a novel Fc receptor. *ASN/ISN World Congress of Nephrology, San Francisco, October 2001.*

McDonald KJ, Cameron AJM, Allen JM, and Jardine AG. Fc receptor expression by human mesangial cells – implications for immune complex deposition in glomerulonephritis. *Medical Research Society, London, November 2001.*

McDonald KJ, Cameron AJM, Allen JM, McLay JS, and Jardine AG. A pathophysiological role for tubular Fc receptors in glomerulonephritis? *American Society of Nephrology, Philadelphia, November 2002.*

McDonald KJ, Allen JM, and Jardine AG. Characterisation of a mesangial Fc alpha/mu receptor splice variant. *NKRF Fellows Meeting, Keele, April 2003.*

McDonald KJ, Allen JM, Novak J, Novak L and Jardine AG. Characterisation of an Fc alpha/mu receptor splice variant. *10th International Symposium on IgA Nephropathy, Saint-Etienne, March 2004.* (Oral presentation)

Abbreviations

ACE..... Angiotensin converting enzyme

Ag..... Antigen

ASGPR..... Asialoglycoprotein receptor

CD.....Cluster of differentiation (antigen)

CDNA.....Complementary deoxyribonucleic acid

CIC.....Circulating immune complex

C_t.....Threshold cycle

dbcAMP..... Dibutyryl cyclic adenosine monophosphate

DNA..... Deoxyribonucleic acid

dNTP..... Deoxynucleotide triphosphate

EDTA..... Ethylenediaminetetraacetic acid

ELISA.....Enzyme-linked immunosorbent assay

ER..... Endoplasmic reticulum

Fab..... Antigen binding fragment (of immunoglobulin)

Fc..... Crystallisable fragment (of immunoglobulin)

Fcα/μR.....Fc alpha/mu receptor

FcαRI.....Fc alpha receptor I

FcγR.....Fc gamma receptor

FcRn..... Neonatal Fc receptor

Gal..... Galactose

GalNAc.....*N*-acetyl galactosamine

GFP.....Green fluorescent protein

GPI.....Glycosylphosphatidylinositol

HLA..... Human leukocyte antigen

HMC..... Human mesangial cell

IgA..... Immunoglobulin A

IgE..... Immunoglobulin E

IgG..... Immunoglobulin G

IgM..... Immunoglobulin M

IL.....Interleukin

ITAM..... Immunoreceptor tyrosine-based activation motif

MHC..... Major histocompatibility complex

mRNA..... Messenger ribonucleic acid

NANA.....*N*-acetylneuraminic (sialic) acid

PBS..... Phosphate buffered saline

PCR.....Polymerase chain reaction

PIGR..... Polymeric immunoglobulin receptor

PMA..... Phorbol 12-myristate 13-acetate

PTC.....Proximal tubular cell

RACE..... Rapid amplification of cDNA ends

RNA.....Ribonucleic acid

RT-PCR..... Reverse transcription polymerase chain reaction

TCR..... T cell receptor

TfR.....Transferrin receptor

TGF.....Transforming growth factor

TNF.....Tumour necrosis factor

Author's declaration

The experimental design of the work presented in this thesis was by me and by my supervisors, Dr Alan Jardine and Professor Janet Allen. All experimental work was performed by me except for immunohistochemistry of tissue sections in chapter 5 which was performed by Dr Barbara Young (Department of Pathology, Western Infirmary, Glasgow)

I declare that this thesis has been composed by me and is a record of work performed by me. It has not previously been submitted for a higher degree.

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Summary

IgA nephropathy is the most common form of glomerulonephritis and is the underlying cause of renal failure in up to 10% of patients in dialysis and renal transplantation programmes. The condition has a variable natural history, leading over a period of several years to end stage renal failure in approximately one third of those affected. The pathogenesis of IgA nephropathy, and the factors determining susceptibility and disease progression are incompletely understood, but abnormalities have been described in both the systemic and mucosal limbs of the immune system. Although circulating levels of IgA may be increased in patients, and the IgA is abnormally glycosylated, the characteristic diagnostic feature is the presence of IgA-containing immune complexes in the glomerular mesangium. However, the mechanism by which these complexes are deposited in the mesangium, and the nature of their pathophysiological role, is unknown. Human mesangial cells (HMCs) cultured *in vitro* can bind monomeric and polymeric IgA in a dose-dependent, specifically inhibitable manner but do not express classical IgA receptors such as the Fc alpha receptor (FcαRI), the polymeric immunoglobulin receptor or the asialoglycoprotein receptor. This evidence has led several authors to suggest that HMCs possess a novel IgA receptor, but its identity has remained elusive. Mesangial cell activation following IgA binding and receptor signalling could underlie the mesangial cellular proliferation and extracellular matrix accumulation seen in IgA nephropathy. A better understanding of these potentially receptor mediated events is an important step toward defining the pathophysiology of IgA nephropathy more clearly.

Initial reports of FcαRI expression by HMCs had yielded conflicting results. In accordance with work subsequently published by other authors we failed to detect FcαRI message or protein expression by HMCs using a variety of techniques. Another report had suggested that HMCs expressed a novel FcαRI variant that perhaps mediated mesangial deposition of IgA. We examined HMCs for expression of such a

variant using real time polymerase chain reaction to provide relative quantification of FcαRI exons. These investigations provided no evidence for expression of an FcαRI variant by cultured HMCs.

Mesangial cells were then assessed for expression of the human homologue of a novel IgA and IgM-binding receptor described on murine macrophages and B lymphocytes. We hypothesised that this Fcα/μR could be involved in the mesangial deposition of IgA if it were expressed in human kidney. Fcα/μR gene expression was detected in human renal cortex, isolated glomeruli and in primary cultures of HMCs. Receptor expression levels were substantially augmented by incubation of HMCs with the inflammatory cytokine, IL-1α. The predicted full length coding sequence for human Fcα/μR was then isolated and cloned from an HMC line and the protein expressed in transfected cells. The human Fcα/μR protein was approximately 58kDa in weight and was minimally *N*-glycosylated. As predicted from the characteristics of the murine homologue, the expressed human Fcα/μR was able to bind IgA and IgM, but not IgG. The genomic organisation of human Fcα/μR was elucidated and two variant transcripts were discovered. One alternatively spliced transcript was the result of an out of frame deletion of an entire exon that resulted in a shift of reading frame and the production of a novel amino acid sequence. Levels of this ΔEC3 transcript in HMCs were also regulated by IL-1α. The coding sequence of this variant was cloned and expressed in a heterologous system. Experimental data confirmed the predictions of *N*-linked glycosylation and a membrane spanning sequence but immunoglobulin binding by this protein was not detected. Localisation of fluorescently tagged fusion proteins suggested that the Fcα/μR ΔEC3 protein was retained within the endoplasmic reticulum (ER) in contrast to wildtype receptor which was expressed on the cell surface. It was hypothesised that retention of the protein within the ER was due to a motif within the cytoplasmic tail and that cell surface expression would only occur under circumstances in which this motif was overridden.

In IgA nephropathy, progressive renal damage is associated with tubulointerstitial fibrosis. In proteinuric glomerulopathies renal tubular cells are exposed to abnormal

types and amounts of filtered protein including immunoglobulins such as IgG and IgA, and it was hypothesised that an interaction between tubular cells and filtered immunoglobulins may involve specific cellular receptors. Expression of potential receptors by cultured proximal tubular cells (PTCs) was investigated. None of the classical Fc receptors for IgG were present in PTCs although expression of the neonatal Fc receptor which may play a role in IgG recycling from tubular filtrate was demonstrated. However, PTCs expressed the Fcα/μR and once again transcript levels were regulated by IL-1α. Expression of Fcα/μR protein by tubular cells in normal kidney sections was confirmed by immunohistochemistry.

In conclusion, expression of the novel human Fcα/μR was demonstrated in renal mesangial cells and proximal tubule. The receptor coding sequence was cloned and characteristics of the Fcα/μR were defined. The Fcα/μR or a variant form could mediate the renal response to injury associated with IgM and IgA immune complexes and is a candidate receptor for the mesangial response to IgA immune complexes in IgA nephropathy.

Chapter 1

Introduction

1.1. IgA Nephropathy

1.1.1. Overview of IgA nephropathy

The renal disease process that was to become known as immunoglobulin A (IgA) nephropathy was first described 38 years ago by Berger and Hinglais (Berger & Hinglais, 1968). The initial short report described intercapillary deposits of IgA present in all glomeruli of renal biopsies from 25 patients with proteinuria and microscopic haematuria. Over the intervening years, IgA nephropathy has been recognised as the most prevalent form of glomerulonephritis on a worldwide basis (Levy & Berger, 1988). Although the clinical course and consequences of the condition have been extensively described, the molecular pathogenesis of IgA nephropathy remains incompletely defined.

1.1.2. Diagnosis of IgA nephropathy

The diagnosis of IgA nephropathy requires histological examination of a renal biopsy specimen. Light microscopic features alone are non-diagnostic and range from the finding of normal kidney to a diffuse or focal, mesangio-proliferative glomerulonephritis. Definitive diagnosis depends on immunofluorescence techniques employing specific antibodies recognising IgA to reveal the characteristic deposits of IgA within the kidney. These IgA deposits are usually present in the glomerular mesangium but can also be seen within the walls of glomerular capillaries in some patients. Electron microscopy examination typically shows the presence of granular electron-dense deposits representing immune complexes located within the

mesangium. Typical light microscopic, immunofluorescent and electron microscopic features of IgA nephropathy are shown in figure 1.1. Immunofluorescence staining often reveals co-localisation of other immune system components with glomerular IgA. Most prominent among these is complement factor C3 although IgG and IgM may also be present. Progressive IgA nephropathy leading to end stage renal disease is associated with atrophy and fibrosis within the tubulointerstitial compartment. These latter changes are common to a range of conditions leading to renal failure and may represent ultimate renal damage along a final common pathway. Although approximately one half of patients with IgA nephropathy have serum concentrations of IgA above the normal range (Layward *et al*, 1993; Leung *et al*, 2001), this measure is insufficiently sensitive or specific to be of diagnostic value.

1.1.3. Clinical features of IgA nephropathy

IgA nephropathy is seen with variable frequency in different racial groups such that it is most common in Asians and Caucasians but seen less commonly in Black populations (Levy & Berger, 1988). This variation is thought to reflect a combination of screening and biopsy policies in different countries together with genetic differences in disease susceptibility. The incidence is highest in young adults and males are more commonly affected than females.

The mode of presentation is variable, with isolated microscopic or dipstick haematuria often in association with a mild to moderate degree of proteinuria being the most frequent. This finding of dipstick haematuria is often detected in asymptomatic subjects following a routine urinalysis examination. An alternative scenario is presentation with a single episode or recurrent episodes of macroscopic haematuria typically during or following an upper respiratory tract infection and this presentation tends to be more prevalent in younger patients. A smaller number of patients will present with nephrotic syndrome or with a rapidly progressive glomerulonephritis and crescent formation. The clinical importance of IgA nephropathy is that up to 25% of patients will develop end stage renal disease over a follow up period of 20 years

(Rekola *et al*, 1991; Johnston *et al*, 1992). None of the clinical factors associated with progressive renal disease is specific for IgA nephropathy and variations between different geographical centres in rates of progression probably reflect an effect of lead time bias and a higher proportion of more mildly affected individuals in some centres (Geddes *et al*, 2003). Predictive factors identified for progression to end stage renal disease include younger age, degree of proteinuria, elevation of serum creatinine at presentation and the presence of hypertension (D'Amico, 1992; Geddes *et al*, 2003). Patients who experience episodic macroscopic haematuria are believed to have a lower risk of progression to end stage renal disease. The histological features most strongly associated with progressive disease are also common to other glomerular pathologies and include tubular atrophy, interstitial fibrosis and the presence of crescents.

It is also likely that a large number of cases of undiagnosed or "silent" IgA nephropathy are present within the general population. A case series of 200 patients undergoing autopsy following death from a traumatic cause and with no prior history of renal disease revealed that eight (4%) had mesangial deposits of IgA on immunofluorescence examination (Sinniah, 1983). The glomeruli appeared normal in all but one case which exhibited mesangial hypercellularity.

Regression of the histological features of IgA nephropathy including mesangial cellular proliferation and interstitial mononuclear cell infiltration has been documented (Hotta *et al*, 2002). It is possible that such improvements could occur spontaneously but in this study they were described following repeat renal biopsy after treatment. Of 35 patients in whom haematuria had resolved, the extent of mesangial IgA had improved in 26 patients, including eight patients in whom mesangial IgA deposits had resolved completely.

Henoch-Schönlein purpura (HSP) is a systemic vasculitis with renal involvement that is histologically indistinguishable from IgA nephropathy. The condition usually appears in childhood or adolescence and other manifestations include cutaneous,

rheumatological and gastrointestinal symptoms. It is unclear which elements of the pathogenesis of HSP may be shared with IgA nephropathy.

1.1.4. Treatment of IgA nephropathy

The principles of treatment in IgA nephropathy are similar to those in other glomerular and progressive renal diseases. These include the control of hypertension and the use of agents that inhibit the renin-angiotensin system to reduce proteinuria (Russo *et al*, 2001). In addition, the use of other immunomodulatory agents has been suggested but no universal consensus exists as to their value. These agents include corticosteroids (Pozzi *et al*, 1999; Katafuchi *et al*, 2003), azathioprine (Yoshikawa *et al*, 1999), cyclosporine (Cattran, 1991) and mycophenolate mofetil. A reduction in proteinuria has been observed following treatment with mycophenolate (Tang *et al*, 2005) but progression to renal replacement therapy was not prevented (Frisch *et al*, 2005). Exceptions to this consensus are the subset of IgA nephropathy patients who present with nephrotic syndrome and who may derive benefit from corticosteroids and patients with rapidly progressive IgA nephropathy with crescent formation who may benefit from immunosuppression with cyclophosphamide.

The role of fish oil in the treatment of IgA nephropathy has been examined in several trials. It has been hypothesised that fatty acids contained in fish oil may alter eicosanoid production and have advantageous effects on cell membrane fluidity. Initial studies reported no benefit (Bennett *et al*, 1989) but a later, larger randomised trial of 106 patients suggested a benefit for fish oil in reducing the rate of renal decline independently of differences in proteinuria and blood pressure control (Donadio *et al*, 1994). Doubts about the validity of this trial centre on the placebo group who received olive oil, and in whom the decline in renal function was steeper than would normally be anticipated. A subsequent meta-analysis concluded that there was a non-significant benefit of fish oil in the treatment of IgA nephropathy (Dillon, 1997).

1.1.5. IgA nephropathy and renal transplantation

IgA nephropathy is associated with a high incidence of histologically recurrent disease in renal allografts with rates of up to 60% reported (Berger, 1988; Odum *et al*, 1994). This process is not without consequence, as recurrent disease is believed to be responsible for graft dysfunction and ultimately graft failure in a proportion of patients. However, an accurate estimate of the contribution to premature graft loss is difficult to establish due primarily to selective biopsy policies and coexisting chronic allograft nephropathy. A recent review of published series estimated that 15% of renal transplant recipients with primary IgA nephropathy developed graft dysfunction due to recurrent disease and that 7% had graft loss attributable to this cause (Floege, 2003). Despite the risk of recurrent IgA nephropathy, the long-term allograft survival rate in these patients is comparable to renal transplant recipients with other primary renal diseases (Ponticelli *et al*, 2001). Cyclosporine-based immunosuppression regimens do not appear to be associated with lower rates of recurrent IgA nephropathy. Similarly, regimens containing mycophenolate mofetil seem to be associated with comparable rates of recurrent IgA nephropathy to azathioprine-based immunosuppression (Chandrakantan *et al*, 2005). Case reports describing inadvertent transplantation of donor kidneys with mesangial IgA deposits provide an interesting insight to mechanisms regulating deposition of IgA. Repeat histological examination has shown that mesangial IgA deposits can resolve promptly when the affected kidney is transplanted into a recipient with a primary renal disease other than IgA nephropathy (Sanfilippo *et al*, 1982; Silva *et al*, 1982). These observations suggest that a systemic abnormality in IgA production or clearance is at least one factor required for mesangial deposition of IgA.

1.2. The Human IgA System

1.2.1. Metabolism and function of IgA

Approximately 70 mg/kg of IgA is produced each day in humans, which is more than any other antibody isotype. However, due to loss of the protein in external secretions and to the longer half-life of circulating IgG, IgA is present at lower serum concentrations (2-4g/l) than IgG (8-16g/l). The molecule is synthesised by B lymphocytes and plasma cells present in the bone marrow, lymphoid tissue, the serum and at mucosal surfaces. Isotype switching to IgA production by naïve B cells is at least partially regulated by the cytokines interleukin-10 and transforming growth factor-β (Defrance *et al*, 1992). IgA is the predominant isotype found at mucosal surfaces such as the respiratory, urogenital and gastrointestinal tracts. The physiological function of IgA is to bind and subsequently neutralise pathogenic bacteria, viruses and toxins. Following specific binding of IgA to a target epitope, attachment of the pathogen to cells of the mucosal epithelial surface may be inhibited or the opsonised particle may be recognised and internalised by immune cells such as neutrophils or monocytes bearing specific IgA receptors. Despite the apparent central importance of IgA in protection of mucosal surfaces from infection, its absence is compatible with a virtually normal life as evidenced by individuals with a selective IgA deficiency. This genetic condition affects approximately 1 in 700 of the population and results in only slightly higher rates of respiratory tract infection in some of those affected.

Hepatic clearance is the predominant route for removal of IgA from the circulation. In humans, this process appears to be mediated by the asialoglycoprotein receptor which is expressed by hepatocytes (Tomana *et al*, 1988). This receptor recognises IgA and other glycosylated proteins through terminal galactose and *N*-acetylgalactosamine moieties and the protein is subsequently internalised and degraded within the hepatocyte prior to excretion in bile. However, expression of the specific IgA receptor FcαRI (CD89) by hepatic Kupffer cells has also been reported and so these cells could also play a role in catabolism of IgA (van Egmond *et al*, 2000). An alternative potential route of clearance is through internalisation and degradation by circulating myeloid cells which bear FcαRI (Launay *et al*, 1999).

1.2.2. Structure of IgA

In common with other antibody isotypes, the monomeric IgA subunit consists of two disulphide-linked immunoglobulin heavy chains each of which is linked by another disulphide bond to an immunoglobulin light chain (figure 1.2.). Heavy chain constant region sequences are common to a particular isotype whereas the two types of light chain (kappa and lambda) are utilised by all isotypes. In humans, two subclasses of IgA exist, IgA1 and IgA2, which have slight differences between the amino acid sequences of the heavy chain constant region. IgA1 possesses a unique proline-rich 18 amino acid "hinge region" between the CH1 and CH2 domains that includes several serine and threonine residues which have the potential to bear *O*-linked carbohydrate groups. IgA2 is considered to be the phylogenetically older type with the subsequent insertion of the hinge region later in evolution to form IgA1. In addition to the monomeric form, IgA can also exist in dimeric and higher polymeric forms. In the circulation, the monomeric form predominates (>95%) and IgA1 is the more abundant subclass (85%).

IgA in the mucosal compartment is almost exclusively in a polymeric form. Dimeric IgA produced by B lymphocytes includes an extra associated protein unit termed the joining (J) chain. This type of IgA (as well as IgM) binds specifically to the polymeric immunoglobulin receptor (PIGR) which is present on the basolateral surface of epithelial cells (Mostov, 1994). The antibody-receptor complex is internalised and translocated across the cell to the apical surface where it is released following proteolytic cleavage. A portion of the PIGR, termed the secretory component, remains in association with the antibody following release and this complex of dimeric IgA, J chain and secretory component is known as secretory IgA.

1.3. IgA Receptors

1.3.1. Overview of Fc receptors

Fc receptors (FcRs) are specialised cellular receptors that recognise and bind the constant region of immunoglobulin heavy chains. They act as crucial links between the humoral and cellular branches of the immune system. Each antibody isotype is recognised by one or more specific Fc receptors. Thus, IgG is recognised by FcγRs, IgA by FcαRs and IgE by FcεRs. Activation of the FcR following binding of the appropriate ligand can initiate a variety of cellular responses including antigen presentation, antibody-dependent cellular cytotoxicity, phagocytosis, production of reactive oxygen species or release of cytokines and other inflammatory mediators. The most extensively characterised FcRs are members of the immunoglobulin superfamily group of receptors and include the high and low affinity receptors for IgG (FcγRI, FcγRII & FcγRIII), the medium affinity receptor for IgA (FcαRI) and the high affinity receptor for IgE (FcεRI). Several of these receptors are dependent on an associated molecule, the FcR γ chain, for signal transduction. The FcR γ chain is a membrane-spanning protein that possesses an immunoreceptor tyrosine-based activation motif (ITAM) which becomes phosphorylated following receptor activation and subsequently recruits additional downstream signalling components.

1.3.2. Fc alpha receptor I (CD89)

Fc alpha receptor I (FcαRI or CD89) is the classical receptor for IgA. The receptor was cloned in 1990 and the gene has been mapped to chromosome 19 (Maliszewski *et al*, 1990; Kremer *et al*, 1992). This type 1 transmembrane protein is predominantly expressed by circulating myeloid cells (neutrophils, monocytes, eosinophils) and has also been found on tissue macrophages and some dendritic antigen presenting cells (Monteiro *et al*, 1990; Geissmann *et al*, 2001). Different immune cells exhibit variable receptor glycosylation resulting in a receptor size between 55 and 100 kDa.

FcαRI is composed of two extracellular immunoglobulin type domains with the membrane distal EC1 domain containing the IgA binding site (de Wit *et al*, 1995; Morton *et al*, 1999; Wines *et al*, 1999) (figure 1.3.). The location of the ligand binding site in the EC1 domain of FcαRI is distinct from the arrangement in FcγRs and the FcεR where the respective ligand binding sites reside in the membrane proximal EC2 domains. The receptor has a single 19 amino acid transmembrane region and a 41 amino acid cytoplasmic tail. The cytoplasmic tail does not contain any recognised signalling motifs but FcαRI has an electrostatic association with the common FcR γ chain homodimer via a positively charged arginine residue in the transmembrane region (Morton *et al*, 1995). The FcR γ chain contains an ITAM motif which mediates downstream receptor signalling. Receptor aggregation following cross-linking by ligand induces tyrosine phosphorylation of the associated FcR γ chain homodimer (Pfefferkorn & Yeaman, 1994). FcαRI has been detected without associated FcR γ chain on the cell surfaces of monocytes and neutrophils. It appears that the presence or absence of FcR γ chain underlies distinct endocytic pathways whereby IgA bound to non-associated FcαRI is recycled towards the cell surface and protected from intracellular degradation (Launay *et al*, 1999).

FcαRI affinity for ligand can be modulated in the presence of certain cytokines. Priming of eosinophils with either interleukin-4, interleukin-5 or granulocyte-macrophage colony stimulating factor resulted in a rapid and transient increase in receptor binding of IgA-coated beads (Bracke *et al*, 1997). The timescale of the observation led to the hypothesis that a direct effect on the receptor was responsible as opposed to increased synthesis or surface expression of FcαRI. Further work using site directed mutagenesis demonstrated that a serine (S263) in the cytoplasmic tail of FcαRI influenced receptor affinity for IgA (Bracke *et al*, 2001). Mutation to an alanine residue resulted in constitutive ligand binding whereas mutation to aspartate significantly reduced binding even in the presence of cytokine. It was proposed that receptor affinity was controlled by cytokines in the extracellular environment through a mechanism (perhaps phosphorylation) involving the intracellular domain of the receptor – a phenomenon termed "inside-out" signalling.

FcαRI is a medium affinity receptor ($K_a \sim 10^6\ M^{-1}$) for both IgA1 and IgA2 and binds polymeric IgA more avidly than the monomeric form (Monteiro *et al*, 1990; Wines *et al*, 1999). Binding of IgA to cells transfected with FcαRI was most efficient with complexes containing five or six IgA molecules (Reterink *et al*, 1997). The crystal structure of the interaction between FcαRI and IgA has been elucidated, demonstrating that two FcαRI molecules associate with a single IgA molecule (Herr *et al*, 2003). This is in contrast to IgG-FcγRIII and IgE-FcεRI interactions which display 1:1 stoichiometry. Various mediators have been reported to increase expression of FcαRI in freshly isolated monocytes. These include lipopolysaccharide, tumour necrosis factor-α, IL-1 and granulocyte-monocyte colony stimulating factor (Shen *et al*, 1994). In each case an increase in FcαRI transcript levels was observed and IgA-mediated phagocytosis was enhanced. In cultured monocytes, FcαRI transcript and cell surface protein levels were negatively regulated by transforming growth factor-β1 (Reterink *et al*, 1996). In addition, treatment of monocytes with TGF-β1 produced reduced amounts of IL-6 in response to stimulation with IgA.

Multiple alternatively spliced forms of FcαRI mRNA have been described in circulating myeloid cells and in alveolar macrophages (Morton *et al*, 1996; Patry *et al*, 1996; Pleass *et al*, 1996). These include transcripts lacking the entire EC2 exon (ΔEC2) and the membrane-proximal 66 nucleotides of the same exon (Δ66EC2). Treatment of alveolar macrophages with *N*-glycanase revealed an FcαRI protein backbone 4 kDa smaller than wildtype consistent with the expression of the Δ66EC2 transcript by these cells (Patry *et al*, 1996). Although the ΔEC2 variant has not been identified as a naturally occurring protein, cells transfected with the cDNA are able to bind secretory but not serum IgA (Pleass *et al*, 1996). The inflammatory cytokine tumour necrosis factor-α (TNF-α) appears to regulate the ratio of ΔEC2/wildtype transcript in neutrophils and monocytes and a reduction in the ΔEC2/wildtype transcript ratio was observed in neutrophils from patients with pneumonia (Togo *et al*,

2003). These alternatively spliced forms of FcαRI and regulation of their expression by inflammatory cytokines may serve to extend the functional diversity of the receptor.

1.3.3. Polymeric immunoglobulin receptor

The polymeric immunoglobulin receptor is present on the basolateral surface of epithelial cells and recognises and binds dimeric IgA and pentameric IgM present in the serum. It functions to transport these molecules across the cell to be released at the apical surface into the mucosal compartment of the immune system (Mostov *et al*, 1984; Mostov, 1994).

1.3.4. Asialoglycoprotein receptor

The asialoglycoprotein receptor (ASGPR) is a heterodimer of H1 and H2 subunits and is expressed on the surface membranes of hepatocytes. It mediates internalisation of circulating glycoproteins bearing terminal galactose and *N*-acetylgalactosamine groups (Stockert, 1995). This hepatic route is the principal mechanism of IgA clearance from the circulation. In addition ASGPR has been described on rat renal mesangial cells and a role in the pathogenesis of human IgA nephropathy has been suggested (Gomez-Guerrero *et al*, 1998).

1.3.5. Fc alpha/mu receptor

The Fc alpha/mu receptor (Fcα/μR) was initially described on murine B lymphocytes and macrophages (Shibuya *et al*, 2000). The existence of an Fc receptor for IgM had been hypothesised and the receptor was identified by probing a T cell leukaemia cell line cDNA library using a mouse monoclonal IgM. The receptor gene codes for an approximately 70kDa membrane-spanning glycoprotein with an extracellular immunoglobulin-like domain. The receptor was able to bind IgM with high affinity ($K_a \sim 10^9\ M^{-1}$) and IgA with lower affinity ($K_a \sim 10^8\ M^{-1}$). Cells expressing Fcα/μR were able to internalise IgM-coated beads and this function was dependent on a di-

leucine motif in the cytoplasmic tail of the receptor. A human homologue of Fcα/μR was also described which mapped to a region of chromosome 1 (1q32.3) in close proximity to the genes for other Fc receptors including FcγRs I-III, FcεR and the polymeric immunoglobulin receptor. Both mouse and human Fcα/μRs share a motif present in the immunoglobulin-like domain with the polymeric immunoglobulin receptor. Expression of mouse Fcα/μR was demonstrated in several tissues including spleen, thymus, kidney, liver and intestine. More recently the expression of Fcα/μR in the mouse central nervous system has been examined as observations had suggested a modulating role for IgM in the pathogenesis of multiple sclerosis (Nakahara *et al*, 2003). Fcα/μR was demonstrated in mouse forebrain using immunocytochemistry as well as in cultured oligodendrocytes and myelin.

1.3.6. Transferrin receptor

The transferrin receptor (TfR) is a 180kDa homodimer which in addition to recognising transferrin, appears to bind IgA1 (Moura *et al*, 2001). Both monomeric and polymeric forms of IgA1, but not IgA2, were initially reported to be recognised by the receptor. TfR expression has been shown to be upregulated on glomerular mesangial cells in patients with IgA nephropathy and IgA1 binding to mesangial cells in culture is specifically reduced by blocking antibodies for the receptor and by transferrin.

1.3.7. Other IgA receptors

In addition to the characterised IgA receptors there is some evidence that other as yet unidentified receptors for IgA may be present on some cell types. The human colonic adenocarcinoma cell line, HT29/19A was shown to bind monomeric and heat aggregated IgA1 in a manner consistent with the presence of a specific low affinity receptor with $K_a \sim 10^7\ M^{-1}$ (Kitamura *et al*, 2000). The association was not inhibited by either IgG, IgM, asialo-orosomucoid or secretory IgA suggesting that the polymeric immunoglobulin receptor, asialoglycoprotein receptor and Fcα/μR were not

responsible. Expression of neither FcαRI mRNA nor protein was detected. This work predated the recognition of TfR as an IgA receptor and although IgA2 binding to HT29/19A cells was not assessed, it is not possible to exclude that IgA1 binding to these cells was mediated by TfR in a similar manner to the initial report by Moura *et al.*

Another report documented binding of IgA to Peyer's patch M cells in a mouse model (Mantis *et al*, 2002). M cells are specialised epithelial cells that deliver mucosal antigens from the intestinal lumen to underlying lymphoid tissue. The apical surfaces of human M cells were also found to be coated with IgA when biopsies of normal terminal ileum were examined after immunofluorescent staining. No specific IgA receptor was identified on murine M cells and expression of candidate receptors was not assessed in human M cells.

1.4. Mechanisms of IgA nephropathy

1.4.1. IgA glycosylation

The most consistent abnormalities described in IgA nephropathy are related to aberrant glycosylation of the IgA1 molecule. IgA1 is unique amongst immunoglobulins and unusual amongst serum proteins in possessing *O*-linked carbohydrate groups attached to amino acids in the hinge region. The molecule also bears *N*-linked carbohydrate groups in common with other immunoglobulin types and many other serum proteins. In IgA1 the *N*-linked carbohydrates are present in the CH2 domain and in the tail-piece extension of the CH3 domain (Yoo & Morrison, 2005). The IgA1 hinge region contains five serine (Ser) and four threonine (Thr) residues per heavy chain, each of which has the potential to bear *O*-linked glycans via hydroxyl groups. Analysis of normal serum IgA1 suggests that Thr^{228}, Ser^{230} and Ser^{232} support *O*-linked glycans while sites at Thr^{225} and Thr^{236} are incompletely occupied (Mattu *et al*, 1998). These

carbohydrate side chains are based on an *O*-linked *N*-acetylgalactosamine (GalNAc) unit which may be β1,3-linked to a galactose (Gal) group. In turn the solitary GalNAc unit or the GalNAc/ β1,3-Gal moiety may be crowned with one or two sialic acid groups. Thus the profile of IgA1 *O*-glycosylation in an individual subject is heterogeneous depending on whether a particular serine or threonine residue possesses a carbohydrate side chain and on the composition and structure of the groups present (reviewed in Novak *et al*, 2001). These variable hinge *O*-glycoforms are illustrated in figure 1.4.

Several groups using different methods have now demonstrated the characteristic glycosylation abnormalities of serum IgA1 in IgA nephropathy. The observation that IgA from patients with IgA nephropathy bound with reduced affinity to the lectin jacalin led to the hypothesis that alterations in IgA glycosylation may be important in disease pathogenesis (Andre *et al*, 1990). The *O*-glycosylation profile of IgA1 has been studied by employing GalNAc-specific lectins such as *Vicia villosa* and *Helix aspersa*. These studies demonstrate lectin binding patterns consistent with increased exposure of the core GalNAc unit on the *O*-linked carbohydrate side chains indicating a reduction in the number of galactose groups in IgA nephropathy patients (Allen *et al*, 1995; Tomana *et al*, 1997). Another study concluded that the deficiency in galactosylation in IgA1 from patients was restricted to the monomeric fraction (Leung *et al*, 1999). The same study showed that the normally galactosylated polymeric IgA1 from IgA nephropathy patients instead showed increased sialylation – consistent with an earlier study which examined sialylation of total IgA1 from patients (Baharaki *et al*, 1996). Further work suggested that the increased sialylation of IgA1 from patients was restricted to λ–IgA1 (Leung *et al*, 2002).

In another study, a polyclonal antiserum was generated against the IgA1 hinge peptide sequence and its reactivity against different preparations of IgA was evaluated (Kokubo *et al*, 1999). Reactivity of the antibody was significantly higher with IgA1 purified from IgA nephropathy patients in comparison with healthy controls and subjects with other renal diseases. These findings were consistent with increased

exposure of the hinge peptide core in IgA nephropathy due to fewer or less bulky *O*-linked oligosaccharide side chains.

More sophisticated techniques to examine carbohydrate structures at the IgA1 hinge have been applied subsequently. Matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry has confirmed heterogeneity of *O*-glycosylation at the hinge region of normal serum IgA1. Analysis of hinge peptides demonstrated between two and five GalNAc groups with variable numbers of galactose residues (Novak *et al*, 2000). Similar heterogeneity of hinge *O*-glycans was observed with a myeloma IgA1 protein. A comparison of serum IgA1 from IgA nephropathy patients and controls using MALDI-TOF mass spectrometry was consistent with results from earlier lectin-binding assays indicating reduced Gal and/or GalNAc groups in hinge glycopeptides from patients (Hiki *et al*, 1998). Similarly, fluorophore-assisted carbohydrate electrophoresis (FACE) demonstrated a significantly higher proportion of lone hinge GalNAc groups in serum IgA1 from IgA nephropathy patients (Allen *et al*, 1999) and electrospray ionization liquid mass spectrometry showed a reduction in GalNAc, Gal and sialic acid groups in IgA1 from patients with IgA nephropathy compared with normal subjects and patients with other primary glomerulonephritides (Odani *et al*, 2000).

The mechanism responsible for the production of underglycosylated IgA1 in IgA nephropathy is not clear. The C1 inhibitor protein is another circulating protein with *O*-linked carbohydrate groups, but no abnormalities in its glycosylation pattern were detected in patients with IgA nephropathy (Allen *et al*, 1995). This makes non-specific abnormal manufacture or degradation of *O*-glycans unlikely explanations for underglycosylation. There are no differences in the peptide sequence at the hinge region of IgA1 in IgA nephropathy patients which could potentially limit the number of sites for *O*-glycans (Greer *et al*, 1998). The only functional abnormality identified thus far is reduced activity of the enzyme, β1,3 galactosyltransferase in B lymphocytes from IgA nephropathy patients (Allen *et al*, 1997). This enzyme mediates the addition of Gal to GalNAc in the manufacture of the *O*-linked carbohydrate side chains of

IgA1. Reduced enzyme activity appears to be confined to B lymphocytes in IgA nephropathy patients as no differences were observed in T lymphocytes or monocytes between patients and controls. However, factors regulating both β1,3 galactosyltransferase activity as well as the activities of many other glycosyltransferase enzymes likely to be involved in the post-translational modification of IgA1 are not well understood. It is also possible that the prevailing local cytokine environment could be a factor involved in the mechanism responsible for plasma cells producing different glycoforms of IgA1 (Chintalacharuvu & Emancipator, 1997). This cytokine profile could be a reflection of the immunoregulatory balance between Th1 and Th2 lymphocyte subsets at a particular point in time.

1.4.2. Functional effects of abnormal IgA glycosylation

There is considerable evidence to suggest that the aberrant glycosylation of serum IgA1 seen in IgA nephropathy has functional consequences which could be relevant to disease pathogenesis. It has been postulated that abnormal glycosylation may affect IgA1 clearance from the circulation, perhaps via the hepatic asialoglycoprotein receptor, or independently promote mesangial deposition either through non-specific interactions with matrix components or via a specific cellular receptor. Additionally, the propensity to form aggregates of IgA1 may be increased by abnormal glycosylation, and exposure of hinge region epitopes which would otherwise be masked could result in the generation of anti-IgA1 antibodies which could alter IgA1 antigen binding or increase the tendency to form macromolecular IgA1.

In experiments where rat kidneys were perfused with fractionated IgA1 from IgA nephropathy patients, the electrochemically neutral fraction and the fraction with high affinity for jacalin were deposited within glomerular capillaries and the mesangium within a few hours (Hiki *et al*, 1999). Analysis of this "glomerulophilic" IgA1 using gas-phase hydrazinolysis and lectin binding was consistent with underglycosylation of the hinge region. In contrast, IgA preparations from healthy controls did not accumulate in the rat kidneys following perfusion. Similar deposition was observed

when rat kidneys were perfused with normal human IgA1 which had been enzymatically treated to remove Gal and sialic acid groups (Sano *et al*, 2002).

Higher molecular weight aggregates of circulating IgA1 may be more susceptible to mesangial deposition and evidence supports the notion that such aggregation may be associated with underglycosylation of the IgA1 hinge region. IgA1 from IgA nephropathy patients formed multimeric aggregates following heating to 63°C *in vitro* more readily than IgA1 preparations from normal subjects (Hiki *et al*, 1996). Enzymatically deglycosylated IgA1 from normal subjects also showed a spontaneous tendency to self-aggregate not observed in the native form (Kokubo *et al*, 1998). In the same study, the multimeric IgA1 produced through enzymatic deglycosylation had significantly higher affinity for the mesangial extracellular matrix components laminin, fibronectin and type IV collagen in an *in vitro* binding assay. Similarly IgA from patients with IgA nephropathy was shown to bind more avidly to collagen compared to IgA purified from patients with HIV infection (van den Wall Bake *et al*, 1992). In separate work it was demonstrated that serum IgA1 from a proportion of IgA nephropathy patients possessed affinity for IgA1 and this interaction was considered to underlie IgA1 self-aggregation and perhaps promote glomerular deposition (Hiki *et al*, 1991; Kokubo *et al*, 1997). This IgA1-IgA1 interaction was tested in competitive inhibition assays using various hinge region components (Kokubo *et al*, 1997). Minor inhibition of the interaction was observed with exogenous Gal and GalNAc but significant inhibition of the IgA1-IgA1 interaction was demonstrated with the addition of exogenous synthesised hinge peptide (20-mer). These findings implicate the hinge region amino acid sequence and these carbohydrate groups in the formation of IgA aggregates. Thus the normal *O*-linked carbohydrate groups of the IgA1 hinge region may function to prevent self-aggregation and to inhibit interaction with extracellular matrix components.

Differential IgA1 glycosylation may also mediate distinct responses at the glomerular level. Mesangial cells express integrins which link the cellular cytoskeleton to components of the extracellular matrix and subsequently modulate several important

activities including cellular proliferation, enzyme production and expression of various genes. When cultured human mesangial cells were incubated with enzymatically deglycosylated IgA, expression of $\alpha_v\beta_3$ integrin was enhanced by up to 5-fold from basal levels (Peruzzi *et al*, 2000). Similar enhancement of mesangial cell integrin expression was also observed following incubation with IgA fractions from IgA nephropathy patients showing increased exposure of GalNAc when compared with IgA from healthy controls. The same authors have also shown a reduction in expression and production of vascular endothelial growth factor (VEGF) by cultured human mesangial cells exposed to enzymatically deglycosylated IgA (Amore *et al*, 2000). VEGF is a vasoactive peptide implicated in capillary repair mechanisms following renal injury and this down regulation by abnormally *O*-glycosylated IgA appeared to be mediated via enhancement of nitric oxide synthase activity. Whether IgA purified from the serum of patients with IgA nephropathy has the same effect has not been reported. Both *in vitro* deglycosylated normal IgA and IgA fractionated glycoforms of molecular weight between 250 and 500kD from IgA nephropathy patients significantly inhibited the proliferation of cultured human mesangial cells and also appeared to exert a pro-apoptotic effect on these cells (Amore *et al*, 2001).

1.4.3. Immune response in IgA nephropathy

The IgA immune response appears to be augmented in IgA nephropathy as when B lymphocytes from IgA nephropathy patients were transformed *in vitro* with Epstein-Barr virus, IgA1 production was significantly higher when compared to controls (Layward *et al*, 1994). Whether the IgA that is ultimately deposited in the glomerular mesangium originates from bone marrow plasma cells or from cells in the mucosal compartment of the immune system is unresolved. The observation that episodic macroscopic haematuria is often associated with respiratory tract infections in subjects with IgA nephropathy led to the suggestion that mucosal IgA produced in response to infection was subsequently routed to the kidney via the systemic immune compartment. However, circumstantial evidence tends to favour the systemic immune system being the origin of glomerular IgA as abnormalities in the systemic immune

response have been documented in patients with IgA nephropathy. A higher proportion of tonsillar plasma cells in IgA nephropathy patients express mRNA for J-chain when compared to cells from normal subjects (Harper *et al*, 1995). This implies a shift towards increased polymeric IgA production in the systemic immune compartment in IgA nephropathy. Similar results were obtained when bone marrow plasma cells were examined from patients with IgA nephropathy (Harper *et al*, 1996). IgA nephropathy patients also produced a higher proportion of polymeric IgA and demonstrated a prolonged IgA immune response following systemic immunisation with tetanus toxoid when compared to normal control subjects (Layward *et al*, 1992).

In contrast, most studies have suggested down regulation of IgA production in the mucosal immune response in patients with IgA nephropathy. The proportion of IgA-producing plasma cells in duodenal biopsies from IgA nephropathy patients appears to be reduced and fewer of these cells express J-chain mRNA when compared to controls (Harper *et al*, 1994). Consistent with this is the response of IgA nephropathy patients following intra-nasal immunisation with cholera toxin subunit B where significantly fewer IgA1-secreting cells and less antigen-specific IgA1 were found in the circulation when compared with controls (de Fijter *et al*, 1996). However, patients with IgA nephropathy and *Helicobacter pylori* infection appeared to have higher levels of *Helicobacter*-specific IgA compared with controls, in addition to altered IgG isotype switching (Barratt *et al*, 1999). These different responses to mucosal antigen may reflect chronic versus transient exposure or may be a function of the specific antigens.

Publication of recent work examining the involvement of mucosally derived secretory IgA in IgA nephropathy may lead to renewed interest in the role of the mucosal immune system in the condition (Oortwijn *et al*, 2006). In this study, serum levels of secretory IgA in patients were not significantly elevated compared with controls but did seem to be associated with the degree of haematuria. Binding of secretory IgA to cultured human mesangial cells was demonstrated and this was associated with production of interleukin-6 by these cells. This observation is at odds with the

perceived anti-inflammatory properties of secretory IgA but may be of significance in the pathogenesis of IgA nephropathy.

Populations of regulatory T lymphocytes are also altered in patients with IgA nephropathy. There was reduced usage of specific variable (V) genes in γδ-T cells from the intestinal mucosae of patients compared to controls (Olive *et al*, 1997). A further study confirmed that V region usage was unaltered in circulating T cells of patients but that the mucosal abnormality was mirrored in V region usage in bone marrow T cells with reduced expression of Vγ3 and Vδ3 in IgA nephropathy patients (Buck *et al*, 2002). It was hypothesised that this abnormality may influence the mucosal and systemic immune responses to certain antigens and could be a factor in the abnormal production of IgA in IgA nephropathy. Alternatively it is possible that the abnormalities arose as a consequence of selective depletion of these particular T cell subsets in response to a specific antigenic challenge associated with disease. In addition to T cell regulation, production of IgA by plasma cells is influenced by antigen presenting cells. Dendritic cells generated from patients with IgA nephropathy had a reduced ability *in vitro* to induce IgA production by naïve B cells in comparison to dendritic cells derived from controls (Eijgenraam *et al*, 2005). This functional hyporesponsiveness of IgA nephropathy dendritic cells was apparent only in the presence of IL-10 and appeared to be dependent on a membrane-bound dendritic cell factor. These findings may provide some insight to the mechanisms underlying reduced mucosal immune responsiveness in IgA nephropathy patients.

Although various dietary and infectious antigens such as gliadin, cytomegalovirus, and *Haemophilus parainfluenzae* have been proposed as the targets of the immune response in IgA nephropathy, the data are conflicting. No consistent exogenous antigen has been described either in circulating immune complexes or in mesangial deposits of IgA. However, it is possible that the immune response generated is the result of exposure to several different antigenic stimuli or to a particular combination of antigens. Alternatively, by the time the disease phenotype is recognised, perhaps

the initiating antigen has disappeared or been cleared by the immune system leading to difficulty in retrospective identification.

1.4.4. Circulating IgA immune complexes

Evidence suggests that a proportion of serum IgA circulates in complexes with other immunoglobulins. IgA was found to be present in association with IgG and IgM in one study, and levels of these IgA circulating immune complexes (CIC) were higher in patients with either IgA nephropathy or Henoch-Schönlein nephritis when compared to healthy controls (Coppo *et al*, 1982). Additionally, IgA CIC levels showed significant correlation with histological disease activity and the degree of microscopic haematuria. Later work with IgA1 from IgA nephropathy patients exhibiting lectin binding properties characteristic of deficient hinge region galactosylation confirmed that IgA1 was present in complexes with IgG (Tomana *et al*, 1997). Importantly this study also suggested that the majority of under-galactosylated IgA1 from patients was present in the high molecular mass IgA1 fractions associated with IgG antibodies as opposed to fractions containing monomeric IgA1. In addition, there appeared to be no difference in galactose content between the uncomplexed IgA1 fractions of patients and controls. The basis of this association between serum IgA1 and IgG in the formation of CICs was defined in further work which demonstrated a specific antibody-antigen interaction where IgG recognised glycan-based epitopes in the hinge region of IgA1 (Tomana *et al*, 1999). The predominant anti-glycan antibody present in the CICs was IgG2 but a small proportion was of IgA1 isotype and subclass. Anti-glycan IgG and IgA1 antibodies were also detected in the sera of healthy subjects but at lower levels than found in IgA nephropathy patients. IgA1 present in CICs appeared to be in a polymeric form as indicated by the presence of J-chain. It was proposed that abnormal exposure of IgA1 hinge region GalNAc groups in IgA nephropathy leads to the generation of antibodies specific for epitopes which would otherwise be masked or that the exposed hinge region epitopes are recognised by preformed naturally occurring antibodies which recognise similar carbohydrate-based epitopes of microorganisms.

Some functional characteristics of galactose–deficient IgA1-containing CICs isolated from IgA nephropathy patients have been assessed in studies using cultured human mesangial cells (HMC) *in vitro*. These CICs bound to HMCs with a higher affinity than uncomplexed IgA1 and were subsequently internalised and metabolised (Novak *et al*, 2002). The CICs also appeared to have a modulatory effect on the *in vitro* proliferation of HMCs. CIC fractions of molecular weight 800-900 kDa isolated from IgA nephropathy patients promoted HMC proliferation as assessed by ^{3}H-thymidine incorporation whereas CICs in the range 700-800 kDa exerted an inhibitory effect on HMC proliferation (Novak *et al*, 2005). IgA-containing CICs of molecular weight 800-900 kDa isolated from healthy controls also stimulated HMC proliferation but by a reduced amount compared with those from IgA nephropathy patients. CICs of molecular weight 800-900 kDa isolated from a single patient with IgA nephropathy during an episode of macroscopic haematuria exerted a greater stimulatory effect on HMC proliferation than CICs isolated from the same subject in the absence of macroscopic haematuria. Thus CICs from IgA nephropathy patients are responsible for distinct functional effects dependent on size and may also possess particular characteristics at different phases of the natural history of the condition.

IgA-CICs containing fibronectin have also been described in IgA nephropathy patients but evidence for a pathogenic function is lacking (Lai *et al*, 1996a). The anti-inflammatory protein, uteroglobin inhibited the formation of these IgA-fibronectin CICs and it was hypothesised that this property may prevent the mesangial deposition of IgA. Supporting evidence for such a role came from two separate uteroglobin "knockout" mouse models which displayed features of human IgA nephropathy with haematuria, proteinuria, glomerular deposition of IgA and IgA-fibronectin CICs (Zheng *et al*, 1999). However, further investigation did not support a role for uteroglobin in human IgA nephropathy as levels of uteroglobin were not significantly altered between patients and controls and in fact there appeared to be increased incorporation of uteroglobin into IgA-fibronectin CICs in IgA nephropathy patients (Coppo *et al*, 2002).

The presence of IgA-containing CICs in IgA nephropathy patients could lead to reduced hepatic clearance of IgA through diminished exposure to ASGPR on hepatocytes. Alternatively, IgA in the complexed form may be preferentially deposited within the glomerular mesangium. This latter mechanism may represent "mesangial trapping" due to the physical size of IgA-CICs or could be due to an altered interaction with mesangial matrix components or a specific mesangial receptor.

1.4.5. FcαRI-IgA complexes

Circulating, soluble complexes of FcαRI and its ligand, IgA have also been implicated in the pathogenesis of IgA nephropathy. A soluble 30kDa version of FcαRI capable of binding IgA in a dose-dependent manner was shed from monocytic cells following either IgA binding or crosslinking with specific antibody (van Zandbergen *et al*, 1999). An active signalling event and the presence of the FcR γ chain molecule were required for FcαRI shedding. It has been suggested that proteolytic cleavage of cell surface receptors and the release of a soluble form in this manner represents a mechanism of fine adjustment resulting in functional receptor down-regulation and avoidance of prolonged receptor activation. The presence of covalently-linked FcαRI-IgA complexes was subsequently demonstrated in human sera but only in high molecular mass fractions (van der Boog *et al*, 2002). It was proposed that this interaction contributed to the formation of circulating polymeric IgA. Dimeric IgA, defined by the presence of J-chain, was not associated with soluble FcαRI.

Two separate research groups have examined the potential role of FcαRI-IgA complexes in IgA nephropathy but with apparently conflicting conclusions. Using an ELISA-based assay, one group found that 40% of IgA nephropathy patients had high levels of circulating FcαRI-IgA complexes and levels in sera from patients with other renal and immune complex diseases were not significantly different from healthy controls (Launay *et al*, 2000). When a transgenic mouse model expressing human FcαRI on monocytes and macrophages was generated, extensive mesangial IgA

deposition in association with proteinuria and microscopic haematuria was observed. The spontaneous development of this phenotype resembling human IgA nephropathy was found to be dependent on soluble FcαRI which was released following interaction of mouse polymeric IgA with the transgenic receptor. However, soluble FcαRI was not detected as a component of mesangial deposits. This group has subsequently reported the development of IgA deposition and microscopic haematuria in this mouse model to be independent of the FcR γ chain (Arcos-Fajardo *et al*, 2004).

A study which quantified levels of soluble FcαRI in human sera performed by a separate group using a dot-blot assay found no differences between controls and IgA nephropathy patients with limited or extensive clinical disease (van der Boog *et al*, 2003). The discrepancy with previous results seemed to be due to the methodology of quantification. It appeared that ELISA systems did not detect covalently linked circulating FcαRI-IgA complexes whereas theses complexes were identifiable and measurable using the dot-blot assay. A significant conformational alteration following covalent linkage was considered to be the reason why ELISA systems were unable to detect soluble FcαRI-IgA complexes. In a different approach taken to test the effect of soluble FcαRI in an animal model, this same group injected soluble recombinant human FcαRI into mice. In contrast to the effects observed in mice expressing the human FcαRI transgene, no mesangial IgA deposition was seen despite high circulating levels of FcαRI (van der Boog *et al*, 2004). Instead, rapid clearance of FcαRI from the circulation with subsequent deposition of FcαRI in the liver occurred. Using three different methods, it appeared in this study that mouse IgA was unable to bind to soluble human FcαRI. These results implied that the presence of circulating human FcαRI alone in the transgenic mouse model was insufficient to explain the renal phenotype or that alternatively there is some crucial difference between recombinant human soluble FcαRI and human circulating FcαRI in the transgenic model. The role of soluble FcαRI in the pathogenesis of human IgA nephropathy therefore remains unclear.

1.4.6. Characteristics of mesangial IgA

Immunohistochemical staining of renal biopsy sections using polyclonal antisera and monoclonal antibodies has been utilised in order to characterise the nature of IgA deposited in the mesangium in IgA nephropathy. IgA1 is consistently present and was the predominant subclass in cases where IgA2 was co-deposited (Conley *et al*, 1980; Lomax-Smith *et al*, 1983). Whether IgA2 was present did not correlate with any clinical or other pathological feature (Hisano *et al*, 2001). Secretory component was not detected thus favouring a systemic as opposed to a mucosal origin of the glomerular IgA. Both kappa and lambda light chains have been detected in mesangial IgA deposits with the latter present invariably and predominantly in cases of co-localisation (Lai *et al*, 1988). It has been demonstrated that multimeric IgA is present in the mesangium although whether this is the exclusive form or what amount of monomeric IgA may also be present is unknown as there have been no quantitative measurements (Tomino *et al*, 1982). This polymeric species of IgA recovered from the mesangium had a more anionic charge profile in comparison with serum IgA (Monteiro *et al*, 1985). A recent report described the recovery of secretory IgA from the transplanted kidney of a patient with recurrent IgA nephropathy (Oortwijn *et al*, 2006). Previous studies using immunohistochemical methods had not detected secretory IgA within renal IgA deposits. Similarly, these investigators were unable to detect secretory IgA in this kidney using these immunohistochemical techniques and suggested that alternative reagents and methodologies should be developed to detect mesangial secretory IgA.

A crucial link in understanding the pathogenesis of IgA nephropathy was facilitated by the detailed examination of mesangial IgA. Previous techniques were insufficiently sensitive to determine the nature of hinge region glycosylation in the small amounts of mesangial IgA1 recoverable from individual needle biopsy specimens from patients with IgA nephropathy. The central role proposed for deficient hinge region galactosylation in circulating IgA1 was validated when mass spectrometry was used to analyse a sample of IgA1 pooled from 290 renal biopsy specimens of patients with IgA nephropathy (Hiki *et al*, 2001). The profile of hinge region glycopeptides from

these subjects showed reduced galactosylation consistent with the abnormality seen in serum IgA1 in IgA nephropathy.

The same abnormality was demonstrated in IgA1 recovered from nephrectomy specimens from three subjects with biopsy proven IgA nephropathy (Allen *et al*, 2001). In this report comparison was made between serum IgA1 and glomerular IgA1 from each subject using lectin binding assays for terminal GalNAc. In each case lectin binding to glomerular IgA1 was higher than that observed with the corresponding serum IgA1 for each patient. Therefore, in addition to supporting the notion of reduced hinge region galactosylation in glomerular IgA1, these findings also suggested that serum IgA1 glycoforms displaying the most profound abnormalities in *O*-glycosylation might be selectively deposited within the kidney. Thus the species of IgA1 of which the disease-defining glomerular deposits are composed appears to share the characteristic aberrant hinge region glycosylation of serum IgA1.

1.4.7. The tubulointerstitium in IgA nephropathy

Tubulointerstitial damage leading to progressive fibrosis is the final common pathway of renal damage in many chronic kidney diseases (D'Amico *et al*, 1995). In IgA nephropathy, the extent of interstitial scarring correlates with progression to end stage renal disease (Freese *et al*, 1998). Possible mechanisms of tubular damage in IgA nephropathy and other glomerulonephritides include a toxic effect of proteinuria, the release of cytokines and other inflammatory mediators into the local microcirculation from glomerular and tubular cells and the subsequent recruitment and infiltration of inflammatory cells. Increased urinary concentrations of IgA have been demonstrated in patients with IgA nephropathy and it is likely that tubular cells are exposed to IgA1 or IgA1-containing immune complexes that appear in the glomerular filtrate due to loss of integrity of the glomerular filtration barrier (Galla *et al*, 1985). Indeed the presence of IgA deposits on renal tubular cells has been demonstrated by immunofluorescence in a small proportion of patients with IgA nephropathy (Frasca *et al*, 1982). In addition to a direct toxic effect, these complexes may specifically

activate tubular epithelial cells via surface receptors initiating or contributing to processes leading to tubulointerstitial damage.

Recent work has demonstrated binding of IgA to cultured proximal tubular cells using IgA isolated from both patients with IgA nephropathy and controls (Chan *et al*, 2005). Although there was increased binding of patient-derived IgA, the overall level of binding was approximately one tenth of that observed with cultured human mesangial cells and neither activation nor proliferation of tubular cells following IgA binding occurred. In contrast, tubular cell proliferation and production of inflammatory mediators was induced following incubation with medium from mesangial cells cultured in the presence of IgA isolated from patients with IgA nephropathy. Synthesis of these mediators appeared to be dependent on the presence of mesangial cell derived tumour necrosis factor-α in the culture medium. These results support the hypothesis of glomerulotubular communication via soluble mediators in the pathogenesis of IgA nephropathy.

1.4.8. Animal models of IgA nephropathy

Various animal models displaying features similar to human IgA nephropathy have been described. The ddY mouse model spontaneously develops a pattern of renal injury analogous to human IgA nephropathy with mesangial deposition of IgA, IgG, IgM and C3 in association with proteinuria and mesangial cell proliferation (Imai *et al*, 1985). The effects of various modulators of the natural history of this model have been examined and reported. The HIGA (high IgA) strain of ddY mice was established by selective outbreeding to generate a similar model with high serum IgA levels. The immune defect in HIGA mice appeared to be multigenic but the glomerular deposition of IgA in these animals was associated with a locus on chromosome 15 (Nogaki *et al*, 2005). As previously discussed, mice with germline disruptions in the uteroglobin gene develop symptoms similar to human IgA nephropathy and the formation of IgA-fibronectin complexes is promoted but these findings do not appear to be relevant to the mechanism of development of human

disease. Similarly, transgenic mice expressing human FcαRI on monocytic cells developed renal disease with mesangial IgA deposition and other features of IgA nephropathy. Over-expression of megsin in transgenic mice induced immune complex deposition, mesangial cell proliferation and enhanced mesangial matrix production (Miyata *et al*, 2002) (see section 1.6.2.1.).

There are significant limitations inherent in applying these findings to the pathogenesis of human IgA nephropathy due to fundamental biological differences between the human and murine IgA systems. No IgA receptor analogous to human FcαRI appears to be present in mice and distinct systems of IgA disposal dependent on the polymeric immunoglobulin receptor are believed to operate. However, the most crucial difference between the species is that mouse IgA lacks the hinge region which characterises human IgA1 so lacks the capacity to bear the *O*-linked glycans which appear to play a central role in human disease. Therefore, it seems unlikely that mouse models of disease will provide the reliable tool required to dissect the complex mechanisms of mesangial IgA1 deposition in IgA nephropathy.

1.5. Mesangial deposition of IgA

1.5.1. Interaction between IgA and the mesangial cell

A key missing link in elucidating the pathogenesis of IgA nephropathy is the failure to identify a mechanism for mesangial IgA deposition and subsequent mesangial cell activation. It therefore follows that the nature of interactions between circulating serum IgA or IgA-containing immune complexes and the mesangial cell is central to this process. Some of the functional consequences of abnormal IgA1 hinge region glycosylation which promote IgA1 self-aggregation or binding to mesangial matrix components as discussed earlier may be significant. An alternative concept is expression by mesangial cells of a specific receptor or multiple types of receptor for

IgA and that the affinity of this specific interaction may be modulated by variations in hinge region glycosylation or the molecular form of IgA1 or by other physiochemical properties of IgA1. It is also conceivable that a mesangial cell receptor binds to a component of IgA1-containing immune complexes distinct from IgA, such as IgG.

IgA1 binds to cultured human mesangial cells (HMC) *in vitro* in a dose-dependent, saturable manner which can be specifically inhibited and this suggests expression of a specific IgA receptor by these cells (Diven *et al*, 1998; Barratt *et al*, 2000; Leung *et al*, 2000). This interaction was specifically inhibited by IgA and the Fc portion of IgA but not by IgG, IgM, asialo-orosomucoid or the Fab portion of IgA. HMCs appear to exhibit a higher affinity for polymeric IgA1 and may preferentially bind polymeric IgA1 bearing lambda light chains (Lai *et al*, 1996b; Barratt *et al*, 2000). One report estimated an affinity constant (K_a) for the interaction between monomeric IgA and HMCs to be 2.3 x 10^6 M^{-1} but showed no difference in the affinity of HMCs for monomeric compared with *in vitro* heat aggregated IgA1 (Diven *et al*, 1998). The electrostatic charge of polymeric IgA1 influences its association with HMCs *in vitro* with the most anionic species displaying increased binding (Leung *et al*, 2001). In this study the higher absolute levels of anionic polymeric IgA1 from sera of patients with IgA nephropathy led to significantly increased binding to HMCs *in vitro* when compared to control sera. This correlates with previous work demonstrating the relatively anionic nature of eluates of mesangial IgA (Monteiro *et al*, 1985).

1.5.2. Mesangial cell receptors

Several responses other than modulation of proliferation can be induced following IgA binding to HMCs. These include release of cytokines such as interleukin-1 and interleukin-6 (Chen *et al*, 1994) and production of the matrix components fibronectin and collagen (Lopez-Armada *et al*, 1996). IgA is capable of activating the alternative complement pathway (Janoff *et al*, 1999) and this may contribute further to glomerular inflammation. Activation of the alternative complement cascade in IgA nephropathy is suggested by the frequent presence of the C3 component in the mesangium and *in*

vivo activation of this system has been demonstrated in 75% of patients on serial measurements (Wyatt *et al*, 1987). The complement system can also be activated through the lectin pathway and the glomerular deposition of mannose-binding lectin (MBL) and MBL-associated serine protease 1 in association with IgA deposits in at least a proportion of patients supports this mechanism of inflammation in IgA nephropathy (Endo *et al*, 1998). The parallel responses of HMC activation and cytokine production together with activation of the complement system probably initiate and maintain the processes of intrarenal inflammation leading to glomerulosclerosis.

An obvious candidate receptor to mediate the interaction between HMCs and IgA is FcαRI (CD89). An early report demonstrated that HMCs could bind IgA and a 60kDa putative IgA receptor was identified in these cells (Gomez-Guerrero *et al*, 1993). Hybridisation of mRNA to a specific FcαRI probe in northern blotting indicated that these HMCs expressed this receptor. Further work extended these findings by showing enhanced expression of FcαRI by HMCs in response to inflammatory cytokines (Bagheri *et al*, 1997). A third study supported these results and described association of FcαRI with the FcR γ chain in HMCs as well as mRNA expression of the ΔEC2 splice variant (Suzuki *et al*, 1999b). Other investigators demonstrated expression of FcαRI in 40% of microdissected glomeruli from IgA nephropathy patients although it is possible that this result reflected expression of receptor by infiltrating blood-borne cells (Kashem *et al*, 1997).

However, these findings were subsequently contradicted by a series of reports which failed to detect mRNA or protein expression of FcαRI or its splice variants by HMCs in culture or in renal biopsy sections while confirming IgA binding by these cells in addition to demonstrating activation following stimulation by IgA (Diven *et al*, 1998; Westerhuis *et al*, 1999; Leung *et al*, 2000). Furthermore, the My43 antibody which competitively inhibits binding of IgA to FcαRI did not reduce IgA binding to HMCs (Westerhuis *et al*, 1999; Leung *et al*, 2000). The explanation for these discrepant results is not entirely clear although pertinent factors may include differences in HMC

preparations and passage numbers of cells used perhaps leading to either loss of receptor expression with increasing passage or contamination of cultures with infiltrating monocytic cells or tissue macrophages bearing FcαRI. A further study failed to detect expression of wildtype FcαRI by HMCs which were able to bind IgA but a novel mRNA transcript related to but distinct from FcαRI was described (Barratt *et al*, 2000).

Other candidate receptors for IgA binding to HMCs include the polymeric immunoglobulin receptor (PIGR) and asialoglycoprotein receptor (ASGPR). However, the inability of their respective alternative ligands, IgM and asialo-orosomucoid, to competitively inhibit IgA binding to HMCs does not support a role for these receptors in mediating IgA binding to HMCs. Furthermore, flow cytometry and nested reverse transcription-PCR did not detect expression of either PIGR or ASGPR by cultured HMCs (Leung *et al*, 2000; Novak *et al*, 2002).

The transferrin receptor (TfR, CD71) has been proposed to function as an IgA1 receptor on HMCs (Moura *et al*, 2001). Although initially reported to have a higher affinity for monomeric compared to polymeric IgA1 it now appears that TfR binds only polymeric IgA1 and that earlier results were confounded by contamination of monomeric IgA1 preparations with transferrin (Moura *et al*, 2004). The TfR did not have affinity for IgA2 in either the monomeric or polymeric form. TfR expressed by HMCs mediated endocytosis following polymeric IgA1 binding and polymeric IgA1 derived from IgA nephropathy patients bound more avidly to TfR. The finding that glycosylation of polymeric IgA1 modulated association with TfR is of particular significance and is the likely reason why IgA2 does not bind to TfR. Deletion of either *N*- or *O*-linked glycosylation sites on polymeric IgA1 abolished binding to TfR completely whereas enzymatic treatment to remove both sialic acid and β1,3-galactose groups significantly enhanced binding to TfR. These results are consistent with and may explain earlier observations of increased binding of higher molecular weight fractions of IgA1 from IgA nephropathy patients. More recent work demonstrated that binding of polymeric IgA1 to HMCs led to a dose-dependent enhancement of TfR

expression and that HMC production of IL-6 and TGF-β dependent on TfR was induced (Moura *et al*, 2005). Augmentation of HMC TfR expression was also observed when cells were incubated for 72 hours with sera from IgA nephropathy patients when compared to HMCs exposed to normal control sera. This effect was reduced when IgA was depleted from the patient sera, excluding the possibility of substantial cell activation by other factors such as cytokines. The effect also appeared to be independent of IgA concentration in the sera.

Expression of TfR has also been examined in renal biopsy specimens from patients with IgA nephropathy and other glomerular diseases (Haddad *et al*, 2003). Glomerular TfR expression was not specific for IgA nephropathy but instead seemed to be associated with mesangial cellular proliferation. However, the pattern of glomerular TfR expression appeared to co-localise with mesangial IgA deposits in patients with IgA nephropathy. The TfR therefore fulfils many of the criteria for a receptor mediating the mesangial deposition of IgA in IgA nephropathy.

Expression by cultured HMCs of Fc receptors for IgG has been described under certain conditions. Treatment of these cells with interferon-γ (IFN-γ) in combination with lipopolysaccharide led to detection of FcγRIII (CD16) transcript and protein (Radeke *et al*, 1994). This receptor appeared to mediate production of interleukin-6 by HMCs following stimulation by IgG immune complexes. Another report by the same group described functional FcγRI (CD64) expression following stimulation of HMCs in culture with IFN-γ (Uciechowski *et al*, 1998). The implication of these findings is that circulating immune complexes comprising IgA together with IgG could be expected to activate intrinsic mesangial cells under appropriate inflammatory conditions and IgG Fc receptors could potentially be a factor in the localisation of IgA to the mesangium. Genetic polymorphisms of FcγRII and FcγRIII have been reported to be associated with severity of, but not susceptibility to, IgA nephropathy in Japanese patients (Tanaka *et al*, 2005). The FcγRII allele related to IgA nephropathy severity was associated with a lower receptor affinity for IgG while the FcγRIII polymorphism was associated with a high affinity receptor. These differences were

hypothesised to reflect distinct underlying effector mechanisms of immune complex clearance.

1.6. Genetics of IgA nephropathy

1.6.1. Racial variation in prevalence of IgA nephropathy

IgA nephropathy does not display features of classic Mendelian inheritance where a single gene locus is responsible for the phenotype. However, population studies suggest that genetic factors are important in the development and perhaps progression of the condition. The prevalence appears highest in eastern Asian populations and lowest among black African and American ethnic groups (Levy & Berger, 1988). In addition, the disease association with male gender observed in Caucasian subjects appears to be reversed in Black populations where females are predominantly affected (Jennette *et al*, 1985). This variation between populations cannot be explained entirely by differences in biopsy policies and is considered to represent genetically associated variability in disease susceptibility.

1.6.2. Candidate gene polymorphisms

Variability in many biologically plausible candidate genes potentially involved in the pathogenesis of IgA nephropathy has been explored in order to elucidate any associations with either disease susceptibility or progression. The earliest studies of genetic polymorphisms in IgA nephropathy examined the genes of the human major histocompatibility (MHC) locus (reviewed in Hsu *et al*, 2000). Initial reports suggested an association with the HLA-Bw35 and HLA-DR4 antigens and susceptibility to IgA nephropathy. However these associations were not consistently demonstrated in subsequent studies probably due to variations in biopsy policy, racial differences and heterogeneity of susceptibility genes. Similarly, an association

between IgA nephropathy and the MHC class II allele HLA-DQB1 was proposed but following refinements in typing methodologies this finding was refuted by all but one study. In this study of Japanese subjects, two HLA-DQB1 alleles were reported to be protective for IgA nephropathy.

Allelic variations in genes encoding the T-cell receptor (TCR) have also been examined in IgA nephropathy patients and controls. In a case-control study of 40 European patients with IgA nephropathy an association was found with a TCR Cβ polymorphism (Rambausek *et al*, 1993) and the same polymorphism was associated with disease progression in a series of 34 Japanese patients (Nagasawa *et al*, 1995). An allele of the TCR Cα gene was associated with susceptibility but not with disease progression in a case control study that included 53 Chinese patients with IgA nephropathy (Li *et al*, 1997).

The insertion/deletion (I/D) polymorphism in intron 16 of the angiotensin converting enzyme (ACE) gene has been extensively characterised and examined in several pathophysiological conditions. Subjects homozygous for the D allele have serum and tissue levels of ACE up to three times higher than those homozygous for the I allele and this has been associated with many aspects of cardiovascular disease including hypertension, left ventricular hypertrophy and myocardial infarction (Danser *et al*, 1995). In renal disease, the higher levels of serum angiotensin II generated as a consequence of the DD genotype are believed to lead to an adverse glomerular reaction to injury through altered intraglomerular haemodynamic responses and promotion of smooth muscle cell and mesangial cell proliferation (Jardine, 1995). Possession of the ACE DD genotype has been associated with earlier progression of both adult polycystic kidney disease and type 1 diabetic nephropathy to end stage renal failure (Baboolal *et al*, 1997; Vleming *et al*, 1999). There are several published series in both Caucasian and Asian populations examining ACE I/D genotype in IgA nephropathy (reviewed in Hsu *et al*, 2000). Overall these earlier studies suggested an association between homozygosity for the D allele and progression to end stage renal disease in patients with IgA nephropathy. In one study the DD genotype conferred an

independent hazard ratio of 3.6 for reduced renal survival in multivariate analysis (Stratta *et al*, 1999). However, later studies performed with larger numbers of patients and with superior stratification of risk yielded opposite results. A series of 274 Caucasian men with IgA nephropathy and another study of 527 Japanese patients, each with mean follow up in excess of five years, found no significant association between the ACE D allele and disease progression (Frimat *et al*, 2000; Suzuki *et al*, 2000). The contradictory results of earlier studies are likely to be explained by various weaknesses including relatively small sample sizes, heterogeneous populations and poor risk stratification of progression. The ACE I/D genotype did not appear to be associated with disease susceptibility. The largest study examining the effect of polymorphisms in the angiotensinogen gene and in the angiotensin II type 1 receptor gene with progression of IgA nephropathy showed no significant association whereas smaller studies have shown conflicting results (Frimat *et al*, 2000).

Polymorphisms in numerous other genetic loci have been reported to be associated with disease susceptibility or progression of IgA nephropathy to end stage renal disease. These include the genes for transforming growth factor-β1, plasminogen activator inhibitor-1, TNF-α, polymeric immunoglobulin receptor, interleukin-10, uteroglobin, nephrin, CD14, E-selectin, L-selectin, interleukin-4, interferon-γ and mannose-binding protein. A well characterised functional polymorphism in the CCR5 chemokine receptor has been reported to be associated with late progression of IgA nephropathy (Berthoux *et al*, 2006). Renal transplant recipients homozygous for this allele were previously shown to have significantly better long term graft survival. As discussed previously (section 1.5.2.), allelic variations in Fc receptors for IgG (FcγRII and FcγRIII) have been associated with severity of IgA nephropathy in Japanese patients (Tanaka *et al*, 2005).

At least two different polymorphisms have been described in the promoter region of the FcαRI gene. In one study of 90 Japanese patients with IgA nephropathy the distribution of genetic polymorphisms was reported to be significantly different in comparison to patients with other primary glomerulonephritides and healthy controls

(Tsuge *et al*, 2001). However, a similar study comparing 151 Japanese patients with IgA nephropathy with healthy controls and those with other glomerular disease showed no association between these polymorphisms in the promoter of FcαRI and disease susceptibility (Narita *et al*, 2001). The significance of all these reported associations is limited by the inherent weakness of small-scale case control studies in a similar manner to the ACE I/D polymorphism story.

1.6.2.1. Megsin gene polymorphisms

The protein megsin is a member of the serine protease inhibitor superfamily first identified in the mesangium where it is primarily expressed. Increased renal expression of megsin has been associated with mesangial proliferation and matrix expansion in IgA nephropathy and other glomerulonephritides although its precise role in these processes is not clear (Suzuki *et al*, 1999a). Over-expression in a transgenic mouse model led to immune complex deposition, mesangial matrix expansion and mesangial cell proliferation (Miyata *et al*, 2002). Two polymorphisms (C2093T and C2180T) have been described in the 3' untranslated region of the megsin gene. An initial case control study of 110 European IgA nephropathy patients failed to show any association between the C2093T polymorphism and disease (Szelestei *et al*, 2000). However, a later study using a more powerful family-based approach in a Chinese population showed an association between both polymorphisms and susceptibility to IgA nephropathy (Li *et al*, 2004). The association was examined in 423 patients with IgA nephropathy by genotyping each patient along with both parents or unaffected siblings when two parents were not available for study. Both megsin polymorphisms were significantly more likely to have been transmitted to a subject who subsequently developed IgA nephropathy thereby implicating these variations in contribution to disease susceptibility. This strategy highlights important differences compared to the case control approach (albeit in different racial backgrounds) and this large family-based cohort should provide a powerful tool to examine the significance of other genetic polymorphisms in this population.

1.6.3. Familial IgA nephropathy

An increased frequency of urinary abnormalities has been reported in relatives of patients with IgA nephropathy. In one series of patients with IgA nephropathy, 61 of 269 asymptomatic first-degree relatives displayed such abnormalities (Schena, 1995). Further evidence for a role of genetic factors in the pathogenesis of IgA nephropathy comes from reports of affected individuals from within the same family. Such parent-child and sibling pairs have been documented in a variety of ethnic backgrounds (reviewed in Hsu *et al*, 2000). No association with HLA type has been observed and this is consistent with results from the later case-control studies examining HLA type and susceptibility to IgA nephropathy. Several multiplex kindreds from a range of geographical and racial backgrounds where several members in different generations have IgA nephropathy have also been described and these familial forms of IgA nephropathy appear to be clinically indistinguishable from sporadic cases of the disease. One such family from Kentucky, where 14 members had biopsy proven IgA nephropathy, had a total of 176 family members clinically assessed (Julian *et al*, 1985). A further 17 had evidence of clinical glomerulonephritis and an additional six family members had "chronic nephritis" noted on death certificates. Six of those with biopsy confirmed disease had a common ancestor. The pattern of inheritance with male-to-male transmission and no gender imbalance suggested autosomal dominant transmission with variable penetrance. In a separate series of familial IgA nephropathy in Italian kindreds the renal survival appeared to be significantly poorer compared with sporadic disease (Schena *et al*, 2002) but no difference in renal survival has been reported in other families (Julian *et al*, 1988). Together these reports indicate that a distinct form of IgA nephropathy with a strong genetic basis is present within some families. Although histologically indistinguishable from the sporadic form, it is unclear what degree of overlap may exist in the pathogenesis of familial IgA nephropathy.

These extended kindreds with familial IgA nephropathy potentially offer an alternative tool to the case-control study to identify genes that may be important in disease susceptibility or progression, thus avoiding the problems inherent in case-control

studies. Twenty-four kindreds from Italy together with six kindreds from the United States were subjected to linkage analysis in order to identify relevant genetic loci (Gharavi *et al*, 2000). These kindreds included 94 members affected with IgA nephropathy (60 with biopsy proven disease), 48 unaffected members and 21 with status unknown due to age being less than 40 years. The initial approach was to examine whether disease was linked to any of a number of candidate genes potentially involved in the pathogenesis of IgA nephropathy including the immunoglobulin genes, the MHC locus, the galactosyltransferase genes and the genes for uteroglobin and FcαRI. No evidence of linkage to any of these loci was found in these families. Subsequently a genome-wide scan using 384 markers distributed at regular intervals throughout the genome was performed. This demonstrated linkage of the disease trait in 60% of kindreds to a locus designated *IGAN1* at 6q22-23 with a maximum lod score of 5.6. The region of *IGAN1* was estimated to contain between 100 and 200 loci of individual genes but there were no known candidates to explain the trait. There were no obvious clinical or demographic features associated with linkage to *IGAN1* compared with absence of linkage in different families. The pattern of inheritance within the kindreds corresponded best to a dominant model with incomplete penetrance.

1.7. Aims of this project

The principal aim of this work was to identify potential immunoglobulin receptors expressed in the kidney which may play a role in the pathogenesis of immune complex-mediated renal disease. In particular it was hoped to clarify whether human mesangial cells expressed the classical IgA receptor, FcαRI or a variant form of this receptor.

Figure 1.1. Histological features of IgA nephropathy

Typical histological features of IgA nephropathy. (A) Light microscopy of a glomerulus. (B) Immunofluorescence of a glomerulus using an anti-IgA antibody showing mesangial distribution of IgA. (C) Electron microscopy showing granular dense deposits representing immune complexes in the mesangium.

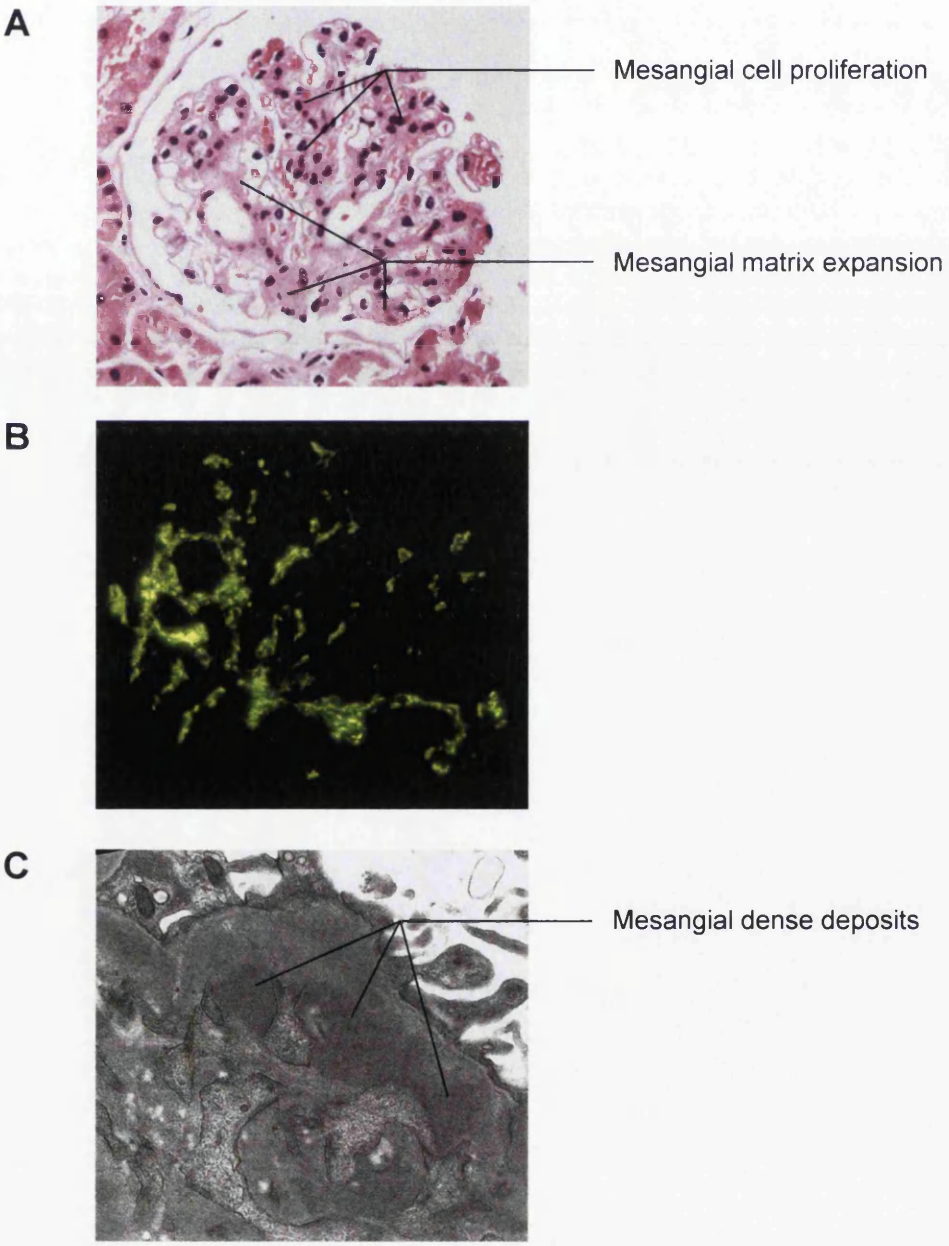


Figure 1.2. Structure of IgA1

Each monomer of IgA1 is composed of two immunoglobulin heavy (H) chains (shaded ovals) and two immunoglobulin light (L) chains (unfilled ovals). Each oval represents an immunoglobulin superfamily domain. The individual chains are linked by disulphide bonds (dashed lines). The molecule can be proteolytically cleaved into the Fc portion which mediates effector functions via specific receptors (FcRs) and the Fab fragment which contains the highly variable antigen binding site (variable (V) domains). IgA1 possesses a unique hinge region which distinguishes it from the phylogenetically older IgA2 subclass. The hinge region peptide sequence can bear *O*-linked carbohydrate groups (filled stars). The positions of *N*-linked carbohydrate groups are indicated by unfilled stars.

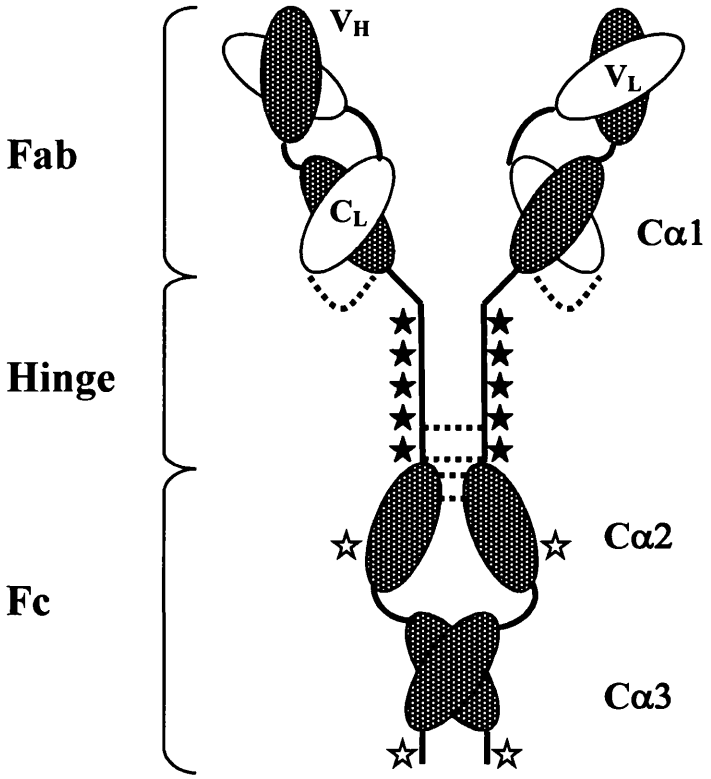
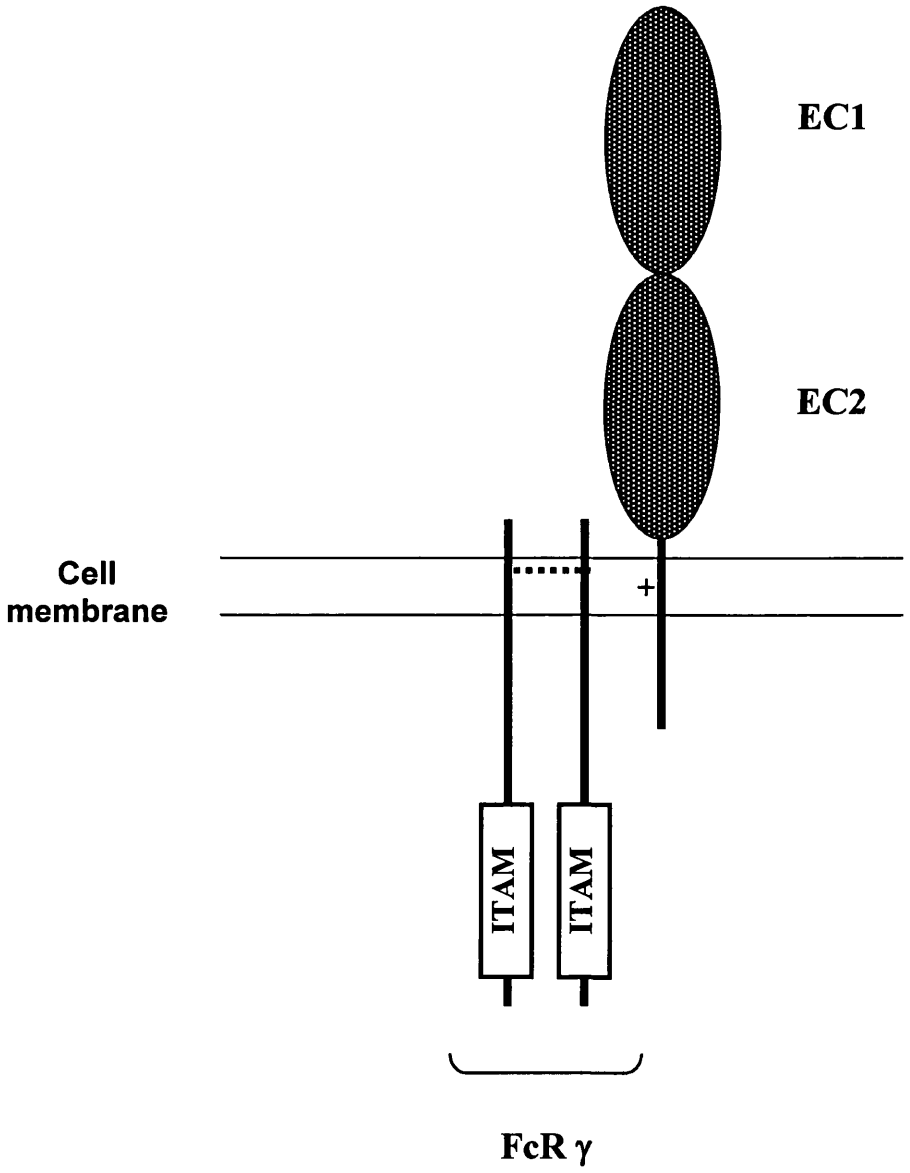


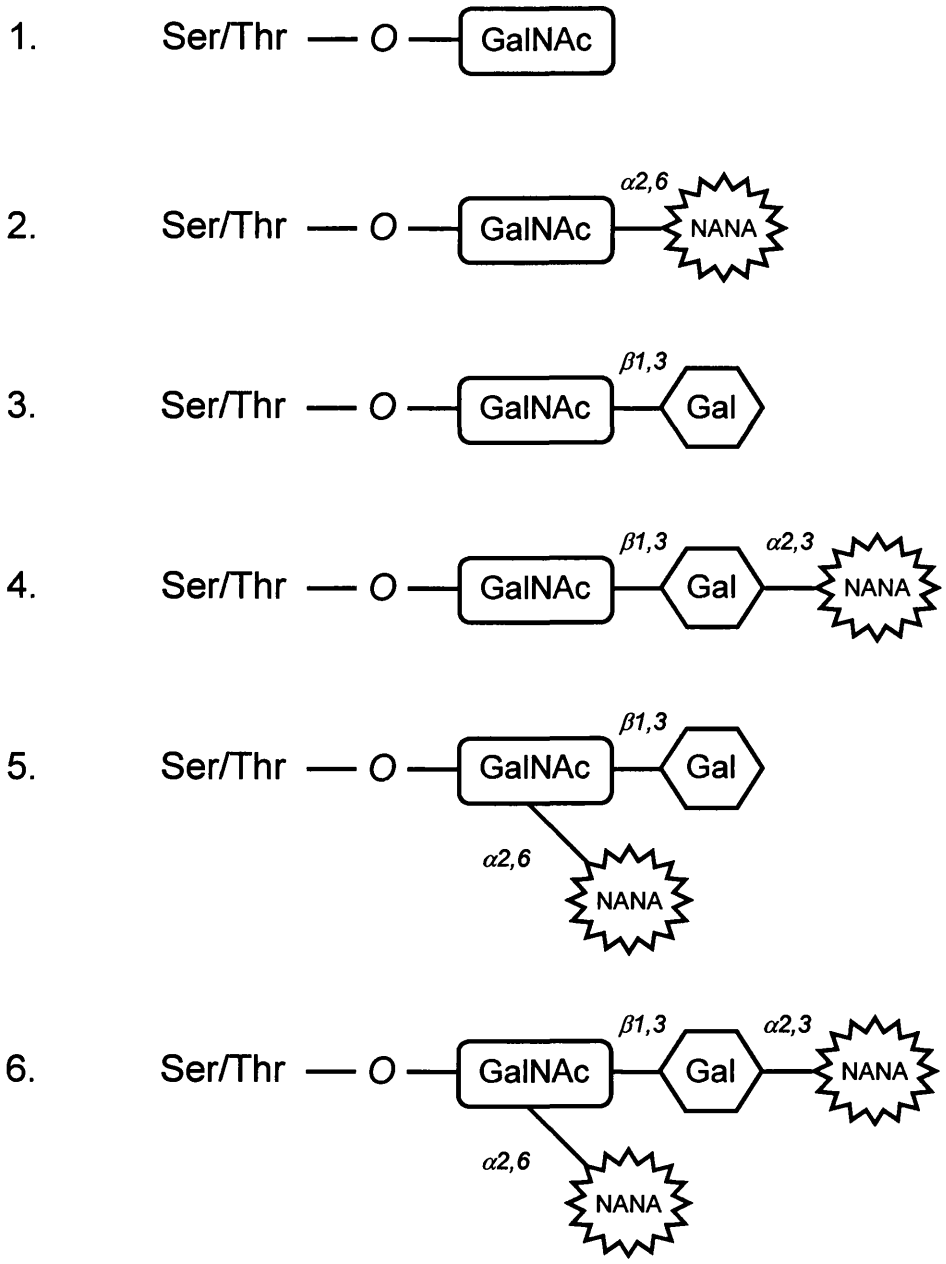
Figure 1.3. Structure of FcαRI (CD89)

FcαRI is a transmembrane protein with 2 extracellular domains (EC1 and EC2). The IgA binding site is located within the EC1 domain. The receptor is associated with the disulphide-linked (dashed line) FcR γ chain homodimer (FcRγ) via a positively charged residue in the transmembrane region (+). Each FcR γ chain possesses an ITAM motif which mediates signal transduction for the receptor complex.



1.4. Possible patterns of *O*-linked glycosylation at the IgA1 hinge region

Each glycan side chain is *O*-linked to either a serine or threonine reside in the IgA1 hinge region. Galactose (Gal) moieties are β1,3-linked to a core *N*-acetyl galactosamine (GalNAc) unit. Sialic acid (NANA) groups may be α2,6-linked to GalNAc or associated with Gal subunits via an α2,3 link.



Chapter 2

Materials and Methods

2.1. Materials

2.1.1. Biochemicals

Unless otherwise stated, all chemicals were obtained form Sigma, Poole, UK. All tissue culture reagents were purchased from Life Technologies, Paisley, UK.

2.1.2. Immunochemicals

The antibodies used in this study are detailed in table 2.1.

2.2. Cell biology techniques

2.2.1. Cell culture

2.2.1.1. Human mesangial cells

Primary human mesangial cell (HMC) cultures were established from glomeruli obtained from the normal pole of tumour nephrectomy specimens using a sequential sieving technique. Ethics approval was obtained from the local hospital ethics committee and patients gave informed consent for the use of tissue. Portions of renal

cortex were dissected and forced through a series of sieves to obtain glomeruli which were seeded into RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine (2mM), penicillin (100 units/ml), streptomycin (10mg/ml), insulin (10μg/ml), transferrin (5.5μg/ml), and selenium (6.7ng/ml) and maintained at 37°C, 5% CO_2 in a water saturated atmosphere. HMCs grew in characteristic clumps and were typically harvested for use between passages 2-4. Purity of cultures was confirmed by positive indirect immunohistochemical staining for actin and vimentin and negative staining for factor VIII related antigen.

In some experiments confluent HMCs were stimulated by the addition of either IL-1α, IL-6 or TNF-α in measured concentrations for 24 hours. These cytokines were obtained from AMS Biotechnology, Abingdon, UK. In other experiments HMCs were incubated with dibutyryl cyclic AMP obtained from Sigma.

2.2.1.2. Other cell lines

The COS-7 human fibroblast cell line (American Type Culture Collection (ATCC), Manassas, VA, USA) was maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum, L-glutamine (2mM), penicillin (100 units/ml) and streptomycin (10mg/ml). Chinese hamster ovary (CHO-K1) cells (European Collection of Cell Cultures, Salisbury, UK) and U937 cells (ATCC) were cultured in Ham's F12 and RPMI 1640 media respectively and supplemented as above. The human hepatocellular carcinoma cell line, HepG2 (ATCC) was cultured in minimum essential medium supplemented as above.

Primary cultures of human proximal tubular cells (PTCs) were a generous gift from Dr James McLay (University of Aberdeen, UK). PTC cultures had been prepared from normal poles of nephrectomy specimens by mincing, collagenase digestion then separation on a Percoll gradient (Mistry *et al*, 2001). PTCs were maintained in DMEM/Ham's F12 supplemented as above and were used after two passages.

Immortalised cell lines were routinely sub-passaged 2-3 times per week. These cell lines were all maintained at 37°C, 5% CO_2 in a water saturated atmosphere.

2.2.2. Transfection of cells

COS-7 or CHO-K1 cells were transfected using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA) when the cells were approximately 50% confluent. A ratio of 3µl transfection reagent to 1µg plasmid DNA was used. Transfection reagent and DNA were mixed in serum free medium before addition to the cells for 48 hours.

2.2.3. Flow cytometry

COS-7 or CHO-K1 cells were detached using a rubber scraper and single cell suspensions formed by passage through a fine bore pipette. Cells were washed with ice cold phosphate buffered saline (PBS, Oxoid, Basingstoke, UK) pH 7.3 comprising 0.16M sodium chloride, 3mM potassium chloride, 8mM disodium hydrogen phosphate and 1mM potassium dihydrogen phosphate containing 0.1% bovine serum albumin (BSA). Between 0.5-1.0 x 10^6 cells per sample were resuspended in 50µl PBS/0.1% BSA with the appropriate amount of biotin labelled primary antibody. Following 45 minutes incubation on ice, cells were washed twice and incubated for a further 30 minutes with streptavidin conjugated to a fluorescent label (streptavidin-DTAF, Jackson Laboratories, West Grove, PA, USA). Cells were then analysed using a Coulter Epics XL FACScan (Beckman Coulter, Fullerton, CA, USA).

2.2.4. Fluorescence microscopy

In antibody binding experiments transfected COS-7 or CHO-K1 cells were grown on glass coverslips for 48 hours. The cells were then washed with ice cold PBS/0.1% BSA and incubated with primary antibody in PBS/0.1% BSA for 45 minutes at 4°C. Following two further washes the cells were incubated with the appropriate secondary

antibody or detection reagent for 30-45 minutes before mounting with DAPI-containing mounting medium (Vector Labs, Burlinghame, CA, USA) to counterstain cell nuclei. In some experiments primary antibodies directly labelled with fluorescein were used. Antibody binding to cells was analysed using a fluorescence microscopy imaging system (Improvision, Coventry, UK).

For immunohistochemistry experiments cells grown on glass coverslips were fixed with 3% paraformaldehyde then permeablised with 0.1% Triton-X100 in PBS for 8 minutes at room temperature. Cells were then incubated with 4% goat serum and 2% BSA to block non-specific binding. Rabbit anti-calnexin and anti-caveolin primary antibodies were incubated overnight at 4°C at concentrations of 1:200 and 2.5μg/ml respectively in blocking buffer. After washing with PBS, a Texas Red-labelled anti-rabbit antibody was applied for one hour at a concentration of 10μg/ml. Nuclei were stained with benzimide (10μg/ml in PBS) for 3 minutes prior to four further washes with PBS.

In some experiments transfected cells were incubated with polymeric myeloma IgA1 (a gift from Dr Jan Novak, University of Birmingham at Alabama, USA) at a concentration of 50μg/ml in 5% normal human serum for one hour on ice. IgA binding was detected with a Texas Red conjugated goat anti-human IgA antiserum at a concentration of 15μg/ml in 4% goat serum. Cells were then fixed with paraformaldehyde and stained with benzimide as above prior to visualisation with fluorescence microscopy.

2.3. Molecular biology

2.3.1. RNA extraction

Total cellular RNA was extracted from cells using TRIzol Reagent (Life Technologies). Cells grown in suspension were pelleted by centrifugation then lysed with repetitive pipetting in TRIzol, using 1ml of reagent per 5-10 x 10^6 cells. Adherent cells were detached by trypsinisation or scraping then pelleted and lysed. RNA was also extracted from a fragment of renal cortex and from microdissected glomeruli in a similar fashion. Samples were incubated for 5 minutes at room temperature to allow dissociation of nucleoprotein complexes before the addition of 0.2ml of chloroform per 1ml of TRIzol. The samples were shaken thoroughly by hand for 15 seconds then incubated for a further 3 minutes at room temperature. Centrifugation at 13,000rpm for 15 minutes was then performed at 4°C in a microfuge. Following centrifugation the upper aqueous layer containing the RNA was transferred to a separate tube and 0.5ml of isopropanol was added to precipitate RNA. Samples were incubated at room temperature for 10 minutes followed by further centrifugation at 13,000rpm for 10 minutes at 4°C. The RNA precipitate was then washed using 1ml of 75% ethanol in diethylpyrocarbonate (DEPC) treated water followed by vortexing then centrifugation at 9,000rpm for 5 minutes at 4°C. The final RNA pellet was allowed to air dry for 5-10 minutes then dissolved in DEPC treated water (typically 30µl volume). RNA concentration was determined by measuring absorbance at 260nm and purity was estimated by checking the 260nm:280nm absorbance ratio. RNA samples were stored at -70°C.

2.3.2. First strand cDNA synthesis

First strand cDNA synthesis was performed using a Superscript first strand synthesis system (Life Technologies). Between 1-3 µg of total RNA was incubated with 1µl of 10mM dNTP mix and 1µl of oligo (dT) (0.5 µg/µl) in a total volume of 10µl for 5 minutes at 65°C then placed on ice. Reaction mixture containing 2µl of 10x reverse transcription buffer, 4µl of 25mM magnesium chloride, 2µl of 0.1M DTT and 1µl of RNase inhibitor per sample was prepared and added. The samples were then

incubated at 42°C for 2 minutes before 1µl (50 units) of Superscript II reverse transcriptase was added. Reverse transcription controls were also performed in some experiments in which case no enzyme was added. The reverse transcription reaction was allowed to proceed at 42°C for 50 minutes before termination by heating to 70°C for 15 minutes. Samples were then diluted 1:5 to a final volume of 100µl with distilled water and stored at -20°C.

2.3.3. Polymerase chain reaction

Polymerase chain reaction (PCR) was performed using a Techne Progene thermocycler, a PTC-150 minicycler or a PTC-200 gradient cycler (MJ Research Inc). Mastermix containing 10x PCR buffer, 25mM magnesium chloride (final concentration 1.5mM), dNTP mix (final concentration 200µM of each dNTP) and forward and reverse primers (final concentration of each 300nM) was prepared and added to each tube. Template cDNA in a volume of 5µl or distilled water as control was then added before the addition of enzyme. For routine PCR, 5 units per reaction of *Taq* DNA polymerase (Promega, Madison, WI, USA) were used. In experiments where high fidelity was required, 2.5 units of *pfu* polymerase (Stratagene, La Jolla, CA, USA) were added to each reaction instead. A "modified hot start" technique was used where the samples were kept on ice after the addition of enzyme then placed in the cycler with the block already heated to a denaturing temperature. The following thermal cycling profile was used. Samples were denatured at 94°C for 90 seconds. Between 30-40 cycles of: denaturation at 94°C for 30 seconds, primer annealing for 60 seconds at a temperature determined by the specific primer combination and DNA extension at 72°C for at least 60 seconds were performed. The extension time was lengthened if the product length was greater than 1000 base pairs or when using *pfu* polymerase. A final extension at 72°C was then performed for 5-10 minutes. Oligonucleotide primers were synthesised by MWG-Biotech, Ebersberg, Germany.

2.3.3.1. Real time polymerase chain reaction

Real time PCR was used to determine relative quantification of mRNA transcripts following reverse transcription. The GeneAMP 5700 Sequence Detection System and SYBR Green Core Reagents were utilised (PE Biosystems, Foster City, CA, USA). In this system primers are designed to generate a small DNA product to maximise reaction efficiency and the reaction takes place in the presence of SYBR Green dye which fluoresces on binding to double stranded DNA. Fluorescence is measured at the end of each PCR cycle and increases exponentially as the reaction progresses. For each reaction the threshold cycle (C_t) is calculated based on the point at which a statistically significant increase in the amount of PCR product is detected. The threshold cycle inversely reflects the number of target sequences present in each sample prior to amplification (Higuchi *et al*, 1993). These kinetics are illustrated in figure 2.1.

Primers for real time PCR were designed using Primer Express software (PE Biosystems) with melting points (T_m) 58-60°C and target amplicons of around 100 base pairs. Reactions were performed in 20µl volumes in triplicate in 96-well optical reaction plates with optical caps. SYBR Green PCR Master Mix (PE Biosystems) supplied at 2x concentration and containing optimised buffer components, deoxynucleotides and AmpliTaq Gold polymerase was used. Each reaction was performed with either 2.5µl diluted cDNA or reverse transcription control as described above. Forward and reverse primers were each present at a concentration of 300nM. The thermal cycling profile started with a 10 minute incubation at 95°C to permit automated "hot start" of enzyme activity followed by 40 cycles of a 15 second melt step at 95°C and a 1 minute anneal/extend step at 60°C. Results were analysed using GeneAmp 5700 SDS software. In order to assess specificity of amplified product and avoid spurious results from "primer-dimer" formation and amplification, dissociation curves showing product melting points were assessed and samples of product were run and visualised on agarose gels.

2.3.4. Agarose gel electrophoresis of DNA

DNA samples were mixed with 6x blue/orange loading buffer (Promega) and run on agarose gels with tris-acetate (TAE) electrophoresis buffer (0.04M Tris-acetate, 0.001M EDTA). Gel concentration varied between 1-2% depending on the degree of separation required. The gels were then stained for 10 minutes in a solution of ethidium bromide in TAE (1μg/ml) and the bands visualised on an ultraviolet transilluminator. Images were recorded either on Polaroid film or on a digital imaging system (EASY plus, Herolab).

2.3.5. Gel extraction and purification of DNA

DNA extraction from agarose was performed using a QIAquick Gel Extraction Kit (Qiagen). Each DNA fragment to be used was excised using a clean scalpel blade and placed in an Eppendorf tube. The gel slices were then incubated at 50°C for 10 minutes in buffer QG until the agarose had dissolved. Approximately 1 gel volume of isopropanol (150μl) was added and each sample placed in a spin column. DNA was applied to the membrane by centrifugation for 1 minute at maximum speed in a microfuge. The flow-through was discarded and the membrane washed with 750μl of buffer PE before further centrifugation. This flow-through was also discarded and the column spun again to remove all traces of wash buffer. The column was then placed into a clean Eppendorf tube and the bound DNA eluted by the addition of 50μl of buffer EB followed by centrifugation for 1 minute.

2.3.6. Subcloning of DNA

2.3.6.1. Ligation of DNA into vector

The TOPO TA cloning system (Invitrogen, Groningen, The Netherlands) was used to subclone PCR products into vectors. Different vectors were used depending on the

application. The pCR4-TOPO (figure A.1.) vector was used to subclone PCR products for routine sequencing and pcDNA3.1/V5-His-TOPO (figure A.2.) was used as an expression vector for full length gene sequences. Constructs expressing a green fluorescent protein tag were generated by subcloning into the pcDNA3.1/CT-GFP-TOPO vector (figure A.3.). The TOPO system exploits the property of *Taq* DNA polymerase to add a single 3' deoxyadenosine (A) overhang to each strand of synthesised DNA. The linearised vectors contain complementary 3' overhanging deoxythymidine (T) residues and the enzyme topoisomerase I to facilitate insertion and ligation of PCR products. Where PCR products were generated using *pfu* polymerase, 3' deoxyadenosine overhangs were added following amplification. This was achieved by incubation of the gel purified product with 0.5 unit of *Taq* polymerase for 10 minutes at 72°C in the presence of deoxyadenosine nucleotides. For each cloning reaction 4µl of fresh PCR product was mixed with 1µl of vector and 1µl of salt solution (1.2M sodium chloride and 0.06M magnesium chloride) and incubated at room temperature for 5 minutes.

2.3.6.2. Transformation of competent *Escherichia coli*

Transformation competent One Shot TOP10 *E. coli* (Invitrogen) were utilised. A single aliquot of competent cells (50µl) was gently mixed with 2µl of plasmid DNA from the ligation step and incubated on ice for 20 minutes. Cells were then heat shocked at 42°C for 30 seconds followed by incubation on ice for 2 minutes. Following the addition of 250µl SOC medium (Invitrogen) cells were gently mixed for 1 hour at 37°C then aliquots spread on prewarmed LB (Luria-Bertani) agar (Life Technologies) selective plates containing 100 µg/ml ampicillin. Plates were incubated overnight at 37°C.

2.3.6.3. Purification of plasmid DNA

For minipreparations of DNA, 4-10 colonies were selected from each ampicillin selective plate and used to inoculate separate 5ml aliquots of LB medium containing

100μg/ml ampicillin. Cultures were grown overnight at 37°C with continuous shaking. DNA was purified using the QIAprep Miniprep system (Qiagen, Crawley, UK) according to the manufacturer's instructions. Briefly, alkaline lysis of pelleted bacterial cells was followed by application of supernatants to columns containing a silica-gel membrane. Plasmid DNA was adsorbed selectively to these membranes and washed prior to elution and collection.

A Qiagen Plasmid Maxi Kit was used for the purification of larger quantities of transfection grade plasmid DNA. Bacterial cultures in 100ml volumes were grown overnight and pelleted cells lysed. Precipitated cell debris including bacterial genomic DNA was separated from soluble plasmid DNA by centrifugation and the supernatants applied to DNA binding membranes. Following washing the DNA was eluted then precipitated by the addition of isopropanol prior to centrifugation. DNA pellets were washed with 70% ethanol then air dried before dissolving in TE buffer pH 8.0 (10mM Tris-Cl pH 8.0, 1mM EDTA). DNA was quantified and assessed using spectrophotometry and agarose gel electrophoresis.

2.3.6.4. Restriction enzyme digests

Typically, 1 μg of plasmid DNA was digested in a 20μl volume containing 2μl of an appropriate 10x reaction buffer and 1 μl of enzyme. Reactions were incubated for a period of time between 2 hours and overnight at 37°C. The products of digestion were resolved on agarose gels and visualised using ultraviolet light as previously described.

2.3.6.5. DNA sequencing

Sequencing of plasmid DNA was performed commercially by MWG-Biotech. For routine sequencing to check the identity of PCR products, DNA was subcloned into the pCR4-TOPO vector (Invitrogen) and the sequencing read was started from either or both of the T3 and T7 priming sites incorporated into the vector. Each sample containing 5-10μg of plasmid DNA was made up to 200μl volume with distilled water

then 20µl 3M sodium acetate pH 5.2 and 500µl 100% ethanol were added. DNA was precipitated by microcentrifugation at 13,000rpm at 4°C for 10 minutes. Pellets of DNA were then washed with 500µl 70% ethanol and either vacuum dried for 10 minutes with gentle centrifugation or air dried for 1 hour. Sequence data were analysed using the GCG software package (Accelrys, Cambridge, UK).

2.3.7. 3' rapid amplification of cDNA ends (RACE)

A 3' RACE system (Life Technologies) was used to identify potential novel transcripts of FcαRI expressed by human mesangial cells. This technique permits the identification of these unknown mRNAs by exploiting the naturally occurring poly (A) tail for use as a priming site for subsequent PCR amplification. First strand cDNA synthesis was performed using 2.5µg of RNA and an oligo-dT adaptor primer then RNase H was added in order to remove the original RNA template. PCR amplification using an FcαRI specific sense primer and an abridged universal amplification primer complementary to the adaptor sequence used to prime the first strand cDNA synthesis was then performed. PCR products were identified on agarose gel electrophoresis and bands of interest were purified, subcloned and sequenced.

2.4. Biochemical assays and methods

2.4.1. Preparation of cell lysates

Adherent cells were lysed by the direct addition of ice-cold lysis buffer (1% (v/v) Triton X-100, 50mM Tris-HCl pH 7.5, 0.25% (w/v) sodium deoxycholate, 150mM sodium chloride, 1mM EDTA, 1mM vanadate and 1mM sodium fluoride) containing protease inhibitors (1mM phenylmethylsulfonyl fluoride and 1µg/ml each of chymostatin, leupeptin, antipain and pepstatin). Cells in suspension were pelleted by centrifugation and medium was removed prior to the addition of lysis buffer.

Following 30 minutes of tumbling motion at 4°C, insoluble debris was removed by centrifugation at 15 000g for 15 minutes at 4°C.

Protein concentrations of lysates were estimated using the Bio-Rad protein assay system which is based on the Bradford Coomassie brilliant blue dye binding system. A range of bovine serum albumin standards was used to construct a standard curve using spectrophotometry and protein samples were diluted to fall within the linear range of this curve. Protein concentrations of all samples were routinely measured in duplicate. Lysates were stored at -70° C prior to further usage.

2.4.2. Polyacrylamide gel electrophoresis

Proteins were resolved using the NuPAGE precast gel system (Invitrogen) which employs bis-tris buffered (pH 6.4) polyacrylamide gels, available at 3 different acrylamide concentrations (10%, 12% and 4-12% gradient gels) allowing a choice of different separation ranges. Additional control over separation can be achieved by selecting from two different running buffers. Protein samples of standardised amount (usually 5-10μg) were denatured in NuPAGE LDS (lithium dodecyl sulphate) sample buffer by heating to 70°C for 10 min. In order to reduce proteins, 50mM dithiothreitol was added immediately prior to sample denaturing.

2.4.3. Immunoblotting

Proteins resolved by polyacrylamide gel electrophoresis were transferred to Protran nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) using the NuPAGE XCell II blotting apparatus and manufacturer's transfer buffer which was supplemented freshly with methanol to a volume of 10% or 20% as appropriate. Transfer was performed at 30 volts for 60-80 minutes. Transfer efficiency and equality of loading were assessed by staining membranes with Ponceau S solution for 5 minutes followed by washing with distilled water. Ponceau S was removed by

washing with TBS (20mM Tris-HCl pH 7.6, 136mM sodium chloride) containing 0.1% (v/v) Tween 20 (TBST).

The nitrocellulose membranes were then blocked in TBST with 4% (w/v) non-fat powdered milk (Marvel) for a minimum of 1 hour at room temperature. Blots were incubated with primary antibodies at the recommended concentration in TBST overnight at 4°C. Three washes of 10 minutes each using TBST were then carried out prior to incubation with the appropriate horseradish peroxidase conjugated secondary antibody for 2-4 hours in TBST at room temperature. Blots were then washed extensively with large volumes of TBST (typically 6 x 10 minute washes) followed by a final wash in TBS prior to developing. Protein bands were visualised by enhanced chemiluminescence (Amersham) and autoradiography.

2.4.4. Membrane preparation

Adherent cells were washed twice in cold nuclear preparation buffer (10mM Tris-HCl pH 7.4, 2mM magnesium chloride, 140mM sodium chloride). Cells were then lysed by the addition of nuclear preparation buffer containing 2% (v/v) Tween 40, protease inhibitors (1mM phenylmethylsulfonyl fluoride and 1μg/ml each of chymostatin, leupeptin, antipain and pepstatin) and phosphatase inhibitors (1mM vanadate and 1mM sodium fluoride) followed by three freeze-thaw cycles using dry ice. Cell debris and nuclei were removed by centrifugation at 15,000g for 5 minutes and membranes were recovered by ultracentrifugation of the supernatant at 100,000g at 4°C for 1 hour (Bunce *et al*, 1988). Following ultracentrifugation the supernatant represents the cytosolic fraction. Membrane pellets were resuspended in cold nuclear preparation buffer.

2.4.5. N-glycanase assay

Lysates of some transfected cells were treated overnight at 37°C with 5 milliunits of recombinant *N*-glycanase (Glyko, Upper Heyford, UK) prior to immunoblotting, in

order to remove any *N*-linked carbohydrate groups. A control lysate without the addition of *N*-glycanase was treated in the same manner.

2.5. Bioinformatics tools

Various bioinformatics tools available through the internet were used to analyse amino acid sequences of proteins of interest. These included the PSORT tool (http://psort.hgc.jp) and the "DAS" Transmembrane Prediction Server (http://www.sbc.su.se/~miklos/DAS/maindas.html) for prediction of features such as signal peptide and transmembrane sequences. The PROSITE database of protein families and domains (http://ca.expasy.org/prosite) was accessed to identify domain similarities and the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc) was used to identify likely sites for *N*-glycosylation. The NetPhos 2.0 server (http://www.cbs.dtu.dk/services/NetPhos) was used to identify potential sites for tyrosine, serine and threonine phosphorylation. A peptide mass calculator at http://ca.expasy.org/tools/peptide-mass.html was also utilised. The GPI Prediction Server (http://mendel.imp.univie.ac.at/sat/gpi/gpi_server.html) was accessed in order to assess the likelihood of a protein possessing a GPI (glycosylphosphatidylinositol) anchor.

Figure 2.1. Kinetics of real time PCR

An amplification plot representing fluorescence signal versus cycle number. The threshold cycle (C_T) is defined as the point when a statistically significant rise in the amount of DNA product is detected.

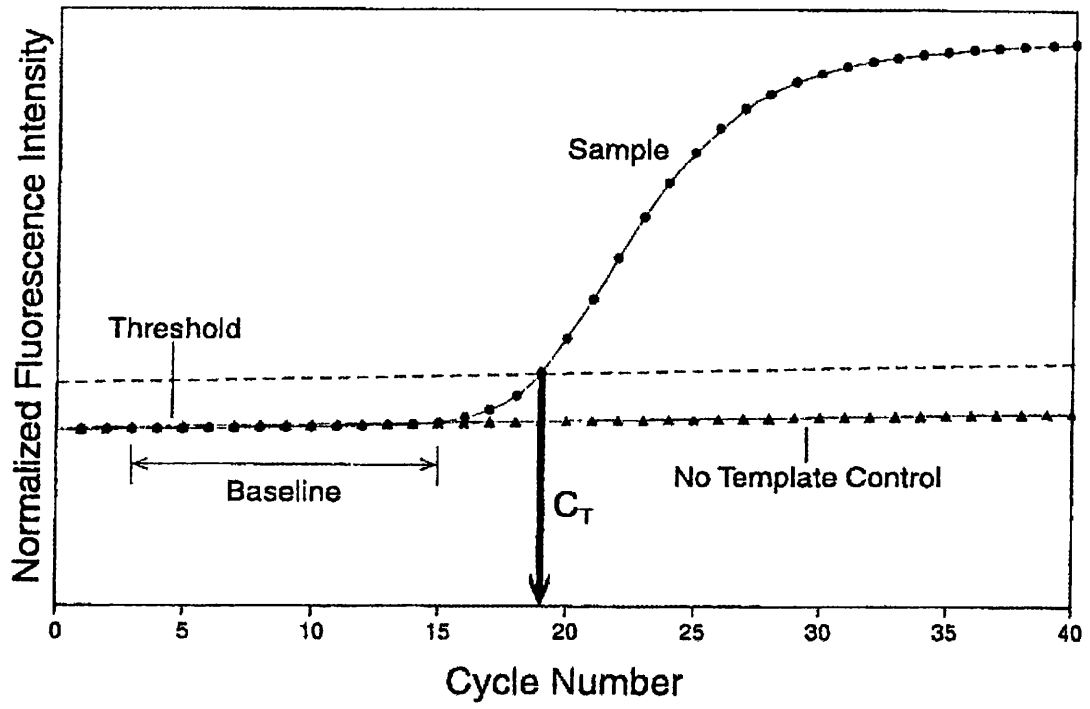


Table 2.1. Antibodies

Antibody (clone)	Application	Manufacturer
biotin-IgA	FACS, IHC	Jackson
biotin-IgM	FACS, IHC	Jackson
biotin-IgG	FACS, IHC	Jackson
FITC-IgA	IHC	Jackson
FITC-IgM	IHC	Jackson
FITC-IgG	IHC	Jackson
anti-CD89 (Mip-8a)	IB	Serotec
anti-V5	IB	Invitrogen
HRP-anti mouse IgG	IB	Amersham
anti-caveolin	IHC	BD Biosciences
anti-calnexin	IHC	Stressgen
Texas Red goat anti-rabbit IgG	IHC	Jackson
Texas Red goat anti-human IgA	IHC	Jackson
anti-FcγRIIa (IV3)	IB	Medarex

FACS: fluorescent activated cell scanning

IHC: immunohistochemistry

IB: immunoblotting

HRP: horseradish peroxidase (conjugated)

Manufacturers

Jackson Laboratories, West Grove, PA, USA

Invitrogen, Groningen, The Netherlands

Serotec, Oxford, UK

BD Biosciences, San Jose, CA, USA

Stressgen Biotechnologies, Victoria, BC, Canada

Medarex, Princeton, NJ, USA

Amersham Pharmacia Biotech, Little Chalfont, UK

Table 2.2. Oligonucleotide sequences

Target	Name	Sequence (5' → 3')	Product
β actin	β actin-for	GGG GTA TGC CCT CCC CCA TGC CAT CCT GCG	482bp
	β actin-rev	TTG GCG TAC AGG TCT TTG CGG ATG TCC ACG	
CD89	89-F2	CGC TTA AGA TGG ACC CCA AAC AGA CCA C	880bp
	89-R2	GCT CTA GAT TAC TTG CAG ACA CTT GGT G	
CD89	89-ec1-for2	CAA ACA AGG CAG GGC GCT ATC	
CD89	EC1-for	TCC CTT GGA TGG ATC TGT GAA	106bp
	EC1-rev	TCT TCT GCC TAT CTC TCG GTA CGT	
CD89	EC2-for	CTC AGC ACA CAT CCC ATT TGA TAG	96bp
	EC2-rev	GAG AAG TTG GCC GGG TGT T	
CD89	TMC-for	CAC AGC CAT ACG GCA CTG AA	101bp
	TMC-rev	GGT GTT CGT GCA AAG GTC AAT	
Fcα/μR	amr-for1	GAC AAC TAC CAA GGC TGA TAG G	702bp
	amr-rev	TCT GTC CCT CAG GGT CCT GGA T	
Fcα/μR	amr-rtf	TGC TAC CTC TGC GGC ATT G	144bp
	amr-rtr	AGA CGC TGT TCC ATA GGA TCT CA	
Fcα/μR	amr-for80	AGG AGG GCA GGA TGG AAA ATG	1614bp
	amr-rev 1693	GGG TCC TGG ATT TCT CTC TG	

Fcα/μR	amr-for54	CTT TAC CAC AAT CTC ACG TCA C	1463bp
	amr-rev1516	CTT CCT TTG CAA TAG AAC CAG AG	
Fcα/μR	amr-rev1559	AGG TGA CCC TTT CTG CCT CCT	
Fcα/μR	amr-rev1559g	GAG GTG ACC CTT TCT GCC TCC T	
Fcα/μR	amr-rev1693g	GGG GTC CTG GAT TTC TCT CTG	
PIGR	PIGR-for	GCC CGA GCT GGT TTA TGA AG	694bp
	PIGR-rev	AGC CGT GAC ATT CCC TGG TA	
FcRn	FcRn-for	CAA AGC TTT GGG GGG AAA AG	359bp
	FcRn-rev	TGC AGG TAA GCA CGG AAA AG	
FcγRI	I-for	CAT GTG GTT CTT GAC AAC TCT GCT CC	876bp
	I-rev	TGA AAC CAG ACA GGA GTT GGT AAC TGG	
FcγRIIb	II-for	GCA CAG GAA ACA TAG GCT ACA CG	287bp
	IIb-rev	GGT GAT TGT GTT CTC AGC CCC	
FcγRIII	III-for	ATG TGG CAG CTG CTC CTC CCA ACT G	567bp
	III-rev	GGT GAT GTT CAC AGT CTC TGA AGA CAC	
ASGPR	H2-for	GCA GGC TGA ATC CCA GGA GA	1072bp
	H2-rev	CCA TTG AAG AGG CTG ACG AT	

Chapter 3

Human mesangial cells do not express FcαRI

3.1. Introduction

FcαRI (CD89) operates as a medium affinity cell surface receptor for IgA1 and IgA2. Expression is predominantly by circulating monocytes, tissue macrophages, neutrophils and eosinophils, although dendritic cells also bearing the molecule have been described (Monteiro *et al*, 1990; Geissmann *et al*, 2001). In common with other Fc receptors, FcαRI assists in the coordination of the immune response by acting as a functional link between antibodies generated by the humoral immune system and the effector cells of the cellular immune system. Engagement of the receptor by ligand may initiate several cellular responses including internalisation and intracellular killing of antibody-coated organisms, presentation of antigens and production of inflammatory mediators including cytokines. The FcαRI glycoprotein is expressed at the cell surface in association with the FcR γ chain homodimer (figure 1.3.) (Pfefferkorn & Yeaman, 1994). The FcR γ chain contains an activating signal motif (ITAM) which modulates signal transduction via FcαRI by tyrosine phosphorylation.

The signalling cascade initiated following FcαRI cross-linking involves recruitment of the Src family non-receptor protein tyrosine kinase, $p56^{lyn}$ to the complex of FcαRI and FcR γ chain (Gulle *et al*, 1998). Phosphorylation of several targets including the FcR γ ITAMs and the cellular protein $p72^{syk}$ then ensues which leads to $p72^{syk}$ association with the receptor complex. Subsequently, modulation of a multimeric adaptor protein complex containing tyrosine phosphorylated Cbl, Shc, SHIP, Grb2 and Crkl occurs with recruitment of Sos and activation of the Ras kinase pathway (Park *et al*, 1999). Activation of the Raf1-MEK-MAP and phosphoinositide 3-kinase pathways then takes place which leads to cellular effector functions including release

of inflammatory mediators, antigen presentation and phagocytosis with intracellular killing.

Following receptor cross-linking by ligand, FcαRI redistributes to cholesterol-rich glycolipid rafts in the plasma membrane (Lang *et al*, 1999). These rafts function as platforms to facilitate the co-localisation of receptor complexes with downstream components of the signalling pathway, and form polar caps in the cell membrane. The process of FcαRI recruitment to rafts was found to be independent of FcR γ chain in contrast to the translocation of $p56^{lyn}$ to rafts which was dependent on the presence of FcR γ chain. In further work examining signalling events after FcαRI cross-linking it was demonstrated that a wide variety of signalling effectors including Blk and Syk tyrosine kinases, protein kinase C and phosphoinositide 3-kinase were recruited to lipid rafts following FcαRI cross-linking (Lang *et al*, 2002).

The gene coding for FcαRI is located on chromosome 19 in contrast to all other FcR genes which are situated on chromosome 1 (Kremer *et al*, 1992). The FcαRI gene comprises five exons spanning approximately 12kB of genome and codes for a type 1 transmembrane protein of 266 amino acids (de Wit *et al*, 1995). The first two exons (S1 and S2) encode the hydrophobic signal peptide which is cleaved to produce the mature protein during translocation from the endoplasmic reticulum to the cell surface. Each of the EC1 and EC2 exons encodes an extracellular immunoglobulin-like domain with the former containing the IgA binding site. A single exon (TM/C) encodes both the 19 amino acid membrane-spanning region and the 41 amino acid cytoplasmic tail.

Several distinct transcripts, based on alternative splicing of FcαRI mRNA have been described (Morton *et al*, 1996; Patry *et al*, 1996; Pleass *et al*, 1996). These include a version with complete deletion of the 288 base pairs comprising the EC2 exon (ΔEC2) and another lacking the membrane-proximal 66 nucleotides of EC2 (Δ66EC2). Although there is some evidence that at least the protein product of the Δ66EC2 transcript is expressed in nature by cells (Patry *et al*, 1996), the precise purpose or

relevance of these variant transcripts is not known. A further variant of FcαRI designated FcαRb has been described in eosinophils and neutrophils (van Dijk *et al*, 1996). The FcαRb transcript encodes a truncated form of receptor lacking the TM/C exon but containing extra sequence coding for an additional 23 amino acids which extend the EC2 exon until a new stop codon is reached. The majority of this protein is released in a soluble form but a proportion was also found to be membrane associated and cells transfected with this cDNA were able to bind IgA. The nature of FcαRb membrane association was not clear but did not appear to be mediated by a glycosyl phosphatidyl inositiol anchor. Although FcαRb appears to bind IgA, no tyrosine phosphorylation or calcium mobilisation was induced following receptor cross-linking despite co-expression of the FcR γ chain homodimer. The absence of a conventional membrane-spanning region in FcαRb is likely to preclude a functional association with the FcRγ chain and this may explain the inability of FcαRb to mediate signal transduction.

Reports of FcαRI expression on circulating immune cells in IgA nephropathy patients have generated conflicting results. Using flow cytometry, a Japanese group described enhanced expression of FcαRI on both neutrophils and monocytes from patients with IgA nephropathy (Kashem *et al*, 1994; Kashem *et al*, 1996). However, later work from a European group showed reduced FcαRI expression in both neutrophils and monocytes from IgA nephropathy subjects (Grossetete *et al*, 1998). Down-regulation of FcαRI by these cells was mediated by IgA1, with polymeric IgA1 exerting a more powerful negative effect on receptor expression than the monomeric form. Also of interest was the finding that FcαRI on patient monocytes and neutrophils was of a higher molecular weight (60-85 kDa) in comparison to FcαRI on cells from controls (55-75 kDa), consistent with altered post-translational modification in IgA nephropathy. Additionally, endogenous IgA1 bound to the higher molecular weight version of FcαRI with greater affinity and this was postulated to be the mechanism of receptor down-regulation.

Whether or not human mesangial cells (HMC) express FcαRI is of considerable significance as this receptor could potentially mediate the mesangial accumulation of IgA1 and activation of HMCs towards an inflammatory phenotype in IgA nephropathy. Several research groups published work between 1993 and 1999 that supported the notion of FcαRI expression by HMCs (Gomez-Guerrero *et al*, 1993; Bagheri *et al*, 1997; Kashem *et al*, 1997; Suzuki *et al*, 1999b) (see section 1.5.2.). However, these findings were contradicted by a report describing cellular activation of HMCs following IgA1 binding in the absence of detectable FcαRI at either the mRNA or protein level (Diven *et al*, 1998).

A further report confirmed dose-dependent binding of IgA by cultured HMCs but again failed to detect expression of either FcαRI protein or wildtype transcript (Barratt *et al*, 2000). The possibility of expression by HMCs of a novel IgA receptor protein was raised by the observation that IgA binding to these cells was not inhibited by the My43 antibody which blocks ligand binding to FcαRI. FcαRI expression was not induced following incubation of HMCs with activating mediators nor was any soluble receptor protein detected in cell culture medium. Neither was expression of the FcαRb isoform detected. Employing a strategy designed to identify the expression of different FcαRI transcripts by using RT-PCR with nine different combinations of primer pairings, these investigators demonstrated expression of three novel FcαRI-related transcripts by HMCs. These transcripts were detected using a sense oligonucleotide primer homologous to sequence in the EC1 exon of FcαRI and an antisense primer to the TM/C exon. The three transcripts detected corresponded to fragments of wildtype FcαRI, and the ΔEC2 and Δ66EC2 splice variants. However, the association between these transcripts and the FcαRI gene was unclear and the natures of the upstream and downstream coding sequences were not determined. The authors concluded that IgA binding to HMCs was mediated by a novel receptor related to, but distinct from, FcαRI.

3.2. Results

3.2.1. FcαRI mRNA is not expressed by human mesangial cells

To determine whether HMCs express wildtype FcαRI, polymerase chain reaction (PCR) was performed using cDNA generated from four separate primary human mesangial cell cultures. U937 cells constitutively express FcαRI and this cDNA was used as a positive control. COS-7 cells do not express any Fc receptors and cDNA from these cells was used as a negative control. The primer combination used was "89-F2" and "89-R2" (table 2.2.)

The expression of FcαRI by U937 cells was confirmed by the presence of a band corresponding to a predicted size of 880 base pairs representing wildtype FcαRI (figure 3.1.). The lower band present in this lane represents the ΔEC2 splice variant at 592 base pairs. The identities of both these products were confirmed by sequencing. No PCR products were seen with any of the four HMC cDNAs or the negative control COS-7 cDNA. Equal expression of the ubiquitous housekeeping gene, β actin was confirmed for all samples indicating comparable quantities and qualities of cDNA in each case (figure 3.1. lower panel). Therefore, HMCs do not express mRNA for either wildtype FcαRI or the ΔEC2 or Δ66EC2 splice variants.

3.2.2. FcαRI protein is not expressed by human mesangial cells

In order to examine FcαRI protein expression, whole cell lysates were prepared using U937, COS-7 and human mesangial cells. The cellular proteins were separated using electrophoresis and immunoblotted using the Mip-8a monoclonal antibody which is specific for FcαRI.

FcαRI protein expression by U937 cells was visualised as a smeared band of approximately 55-70 kDa (figure 3.2.). The smeared appearance of the band is

consistent with variable glycosylation of the receptor. No FcαRI protein expression was detected with either COS-7 cells or HMCs.

3.2.3. FcαRI EC2-related novel transcripts are not expressed by human mesangial cells

3.2.3.1. 3' RACE

If HMC binding of IgA and activation by IgA is independent of wildtype FcαRI then these cells may express a novel variant form of the classical receptor. The partial FcαRI–related transcripts based on the EC2 exon of the receptor and described by Barratt could represent such novel receptors distinct from wildtype FcαRI and therefore would not be detected by either standard PCR approaches or by antisera to wildtype FcαRI. We used 3' rapid amplification of cDNA ends (RACE) in conjunction with a sense primer complementary to sequence in the distal portion of the EC1 exon of FcαRI in an attempt to characterise the downstream sequences of any novel transcripts expressed in HMCs. RNA from four HMC lines, COS-7 cells and U937 cells was reverse transcribed using the adaptor primer supplied in the 3' RACE system. PCR was subsequently performed using an FcαRI gene-specific primer (89-ec1-for2; table 2.2.) and the antisense universal adaptor primer.

A band of approximately 1,600 base pairs was present in each of the four HMC samples and the U937 cell sample (figure 3.3.). In addition these samples all generated a second product of approximately 400 base pairs. In contrast COS-7 cells demonstrated a different pattern with the presence of a band of approximately 200 base pairs and no 1,600 base pair product. The presence of these bands in HMCs suggested possible expression of FcαRI-related transcripts by these cells. As expected these bands were absent in reverse transcription control samples and were dependent on the presence of the gene-specific primer (data not shown). The 1,600 base pair

products from an HMC line and from the U937 cells and the 400 base pair product from the HMC line were excised and gel purified prior to subcloning and sequencing.

Sequencing of the 1,600 base pair product from U937 cells yielded partial FcαRI sequence comprising the EC1 and EC2 exons as expected. The 1,600 base pair band from the HMC line comprised sequence from a proto-oncogene designated UFO (NCBI accession number X66029) which encodes a tyrosine kinase receptor (Schulz *et al*, 1993). The 400 base pair band from HMCs represented sequence from the 3-phosphoglycerate dehydrogenase (PHGDH) gene (Cho *et al*, 2000) (NCBI accession number NM 006623) which is known to be expressed at high levels in human kidney. No FcαRI-related sequence was detected in the products generated from HMCs by 3' RACE. The identification of sequence for the UFO and PHDGH genes in HMCs was likely to be artefactual owing to low stringency priming in the PCR step due to the presence of a single gene-specific primer, combined with extremely low or absent expression of FcαRI-related transcripts in these cells.

3.2.3.2. Real time PCR

We next employed an alternative strategy to identify any novel FcαRI-related transcripts in HMCs. Real time PCR was used to determine relative quantification of different FcαRI exons expressed within the same cell line and between different cells. In order to maximise efficiency, primers were designed to amplify short (approximately 100 base pairs) sequences of different FcαRI exons and these were quantified and compared using real time PCR. It was hypothesised that if a variant transcript containing the EC2 exon of FcαRI but with novel upstream and downstream sequence was expressed by HMCs that the relative expression of the amplicon for the EC2 exon would be higher than for the EC1 and TM/C exons. A diagrammatic representation of the target sequences generated by the primers is illustrated in figure 3.4. Amplicons A, B and C correspond to sequence in the EC1, EC2 and TM/C exons of FcαRI respectively. Primer sequences (EC1-for, EC1-rev, EC2-for, EC2-rev, TMC-for and TMC-rev) are shown in table 2.2.

First strand cDNA reverse transcribed from four primary HMC lines was used for real time PCR together with cDNA from U937 cells and COS-7 cells as positive and negative controls respectively. Matching reverse transcription controls where reverse transcriptase enzyme had been omitted were included for each sample. Each reaction was performed in triplicate and the mean threshold cycle (C_t) determined. If a reaction efficiency of virtually 100% is assumed then the relative amount of transcript in the original sample is halved for each single unit increase in the threshold cycle. To determine whether a particular mean C_t was of biological significance in this highly sensitive system it was compared with the mean C_t of the matching reverse transcription control by subtraction and the difference expressed as ΔC_t. As the amplicons generated for the FcαRI exons were intra-exonic and did not cross exon boundaries, calculation of the ΔC_t controlled for the presence of trace amounts of genomic DNA in the starting RNA samples. This approach also controlled for other potential confounding factors including any reverse transcriptase activity of the DNA polymerase, contamination of the system by product and formation and amplification of primer dimers.

The results of real time PCR for the FcαRI exons are expressed as mean C_t and ΔC_t in table 3.1. For U937 cells the ΔC_t for each amplicon was approximately 7 in comparison to COS-7 cells where the difference between cDNA and reverse transcription controls was around zero for each amplicon. This is consistent with expression of FcαRI by U937 cells but not COS-7 cells as expected. Mean threshold cycles for U937 cells were around 20 whereas those for the four HMC lines ranged from 31.1 to 36.6. Therefore, if HMCs do express transcript for FcαRI then levels must be extremely low at between 2000 and 65,000-fold less than the constitutive expression level in U937 cells.

When the mean C_t for expression of each FcαRI amplicon in the four HMC lines was compared with the corresponding reverse transcription control to generate the ΔC_t value the results were close to zero, ranging from –0.5 to 2.1. Combined with the

finding of high mean threshold cycles for these samples it was concluded that these HMCs did not express FcαRI transcript at biologically significant levels. In addition, these data provided no support for expression of a novel EC2-related FcαRI transcript by these HMCs as the ΔC_t values for amplicon B (EC2 exon) were close to zero for each HMC line.

HMCs and U937 cells were next stimulated for 24 hours with either 1mM dibutyryl cyclic AMP (dbcAMP) or with a combination of IL-1α and TNF-α each at a concentration of 50ng/ml in an attempt to induce expression of FcαRI. IL-1 and TNF-α have both been implicated in the pathogenesis of IgA nephropathy (Yoshioka *et al*, 1993) and dbcAMP has been shown to enhance expression of FcαRI in U937 cells (Cameron *et al*, 2001). Table 3.2. shows the results of real time PCR for the TM/C exon of FcαRI (amplicon C) for these stimulated cells. Expression of FcαRI by U937 cells was increased by approximately 2-fold following incubation with dbcAMP with ΔC_t increasing from 8.2 to 9.4 while the combination of IL-1α and TNF-α reduced the ΔC_t value to 7.6. Once again high mean C_t values were observed with HMCs and no significant effect following stimulation was seen. These results demonstrate that these biological modulators did not induce expression of FcαRI by HMCs.

3.3. Discussion

Using a variety of techniques and approaches our results demonstrate that HMCs express neither message nor protein for the classical IgA Fc receptor, FcαRI. Additionally we could find no evidence for the expression of a novel transcript or variant form of the receptor by these cells.

These results are in disagreement with the series of initial reports from different research groups that documented FcαRI expression by HMCs (Gomez-Guerrero *et al*, 1993; Bagheri *et al*, 1997; Kashem *et al*, 1997; Suzuki *et al*, 1999b). Collectively these authors also described enhanced expression of FcαRI in response to inflammatory cytokines, correlation between glomerular FcαRI expression and histological renal damage and a physical association between FcαRI and the FcR γ chain. Our results are in accordance with later published reports that described IgA binding by HMCs and subsequent cellular activation in the absence of FcαRI expression (Diven *et al*, 1998; Westerhuis *et al*, 1999; Barratt *et al*, 2000; Leung *et al*, 2000). There are several possible explanations for these discrepancies based primarily on likely differences in HMC harvesting techniques, culture and propagation. It could be hypothesised that prolonged culture or varying culture conditions lead to loss of the receptor from the cell surface, although one of these later studies failed to detect FcαRI expression in renal biopsy sections (Westerhuis *et al*, 1999). Another possibility is the presence in HMC cultures of contaminating cells such as tissue macrophages that express FcαRI. Even a very low number of such cells could lead to a spurious positive result in a sensitive RT-PCR system.

Using RT-PCR and immunoblotting we were unable to detect FcαRI expression by HMCs. These findings were extended by the use of real time PCR which permitted a relative quantification of expression. In comparison with the clear constitutive levels of FcαRI expression by U937 cells, the threshold cycles obtained with HMC cDNA were extremely high and did not differ in biological significance from the reverse transcription reverse controls. By applying the same methodology to quantify each of

three FcαRI exons it was possible to exclude significant expression of a novel transcript containing these elements of FcαRI sequence.

Similarly, we attempted to directly identify any novel sequence downstream from the distal part of the EC1 exon that was expressed by HMCs by using 3' RACE. Although this approach was speculative it would be expected to yield useful sequence information in the presence of the novel variants described by Barratt *et al.* Using control U937 cells, elements of FcαRI sequence were detected by 3'RACE but material from HMCs yielded only artefactual gene sequences almost certainly due to the absence of specific target expression by these cells which permitted subsequent low stringency priming and amplification.

FcαRI expression by HMCs was an attractive initial contender to mediate the mesangial deposition of IgA in IgA nephropathy. Despite support for its candidacy from multiple early data, our results together with findings subsequently published in the literature effectively discount this possibility. It remains possible that FcαRI does play a role elsewhere in the pathogenesis of IgA nephropathy either on blood-borne cells such as neutrophils and monocytes or on tissue macrophages involved perhaps in a clearance mechanism or as a soluble circulating IgA receptor. However, it appears almost certain that alternative mechanisms or receptors are responsible for IgA-induced activation of HMCs and the mesangial accumulation of IgA that defines IgA nephropathy.

Figure 3.1. RT-PCR for FcαRI showing expression in U937 cells but not in human mesangial cells.

FcαRI mRNA expression was examined in four separate human mesangial cell (HMC) lines using RT-PCR. U937 cell cDNA was used as a positive control and cDNA from COS-7 cells as a negative control. The lower panel shows expression of the housekeeping gene β actin for each sample. The figures to the left represent molecular size in base pairs.

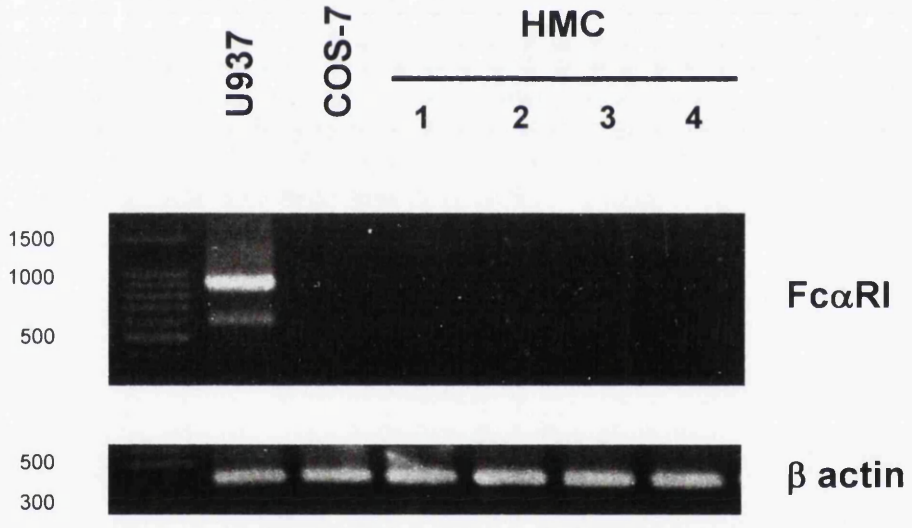


Figure 3.2. Immunoblot for FcαRI showing expression in U937 cells but not in a human mesangial cell line.

FcαRI protein expression was examined in U937, COS-7 and cultured human mesangial cells (HMC). Cell lysates were separated by electrophoresis and immunoblotted using the Mip-8a antibody. The figures to the left represent molecular weight in kDa.

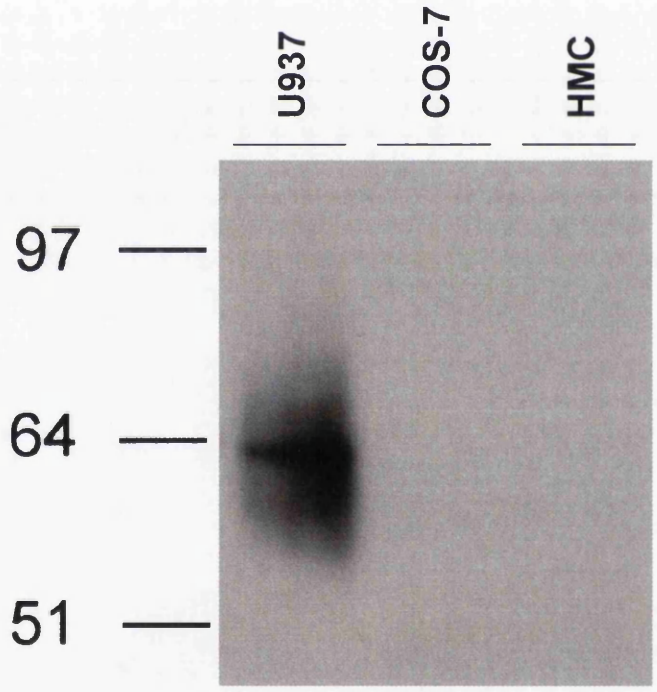


Figure 3.3. 3' RACE using FcαRI gene-specific priming showing a product of approximately 1,600 base pairs in U937 cells and in human mesangial cells.

RNA from U397 cells, COS-7 cells and four human mesangial cell lines was subjected to 3' RACE using a primer specific for sequence in the distal part of the EC1 exon of FcαRI. The figures to the sides represent molecular size in base pairs.

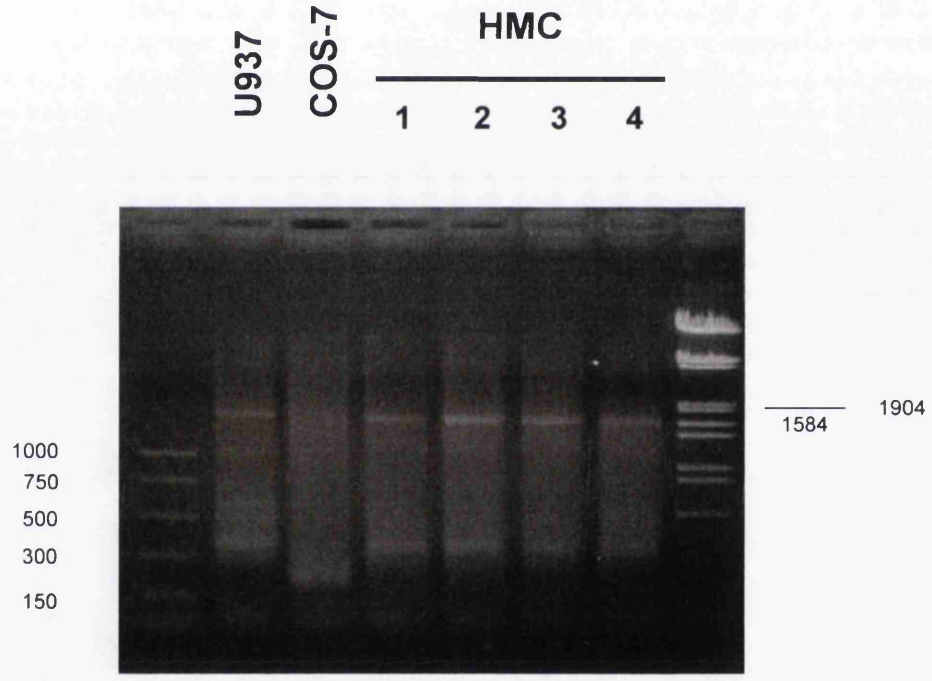


Figure 3.4. Diagrammatic overview of FcαRI sequences targeted in real time PCR

Amplicons A, B and C represent target sequences in the EC1, EC2 and TM/C exons of FcαRI respectively. The size molecular size (base pairs) of each exon is shown underneath.

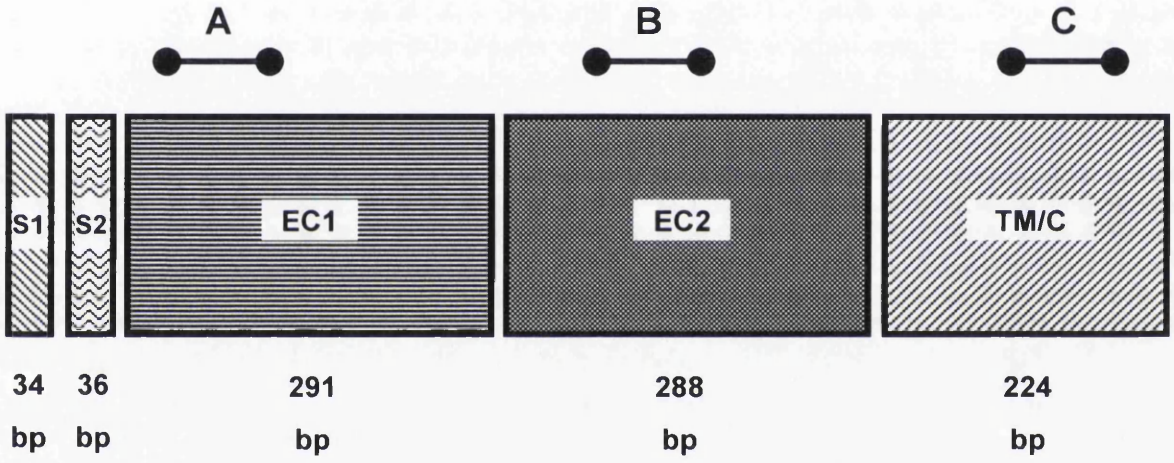


Table 3.1. Real time PCR for EC1, EC2 and EC3 exons of FcαRI

Real time PCR for each exon was performed using cDNA from U937 cells, COS-7 cells and four separate HMC cell lines in parallel with the appropriate reverse transcription controls (RT-CON). Reactions were performed in triplicate and mean threshold cycles (C_t) are shown for each amplicon along with the difference in mean C_t between samples and controls (ΔC_t).

	Amplicon	cDNA	RT-CON	ΔC_t
U937	A	19.8	26.7	6.9
	B	19.3	26.0	6.7
	C	20.3	27.4	7.1
COS-7	A	37.9	37.6	-0.3
	B	30.9	31.2	0.3
	C	40	40	0
HMC-1	A	34.4	34.8	0.4
	B	31.7	31.3	-0.4
	C	34.7	34.2	-0.5
HMC-2	A	36.6	38.6	2
	B	32.2	32.8	0.6
	C	36.4	38.5	2.1
HMC-3	A	33.6	33.6	0
	B	31.1	32.2	1.1
	C	33.7	33.3	-0.4
HMC-4	A	33.0	34.1	1.1
	B	31.6	31.6	0
	C	33.5	34.1	0.6

Table 3.2. Real time PCR for TM/C exon of FcαRI following stimulation of U937 cells and HMCs

Real time PCR for the TM/C exon of FcαRI (amplicon C) was performed using cDNA from U937 cells and from an HMC line following 24 hours stimulation with either 1mM dibutyryl cyclic AMP (dbcAMP) or a combination of IL-1α and TNF-α each at 50ng/ml. Reactions were performed in parallel with the appropriate reverse transcription controls (RT-CON) and in triplicate. Mean threshold cycles (C_t) are shown along with the difference in mean C_t between samples and controls (ΔC_t).

		cDNA	RT-CON	ΔC_t
U937	Unstimulated	21.3	29.5	8.2
	dbcAMP	19.1	28.5	9.4
	IL-1α + TNF-α	21.0	28.6	7.6
HMC	Unstimulated	37.3	38.8	1.5
	dbcAMP	37.3	37.4	0.1
	IL-1α + TNF-α	34.8	34.7	-0.1

Chapter 4

Human mesangial cells express Fcα/μR

4.1. Introduction

Genes encoding Fc receptors for IgG, IgE and IgA had been identified and their products extensively characterised for several years before a specific Fc receptor for IgM was discovered in 2000. IgM is the antibody isotype produced in the primary humoral immune response following immunisation or after the immune system has encountered a new microbial antigen and the existence of an Fc receptor for IgM had been predicted. Numerous so called "natural antibodies" many of which have specificity for carbohydrate epitopes of microorganisms are also of the IgM isotype but the mechanism of downstream effector functions following antibody-antigen interaction was unclear. The polymeric immunoglobulin receptor (PIGR) expressed on epithelial cells and performing the specialised function of polymeric IgA and IgM transport across epithelial surfaces was the only receptor for IgM identified prior to this time. However, although the PIGR is closely related in structure and in chromosomal location to the FcγRs and the FcεR, it is not a true Fc receptor as its function depends on the presence of the joining (J) chain in the structure of its ligands rather than the Fc portion of immunoglobulin. The newly described Fc receptor for IgM was also able to bind IgA and was designated as the Fc alpha/mu receptor (Fcα/μR) (Shibuya *et al*, 2000).

The Fcα/μR was discovered as the consequence of a deliberate attempt to identify an FcR for IgM. A cDNA library derived from a mouse T cell leukaemia line was transfected into COS-7 cells and screened using a mouse monoclonal IgM as a probe. An open reading frame from the cDNA clone isolated predicted a type 1 transmembrane protein with a 32 amino acid signal peptide, a 423 amino acid extracellular region and a 60 amino acid intracellular segment. The presence of a

single extracellular immunoglobulin domain was predicted, as were four potential sites for *N*-linked glycosylation. The extracellular region contained an amino acid sequence motif common to the polymeric immunoglobulin receptor and this was postulated to be involved in ligand binding. The cytoplasmic tail of the Fcα/μR contained a di-leucine motif but was devoid of any tyrosine-based signalling elements. Using RT-PCR, Fcα/μR transcript was detected in several mouse tissues including thymus, spleen, liver, kidney, small and large intestines, testis and placenta. Using a monoclonal antibody raised against mouse Fcα/μR, surface expression of the receptor was demonstrated on the majority of B lymphocytes and macrophages but not on granulocytes, NK cells or T lymphocytes. It was unclear whether the pattern of tissue expression described was a consequence of receptor expression by intrinsic cells or alternatively, was due to expression by infiltrating or resident haemopoietic cells bearing the receptor.

Expression of the mouse Fcα/μR cDNA in transfected cells yielded a protein of approximately 70kDa present on the cell surface. Cells stably transfected with the receptor were able to specifically bind IgM in addition to monomeric and dimeric forms of IgA but were unable to bind any subclasses of IgG. Fcα/μR binding affinity was determined using ^{125}I-labelled mouse IgM and IgA with the receptor demonstrating high affinity for IgM ($K_a = 2.85 \times 10^9\ M^{-1}$) and intermediate affinity for IgA ($K_a = 3.01 \times 10^8\ M^{-1}$). A functional property for the receptor was confirmed with endocytosis of IgM-coated beads by stably transfected cells. The capacity of the receptor to mediate endocytosis was abolished by mutation of the di-leucine motif present in the cytoplasmic tail of Fcα/μR. Finally it was also demonstrated that Fcα/μR could mediate internalisation of IgM-coated bacteria by primary B lymphocytes in culture and that this process could be inhibited by pre-incubation with a blocking antibody against the receptor. The authors hypothesised that processing of opsonised microbes at an early stage of the immune response may be an important physiological function of Fcα/μR and that it may also play a role in the subsequent presentation of antigen to T lymphocytes.

Shibuya *et al* also identified a human homologue of Fcα/μR which shared 49% amino acid identity to the mouse receptor. The gene for human Fcα/μR mapped to a region of chromosome 1 (1q32.3) close to the genes for the PIGR, the FcγRs and the FcεR. The predicted structure of the human Fcα/μR was similar to the mouse receptor in that it contained a single extracellular immunoglobulin-like domain with potential *N*-linked glycosylation sites and sequence motif shared with the PIGR together with a transmembrane region and cytoplasmic tail. However, in contrast to the mouse receptor the human Fcα/μR did not possess the intracytoplasmic di-leucine motif necessary for endocytosis by the mouse Fcα/μR. No other recognised signalling motifs were apparent in the cytoplasmic tail of the human Fcα/μR. No functional data were described for the human Fcα/μR and no information was presented on its tissue distribution.

We hypothesised that if the human form of Fcα/μR also possessed the ability to bind IgA and was also expressed in human kidney, perhaps by mesangial cells, then this receptor could potentially play a role in the molecular pathogenesis of IgA nephropathy.

4.2. Results

4.2.1. Fcα/μR mRNA is expressed in human kidney and by human mesangial cells

To determine whether Fcα/μR message could be detected in human renal tissue, polymerase chain reaction (PCR) was performed using cDNAs generated from normal renal cortex and from microdissected glomeruli from normal human kidney. PCR was also carried out using cDNAs from four separate primary HMC cultures, U937 cells and COS-7 cells. The primer combination used was "amr-for1" and "amr-rev" (table 2.2.) producing a predicted amplicon of 702 base pairs for wildtype Fcα/μR.

The expression of Fcα/μR mRNA in human renal cortex and in microdissected glomeruli was demonstrated by the presence of an appropriately sized band following PCR (figure 4.1.A). The upper band observed in this gel is the predicted size for the genomic fragment of DNA (2,328 base pairs). Figure 4.1.B confirms expression of Fcα/μR mRNA by all four HMC lines examined and by U937 cells. No Fcα/μR expression was detected in COS-7 cells. Full length sequencing of the PCR product from one HMC line was performed and this confirmed the presence of Fcα/μR sequence.

Real time PCR was also used to assess expression of Fcα/μR in these different cell types by comparing the difference in mean threshold cycles (ΔC_t) between cDNA samples and reverse transcription controls as described in section 3.2.3.2. The primer combination used was "amr-rtf" and "amr-rtr" (table 2.2.) and reactions were performed in triplicate. These results (table 4.1.) confirm expression of Fcα/μR transcript by U937 cells and by all four HMC lines with ΔC_t in the range from 3.1 to 5.5 compared with a ΔC_t for COS-7 cells of –0.7 consistent with no expression of Fcα/μR.

4.2.2. Fcα/μR expression by human mesangial cells is specifically increased by IL-1α

To determine whether Fcα/μR message could be regulated by inflammatory cytokines, cultured HMCs were stimulated for 24 hours with either IL-1α or TNF-α at a concentration of 50ng/ml. Semi-quantitative RT-PCR was performed by reducing the number of PCR cycles to 35. Treatment with IL-1α, but not with TNF-α, resulted in upregulation of Fcα/μR expression when compared with unstimulated cells (figure 4.2.). There was no synergistic effect of both cytokines in combination and upregulation of receptor mRNA by IL-1α was confirmed in a second HMC line. Therefore, Fcα/μR expression by HMCs is specifically upregulated by IL-1α.

Real time PCR was next used to determine the magnitude of Fcα/μR upregulation by IL-1α. HMCs and U937 cells were stimulated for 24 hours with either 1mM dibutyryl cyclic AMP (dbcAMP) or with 50ng/ml IL-1α. The results in table 4.2. demonstrate no significant biological effect of either IL-1α or dbcAMP on Fcα/μR expression by U937 cells. However, treatment of HMCs with IL-1α was associated with an increase in ΔC_t value from 0.8 to 8.3 consistent with an approximately 180-fold increase in Fcα/μR expression by these cells. Treatment of HMCs with dbcAMP caused a small increase in ΔC_t value from 0.8 to 2.2.

4.2.3. Cloning and genomic organization of human Fcα/μR

To further characterise the human homologue of Fcα/μR the full length open reading frame was cloned from an HMC line. Primers were designed based on the database sequence (accession number E15470; http://www.ncbi.nlm.nih.gov) predicted to containing the human receptor (Shibuya *et al*, 2000) and are shown in table 2.2. as "amr-for80" and "amr-rev1693". The reverse primer was designed to exclude the predicted native stop codon from the amplified sequence. RT-PCR using *pfu* polymerase was used to amplify a 1614 base pair fragment of DNA. This product was purified from an agarose gel and 3' adenine overhangs were added by incubation with

Taq polymerase. The fragment was then subcloned into the pcDNA3.1/V5/His/TOPO expression plasmid in frame with a V5 epitope tag. Identity and orientation of the insert were confirmed by full length sequencing and restriction digest. The nucleotide sequence of the insert is shown in figure 4.3. and this was submitted to GenBank under accession number AY063125. The ATG translation initiation codon is at position 19 of this sequence. The nucleotide sequence of the cloned receptor, combined with data from the NCBI E15470 sequence, was used to deduce the exon-intron structure of the gene by comparison with data from the NCBI human genome database (http://www.ncbi.nlm.nih.gov/genome/guide/human; reference number NT_021877.3 GI:13635806; accessed 21/05/2001). The genomic organisation of the human Fcα/μR gene is illustrated in figure 4.4. The location of the human Fcα/μR gene was confirmed to be at chromosome 1q32. The gene comprises 7 exons and 6 introns spanning approximately 10,000 bases of genome. All of the intron-exon boundaries obey the splice site consensus rule (5' GT/AG 3') (Breathnach & Chambon, 1981) as shown in table 4.3.

4.2.4. Predicted amino acid sequence of human Fcα/μR

Translation of the open reading frame cloned from the human mesangial cell line predicted a protein composed of 532 amino acids and this sequence is shown in figure 4.5. Analysis of the sequence suggested a hydrophobic signal peptide comprising the first 16 amino acids and this would produce a mature protein of 516 amino acids after cleavage of the signal peptide. The nucleotide sequence for the signal peptide is contained within exon "SP" and into the start of exon "EC1". A transmembrane region composed of 17 amino acids (positions 454-470) was also predicted with the resulting mature membrane-bound protein possessing a 62 amino acid cytoplasmic tail and an extracellular portion of 437 amino acids. Exons "TM" and "CY" contained the nucleotide sequences for the transmembrane and cytoplasmic regions respectively. The extracellular portion of the protein was coded by sequence in exons "EC1", "EC2" and "EC3" with a single immunoglobulin-like domain present at amino acid positions 61-169 encoded by sequence in exon "EC2". Two cysteine residues at

positions 82 and 153 were predicted to form the disulphide bridge of this domain. Two potential sites for *N*-linked glycosylation were identified at amino acid positions 167 and 276 in the extracellular portion of the protein. A threonine residue at position 479 and a serine residue at position 480 were detected as possible sites for phosphorylation in the cytoplasmic tail. No potential sites for tyrosine phosphorylation were identified in this intracellular portion of the protein. The predicted molecular weight of the mature protein following cleavage of the signal peptide was 55,409 Da.

4.2.5. Expression of the human Fcα/μR protein

The cloned Fcα/μR cDNA was used to transiently transfect COS-7 cells and lysates were prepared for immunoblotting. An antibody directed against the V5 epitope tag encoded by the expression plasmid was used to detect the protein. Expression of V5 epitope-tagged Fcα/μR fusion protein by transfected cells was demonstrated by immunoblotting (figure 4.6.A). The molecular weight of the tagged Fcα/μR protein was approximately 63kDa under reducing conditions and there was no significant mobility shift observed under non-reducing conditions indicating that the receptor was expressed as a monomer. As the V5 tag accounts for about 5kDa of weight, the molecular mass of the native protein would be approximately 58kDa.

As the presence of sites for *N*-linked glycosylation was predicted from the Fcα/μR amino acid sequence, a lysate of COS-7 cells transfected with Fcα/μR was incubated with *N*-glycanase to remove any *N*-linked carbohydrate groups. Figure 4.6.B shows that the V5-tagged Fcα/μR protein was only minimally *N*-glycosylated, with *N*-glycanase treatment resulting in a mobility shift of around 3 kDa revealing a protein core of approximately 60 kDa.

4.2.6. Human Fcα/μR specifically binds IgA and IgM

In order to define the antibody binding characteristics of the human Fcα/μR, transfected COS-7 or CHO-K1 cells were incubated with labeled IgA, IgM, or IgG antibodies and examined using fluorescence microscopy and flow cytometry. Cells transfected with irrelevant DNA in the same vector were used as controls. Fluorescence microscopy showed that cells transfected with Fcα/μR cDNA were able to bind IgA and IgM but did not bind IgG (figure 4.7.), whereas cells transfected with irrelevant DNA did not bind immunoglobulin of any isotype (data not shown). The binding of IgM by transfected cells appeared to be stronger than that of IgA.

Flow cytometry of CHO-K1 cells transfected with human Fcα/μR confirmed receptor binding of IgA and IgM (figure 4.8.) in the population of transiently transfected cells. Cells transfected with irrelevant DNA were used as negative controls for antibody binding.

4.2.7. Human mesangial cells express variant Fcα/μR transcripts

An additional product of approximately 1,300 base pairs in size can be seen in figure 4.2. following stimulation of HMCs with IL-1α. This band could also be seen very faintly following RT-PCR of unstimulated HMCs (data not shown). The band was excised from agarose, gel purified, subcloned and sequenced. Sequencing revealed a distinct Fcα/μR transcript with an in frame insertion of 624 base pairs of sequence which comprised the complete intron 5 of the genomic sequence. This intron separates exons EC3 and TM and is spliced out in the wild-type transcript. The sequence of this variant predicts that the first codon following divergence from the wild type sequence is TAA, a stop codon. As the exon coding for the transmembrane domain is downstream from this stop codon, translation of this larger transcript would theoretically result in the production of a soluble, circulating form of the receptor. This would be consistent with the existence of soluble forms of other Fc receptors which have been described, including FcαRI (van Zandbergen *et al*, 1999). The

nucleotide sequence of this variant transcript was submitted to GenBank under accession number AY063126.

Following stimulation of two HMC lines with 50ng/ml IL-1α for 24 hours, RT-PCR for Fcα/μR using an alternative set of primers demonstrated an additional, smaller product to that of the expected wildtype receptor as observed in figure 4.9.A. The forward primer used was complementary to sequence in exon "5'UTR" (amr-for54) and the antisense primer annealed to DNA in exon "TM" (amr-rev1516) (table 2.2.). The amplicon representing wildtype Fcα/μR was anticipated to be 1463 base pairs in size, and after subcloning and sequencing, the upper band in figure 4.9.A. was confirmed as the wildtype transcript. One of the lower bands in figure 4.9.A was excised, gel purified and subcloned into the pCR4-TOPO vector. Sequencing of this product revealed expression of an alternatively spliced transcript of the Fcα/μR gene with an 802bp deletion corresponding to the entire EC3 exon. This Fcα/μR ΔEC3 variant transcript is represented in figure 4.9.B. Detection of the wildtype and ΔEC3 transcripts with this primer set was optimal following stimulation of HMCs with IL-1α. The failure to detect expression of these transcripts in the absence of IL-1α stimulation is likely to be a consequence of reduced sensitivity of this particular primer combination.

4.2.8. Predicted amino acid sequence of the Fcα/μR ΔEC3 transcript

An open reading frame was identified within the Fcα/μR ΔEC3 transcript sequence where the initial 516 nucleotides from the ATG start codon were shared with the sequence of the wildtype Fcα/μR transcript. Due to deletion of the 802 base pairs comprising the EC3 exon, a shift in reading frame occurred starting from the DNA sequence of the TM exon and continuing distally. A stop codon was encountered 661 base pairs distal to the start codon.

The predicted sequence of the Fcα/μR ΔEC3 variant is identical to the first 172 amino acids of the wildtype receptor (figure 4.10.). Subsequently the out of frame deletion

results in divergence of the predicted amino acid sequence from the wildtype receptor. The stop codon identified within the transcript sequence was located in exon "CY" at an earlier position than the stop codon in the frame coding for the wildtype receptor, resulting in a protein sequence shorter than wildtype receptor. The predicted product of the Fcα/μR ΔEC3 variant is a protein of 220 amino acids which includes the same 16 amino acid signal peptide of the wildtype receptor. The same extracellular immunoglobulin-like domain of the wildtype receptor was also maintained prior to sequence divergence. A single potential site for *N*-linked glycosylation was anticipated at position 167. The mature protein has a predicted molecular weight of 23,112 Da following cleavage of the signal peptide. Sequence analysis also anticipated a distal membrane-spanning region (amino acids 184-202) encoded by the same exon (TM), but in an alternative reading frame from, the transmembrane region of the wildtype receptor. The resulting intracellular region is composed of 18 amino acids. Three potential sites for serine phosphorylation only (amino acids 206, 207 and 219) were identified in the cytoplasmic tail. No potential sites for a glycosylphosphatidylinositol (GPI) anchor were identified.

The full length open reading frame of the Fcα/μR ΔEC3 variant was subsequently cloned from a primary isolate of HMCs. Nucleotide sequences of the primers used are shown in table 2.2. as "amr-for80" and "amr-rev1559". The reverse primer was designed to exclude the predicted native stop codon from the target sequence and RT-PCR using *pfu* polymerase was used to amplify a 678 base pair fragment of DNA. This product was purified from an agarose gel and 3' adenine overhangs were added by incubation with *Taq* polymerase. The fragment was then subcloned into the pcDNA3.1/V5/His/TOPO expression plasmid in frame with a V5 epitope tag. Identity and orientation of the insert were confirmed by full length sequencing and restriction digest.

4.2.9. Expression of the human Fcα/μR ΔEC3 protein

The human Fcα/μR ΔEC3 protein was expressed by transient transfection of CHO-K1 cells. The protein was identified by immunoblotting of lysates using an antibody specific for the V5 epitope of the tagged protein. Cells transfected with either irrelevant DNA in the same vector or with wildtype Fcα/μR cDNA were used as negative and positive controls respectively (figure 4.11.). As seen previously, the wildtype Fcα/μR protein was present as a band of approximately 62 kDa. The Fcα/μR ΔEC3 protein was detected as a pair of bands at approximately 29 and 26 kDa. The V5 tag contributes around 5kDa to the molecular weights of these proteins.

The Fcα/μR ΔEC3 protein was predicted to possess a single *N*-linked glycosylation site (figure 4.10.). In order to test this prediction a lysate of CHO-K1 cells transfected with Fcα/μR ΔEC3 cDNA was treated with *N*-glycanase to remove any *N*-linked carbohydrate groups. The protein was detected using the anti-V5 antibody and compared with lysates of equal total weight from Fcα/μR ΔEC3 cDNA transfected cells which had not been enzymatically treated and with cells transfected with irrelevant DNA. Figure 4.12. shows that the double band observed with cells transfected with Fcα/μR ΔEC3 cDNA was resolved to a single band corresponding to the molecular weight of the lower band of the doublet at around 26kDa. Thus in cells transfected with Fcα/μR ΔEC3 cDNA, the expressed protein exists in distinct non-glycosylated and *N*-glycosylated forms. The *N*-glycosylated fraction appeared to be more abundant.

In order to test the prediction of a transmembrane region within the Fcα/μR ΔEC3 protein, membrane preparations of transfected CHO-K1 lysates were prepared. The membrane fractions were separated from cellular lysates using ultracentrifugation and these preparations were immunoblotted with the anti-V5 antibody. Figure 4.13. shows that wildtype Fcα/μR and the Fcα/μR ΔEC3 protein were present within the membrane fractions of lysates prepared from transfected cells. However, only a single band of approximately 29kDa (rather than a doublet) was detected in the

membrane fraction of cells transfected with Fcα/μR ΔEC3 cDNA, suggesting that only the *N*-glycosylated form of the protein was present in association with a cellular membrane. The lower molecular weight unglycosylated form appeared in neither the membrane nor cytosolic fractions. The absence of this band could be explained by its localisation to the cell nucleus or to a nuclear membrane which would be removed with other cell debris early in the membrane preparation process. The 29kDa membrane-associated band representing Fcα/μR ΔEC3 protein did not appear in the cytosolic fraction.

4.2.10. Fcα/μR ΔEC3 protein does not bind IgA or IgM

To determine whether the Fcα/μR ΔEC3 protein was capable of functioning as a cell surface immunoglobulin receptor, CHO-K1 cells transfected with Fcα/μR ΔEC3 cDNA were incubated with either IgA or IgM and examined for binding using flow cytometry and fluorescence microscopy. No significant antibody binding to transfected cells was observed as shown by flow cytometry in figure 4.14. Similarly, when cells transfected with Fcα/μR ΔEC3 cDNA were incubated with IgA or IgM and examined using fluorescence microscopy, no antibody binding to cells was detected (data not shown).

4.2.11. Fcα/μR ΔEC3 protein is not expressed at the cell surface in transfected cells

The apparent inability of Fcα/μR ΔEC3 protein to bind IgA and IgM could be explained by the protein fulfilling a function distinct from the wildtype receptor. Alternatively, it was hypothesised that Fcα/μR ΔEC3 protein produced by transfected cells may not be expressed at the cell surface. Therefore, in order to assess the cellular localisation and distribution of the protein, a green fluorescent protein (GFP) construct was engineered which would enable the tagged protein to be detected within transfected cells using fluorescence microscopy. Primers for PCR were designed to

generate appropriate DNA inserts from the pcDNA3.1/V5/His/TOPO expression vectors containing the coding sequences for wildtype Fcα/μR and the Fcα/μR ΔEC3 variant to subclone into the pcDNA3.1/CT-GFP-TOPO vector. The inserts were cloned into this latter vector in frame with a C-terminal GFP label. The forward primer sequence used for each construct was "amr-for80" (table 2.2.). The reverse primers were "amr-rev1693g" and "amr-1559g" for the wildtype and ΔEC3 variant constructs respectively. The orientation of each insert was determined by restriction digest analysis.

CHO-K1 cells were transiently transfected with the constructs coding for GFP-labelled Fcα/μR and Fcα/μR ΔEC3 proteins and examined using fluorescence microscopy. Figure 4.15.A shows that the GFP-labelled wildtype Fcα/μR was distributed in a diffuse pattern throughout the cytoplasm of transfected cells. In contrast, the GFP-labelled Fcα/μR ΔEC3 protein was observed in a different distribution with expressed protein localised in discrete polar caps at either end of the nucleus. In order to correlate these findings with ligand binding, cells transfected with each GFP-labelled construct were incubated with 50μg/ml of polymeric myeloma IgA1 and examined using three fluorescence microscopy image overlays (figure 4.15.B). This confirmed the widespread cytoplasmic and membrane distribution of wildtype Fcα/μR and IgA binding associated exclusively with cells expressing labelled Fcα/μR. No IgA binding was observed with cells expressing Fcα/μR ΔEC3 protein in the characteristic dense perinuclear caps. It was concluded that the cellular distribution of Fcα/μR ΔEC3 protein in transfected cells was distinct from that of wildtype receptor and that the variant protein was not expressed at the surface of transfected cells.

4.2.12. Fcα/μR ΔEC3 protein expression is restricted to the endoplasmic reticulum in transfected cells

In order to define the intracellular localisation of the Fcα/μR ΔEC3 protein in more detail, transfected CHO-K1 cells expressing either GFP-labelled wildtype Fcα/μR or

GFP-labelled Fcα/μR ΔEC3 were stained with antibodies for calnexin and caveolin. These primary antibodies were detected using a Texas Red-labelled secondary antibody and visualised using fluorescence microscopy. These images are shown in figures 4.16. and 4.17. for calnexin and caveolin respectively. Calnexin is a resident transmembrane endoplasmic reticulum (ER) protein which plays a role in the folding of newly synthesised proteins which are exported along the secretory pathway. Figure 4.16. shows a degree of co-localisation between wildtype Fcα/μR and calnexin. However, this was not exclusive and Fcα/μR expression was apparent in areas other than the ER. In contrast, expression of GFP-labelled Fcα/μR ΔEC3 protein by transfected cells was restricted to areas where calnexin was also detected.

Caveolin is a transmembrane protein present in membrane invaginations (caveolae) on the inner surface of the plasma membrane. It is also expressed in the trans-Golgi network and in intracellular transport vesicles. Figure 4.17. shows a difference between caveolin expression and the intracellular distribution of the Fcα/μR and Fcα/μR ΔEC3 proteins. Wildtype Fcα/μR co-localised with areas of caveolin expression whereas the distribution of Fcα/μR ΔEC3 protein was to distinct regions within the cell.

That wildtype Fcα/μR protein was present with the ER in addition to co-localising to areas where caveolin is expressed is to be expected for a protein that is transported along the secretory pathway and destined for expression on the plasma membrane. In contrast, Fcα/μR ΔEC3 protein expression appeared to be restricted to the ER and was not detected in areas where caveolin was detected. It was concluded that the Fcα/μR ΔEC3 protein is specifically retained within the ER and is not transported any further along the secretory pathway towards the plasma membrane in this cell type under the conditions described.

4.3. Discussion

These results document the expression of mRNA for the Fcα/μR, together with two alternatively spliced forms, by human mesangial cells in culture. This is the first report to describe the cloning and subsequent expression of the human form of the receptor. The finding of a novel human IgA-binding receptor expressed by HMCs is of importance as such a receptor could potentially be involved in mediating immune complex deposition in IgA nephropathy.

Arguably, by the time Shibuya and colleagues had described the murine Fcα/μR, the definitive identification of an Fc receptor for IgM had become overdue. Several reports dating from the late 1980s onwards had described specific binding of IgM by a number of haemopoietic cell types, while Fc receptors for the other main immunoglobulin isotypes had been identified, cloned and characterised in this period. A 60kDa receptor capable of binding IgM was identified on activated human B lymphocytes but expression was not detected on T lymphocytes nor on cells from the monocyte-macrophage lineage (Sanders *et al*, 1987). This molecule was further characterised as an Fc receptor for IgM with *O*-linked, but no *N*-linked carbohydrate groups and appeared to be coupled to the cell membrane of B cells via a GPI-anchor (Ohno *et al*, 1990). Furthermore, this FcμR did not have affinity for IgA so was probably distinct from the yet to be described Fcα/μR. Expression of an FcμR by natural killer (NK) cells had also been described (Pricop *et al*, 1993). This receptor was detected on the majority of resting NK cells and was shown to mediate signal transduction. It is unclear whether any characteristic other than IgM binding was shared with the previously described 60kDa FcμR. Similarly, a receptor that bound specifically to IgM and not to other immunoglobulin isotypes was described on T lymphocytes (Nakamura *et al*, 1993). The molecular size of this molecule was also around 60kDa but as it appeared not to be GPI-anchored the authors concluded that it was similar to but distinct from the FcμR present on activated B lymphocytes.

The initial description of Fcα/μR by Shibuya and colleagues had shown expression of the receptor by murine macrophages and B lymphocytes. In addition, transcript for the receptor was detected in several mouse tissues including kidney, leading us to speculate that human Fcα/μR could be expressed by renal mesangial cells. This hypothesis was confirmed by the detection of message for Fcα/μR in human renal cortex, in isolated human glomeruli and by all HMC lines examined. Our finding of Fcα/μR transcript expression by HMCs has subsequently been confirmed by at least two other research groups (Chan *et al*, 2005; Hu *et al*, 2005). Expression of the receptor by HMCs was specifically enhanced by the inflammatory cytokine, IL-1α. Using quantitative real time PCR we estimated that IL-1α increased HMC Fcα/μR expression by a factor in excess of 100. Increased intrarenal levels of IL-1 have been described in IgA nephropathy and appear to correlate with the degree of tubulointerstitial damage (Yoshioka *et al*, 1993). Therefore, the augmentation of HMC Fcα/μR expression by IL-1α is likely to be of biological significance.

In the original description of the mouse Fcα/μR, expression was demonstrated in haemopoietic and non-haemopoietic tissues but it was unclear whether detection of the receptor in the latter merely represented expression by infiltrating monocytes or B lymphocytes. A further study documented Fcα/μR expression in non-haemopoietic central nervous system tissue where an antibody against the mouse receptor was used to show expression by oligodendrocytes, oligodendrocyte precursor cells and myelin fractions (Nakahara *et al*, 2003). Immunohistochemistry demonstrated staining for Fcα/μR in mouse forebrains. It was hypothesised that Fcα/μR could perhaps mediate a response to intrathecal IgM present in demyelinating conditions such as multiple sclerosis. Thus it was established that Fcα/μR expression by non-haemopoietic cells such as HMCs was possible.

After we had cloned the receptor from an HMC line, the genomic organisation of human Fcα/μR was elucidated by comparison of the cloned cDNA with the human genome sequence. These findings were in general agreement with data subsequently published by the researchers who originally described the murine Fcα/μR, with the

coding sequence for the human receptor being contained within six exons (Shimizu *et al*, 2001). However, one of the exon-intron boundaries derived from our cloned cDNA sequence (intron 6; 3' splice acceptor) differs from that suggested by these other authors. The boundary site suggested by our data obeys the splice site consensus rule (5' GT/AG 3') whereas the exon boundary proposed by the other investigators does not.

Similarly, the features predicted by the amino acid sequence of our cloned cDNA for human Fcα/μR were in broad agreement with those proposed by Shibuya and colleagues. These included a single extracellular immunoglobulin-like domain, two potential sites for *N*-glycosylation and a membrane-spanning region. However, whereas Shibuya *et al.* suggested a 10 amino acid signal peptide, our bioinformatics data predicted a signal peptide comprising the first 16 amino acids of the sequence, encoded by exons "SP" and "EC1". The nucleotide sequence of a signal peptide composed of the first 10 amino acids would be contained entirely in exon "SP". Our findings subsequently demonstrated that the human Fcα/μR was membrane bound and expressed on the surface of transfected cells together with confirmation of the presence of *N*-linked carbohydrate modifications. Immunoblotting suggested a molecular weight for the deglycosylated mature protein of approximately 55kDa which corresponded with that predicted on the basis of amino acid sequence. The relatively light *N*-glycosylation of Fcα/μR is in contrast to FcαRI which bears considerably more *N*-linked carbohydrate groups (Toyabe *et al*, 1997). These results were also the first description of the ligand binding specificity of the human Fcα/μR wherein we observed IgM and IgA but not IgG binding in accordance with the characteristics described for the mouse receptor. Fcα/μR is therefore unique among the known Fc receptors in that it displays dual affinity for different immunoglobulin isotypes.

We also identified two variant transcripts of the human Fcα/μR gene which were both expressed by HMCs and upregulated following treatment with IL-1α in parallel with the effect of this cytokine on levels of wildtype receptor transcript. The significance

of the intron 5 insertion variant, whose sequence would predict a soluble form of the receptor, is not clear and we did not extend this observation. This transcript is more appropriately considered to be a variant form rather than a true alternatively spliced transcript and may simply be an artefact of mRNA processing. In contrast, the Fcα/μR ΔEC3 transcript is a true alternatively spliced product of the gene where the out of frame deletion results in a change of reading frame which produces a novel protein sequence.

Many of the features predicted for the wildtype Fcα/μR were maintained for the Fcα/μR ΔEC3 protein including the same signal peptide and immunoglobulin-like domain. As a consequence of the deleted sequence, only a single site for *N*-glycosylation was predicted for the Fcα/μR ΔEC3 protein. Sequence analysis of the Fcα/μR ΔEC3 protein also anticipated a distal membrane-spanning region encoded in the same exon (TM) as the wildtype receptor but in an alternative reading frame. Our experimental data derived after expression of the Fcα/μR ΔEC3 protein in transfected cells confirmed the presence of *N*-linked carbohydrate groups and that the protein was membrane associated. Thus the same stretch of nucleotide sequence of the Fcα/μR gene contains code for two distinct membrane-spanning regions within the two separate reading frames utilised by the wildtype receptor and the Fcα/μR ΔEC3 protein.

We were however, unable to detect immunoglobulin binding to cells transfected with the Fcα/μR ΔEC3 cDNA. Expression by transfected cells of Fcα/μR ΔEC3 protein bearing a GFP tag showed a distinct pattern of distribution when compared with GFP-linked wildtype Fcα/μR. This pattern was consistent with absence of expression of the protein on the cell surface. Co-localisation studies employing immunofluorescent staining for calnexin and caveolin suggested that in contrast to wildtype Fcα/μR, expression of Fcα/μR ΔEC3 protein by these cells was restricted to the endoplasmic reticulum (ER). The membrane association of Fcα/μR ΔEC3 protein demonstrated earlier must therefore be with ER membrane as opposed to plasma membrane.

An unresolved issue of significance is whether the Fcα/μR ΔEC3 protein has the potential to be expressed on a cell surface membrane either in a particular cell type or in specific circumstances. Until this question can be answered it will be difficult to determine whether the protein can also function as an immunoglobulin receptor akin to the wildtype Fcα/μR. Due to its shorter length and the proximity of the immunoglobulin domain to the anchoring membrane, it was speculated that steric interference might preclude binding of IgM to the Fcα/μR ΔEC3 protein due to the molecular mass and structure of the IgM pentamer. A property such as this may be the basis for functional diversification of the Fcα/μR.

Retention of a protein in the ER can be mediated by specific retention or retrieval signals in the amino acid sequence (reviewed in Teasdale & Jackson, 1996). For example, the CH1 domain of the IgM heavy chain bears a sequence that acts to hold the protein within the ER until association with an immunoglobulin light chain masks the motif and permits export from the ER (Cherayil *et al*, 1993). In this way the transport of aberrantly folded proteins or incompletely assembled multimeric complexes outwith the ER and along the exocytic pathway is prevented. Therefore, several potential explanations for restriction of the Fcα/μR ΔEC3 protein to the ER could be advanced. The protein may be a constituent part of a larger multimeric complex expressed on the plasma membrane and absence of expression of the other (unidentified) components in these transfected cells may thus prevent export from the ER. When expressed in a native cell type, Fcα/μR ΔEC3 protein transport to the cell surface could be under the control of signalling pathways leading to induction of expression of the requisite associated molecules. These events could be under the control of activating signalling pathways initiated by specific mediators such as cytokines or by a particular extracellular environment. The absence of either the associated molecules or of a specific extracellular signal may be responsible for ER retention or retrieval to the ER of the protein in these transfected cells. Examination of the amino acid sequence of the Fcα/μR ΔEC3 protein cytoplasmic tail reveals a di-arginine (RR) motif at position 214-215 (figure 4.10.). This motif has been reported

to direct the retrieval of type II membrane proteins to the ER and would be a candidate retention/retrieval motif for the Fcα/μR ΔEC3 protein (Schutze *et al*, 1994).

Figure 4.1. RT-PCR for Fcα/μR showing expression in renal tissue and in four human mesangial cell lines.

(A) Fcα/μR mRNA expression was assessed in specimens of renal cortex and microdissected glomeruli using RT-PCR. The predicted Fcα/μR amplicon was 702 base pairs in size. (B) Expression of FcαμR mRNA was examined using RT-PCR in four separate human mesangial cell (HMC) lines, U937 cells and COS-7 cells. The figures to the left represent molecular size in base pairs.

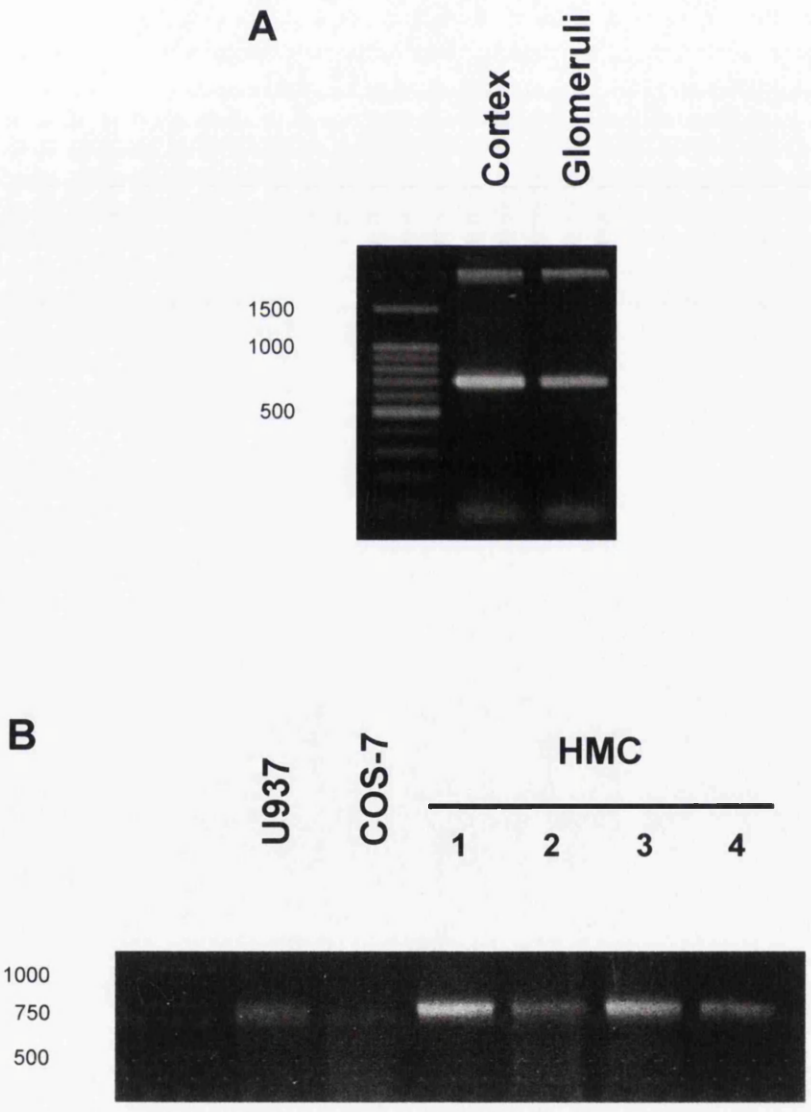


Figure 4.2. Semi-quantitative RT-PCR for Fcα/μR demonstrating increased receptor expression by human mesangial cells after treatment with IL-1α

Cultured human mesangial cells were stimulated for 24 hours with IL-1α and TNF-α either alone or in combination, each at a concentration of 50ng/ml and semi-quantitative RT-PCR for Fcα/μR was performed. The lower panel shows β actin expression for each sample. The figures to the left represent molecular size in base pairs.

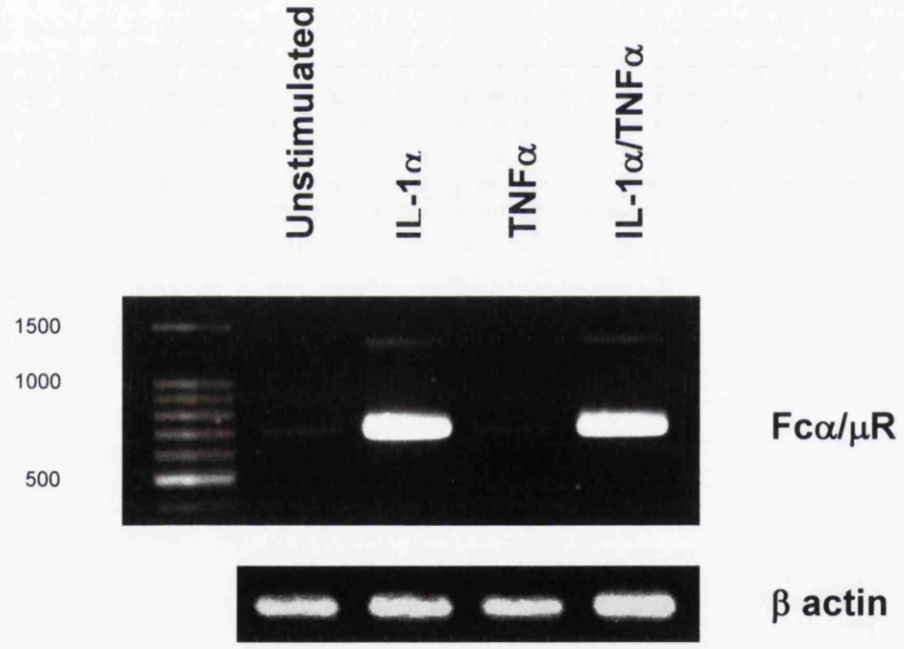


Figure 4.3. Nucleotide sequence of human Fcα/μR cloned from an HMC line

There was a single nucleotide difference at position 745 (A → C) when compared to the E15470 (immunity related factor) database sequence. This change represents a silent mutation for arginine.

```
1       AGGAGGGCAG GATGGAAAAT GCCCCTCTTC CTCATACTGT GCCTGCTACA
51      AGGTTCTTCT TTCGCCCTTC CACAAAAAAG ACCCCATCCG AGATGGCTGT
101     GGGAGGGCTC TCTCCCCTCC AGGACCCATC TCCGGGCCAT GGGAACACTC
151     AGGCCTTCCT CGCCCCTCTG CTGGCGGGAG GAGAGCTCCT TTGCAGCTCC
201     AAATTCATTG AAGGGCTCAA GGCTGGTGTC AGGGGAGCCT GGAGGAGCTG
251     TCACCATCCA GTGCCATTAT GCCCCCTCAT CTGTCAACAG GCACCAGAGG
301     AAGTACTGGT GCCGTCTGGG GCCCCCAAGA TGGATCTGCC AGACCATTGT
351     GTCCACCAAC CAGTATACTC ACCATCGCTA TCGTGACCGT GTGGCCCTCA
401     CAGACTTTCC ACAGAGAGGC TTGTTTGTGG TGAGGCTGTC CCAACTGTCC
451     CCGGATGACA TCGGATGCTA CCTCTGCGGC ATTGGAAGTG AAAACAACAT
501     GCTGTTCTTA AGCATGAATC TGACCATCTC TGCAGGTCCC GCCAGCACCC
551     TCCCCACAGC CACTCCAGCT GCTGGGGAGC TCACCATGAG ATCCTATGGA
601     ACAGCGTCTC CAGTGGCCAA CAGATGGACC CCAGGAACCA CCCAGACCTT
651     AGGACAGGGG ACAGCATGGG ACACAGTTGC TTCCACTCCA GGAACCAGCA
701     AGACTACAGC TTCAGCTGAG GGAAGACGAA CCCCAGGAGC AACCCGGCCA
751     GCAGCTCCAG GGACAGGCAG CTGGGCAGAG GGTTCTGTCA AAGCACCTGC
801     TCCGATTCCA GAGAGTCCAC CTTCAAAGAG CAGAAGCATG TCCAATACAA
851     CAGAAGGTGT TTGGGAGGGC ACCAGAAGCT CGGTGACAAA CAGGGCTAGA
901     GCCAGCAAGG ACAGGAGGGA GATGACAACT ACCAAGGCTG ATAGGCCAAG
951     GGAGGACATA GAGGGGGTCA GGATAGCTCT TGATGCAGCC AAAAAGGTCC
1001    TAGGAACCAT TGGGCCACCA GCTCTGGTCT CAGAAACTTT GGCCTGGGAA
1051    ATCCTCCCAC AAGCAACGCC AGTTTCTAAG CAACAATCTC AGGGTTCCAT
1101    TGGAGAAACA ACTCCAGCTG CAGGCATGTG GACCTTGGGA ACTCCAGCTG
1151    CAGATGTGTG GATCTTGGGA ACTCCAGCTG CAGATGTGTG GACCAGCATG
1201    GAGGCAGCAT CTGGGGAAGG AAGCGCTGCA GGGGACCTAG ATGCTGCCAC
1251    TGGAGACAGA GGTCCCCAAG CAACACTGAG CCAGACCCCG GCAGTAGGAC
1301    CCTGGGGACC CCCTGGCAAG GAGTCCTCCG TGAAGCGTAC TTTTCCAGAA
1351    GATGAAAGCA GCTCTCGGAC CCTGGCTCCT GTCTCTACCA TGCTGGCCCT
1401    GTTTATGCTT ATGGCTCTGG TTCTATTGCA AAGGAAGCTC TGGAGAAGGA
1451    GGACCTCTCA GGAGGCAGAA AGGGTCACCT TAATTCAGAT GACACATTTT
1501    CTGGAAGTGA ACCCCCAAGC AGACCAGCTG CCCCATGTGG AAAGAAAGAT
1551    GCTCCAGGAT GACTCTCTTC CTGCTGGGGC CAGCCTGACT GCCCCAGAGA
1601    GAAATCCAGG ACCC
```

Figure 4.4. Genomic organisation of human Fcα/μR

The full length receptor was cloned from a human mesangial cell line and the sequence compared with the NCBI human genome database. The seven exons (shaded boxes) and six introns (numbered) are shown to scale, and the size of each exon is indicated. Exon names were derived following analysis of the predicted amino acid sequence. 5'UTR represents the 5' untranslated region and exon SP contains sequence for the signal peptide. Exons EC1, EC2 and EC3 code for the extracellular portion of the receptor while exons TM and CY contain sequence for the transmembrane and cytoplasmic regions respectively.

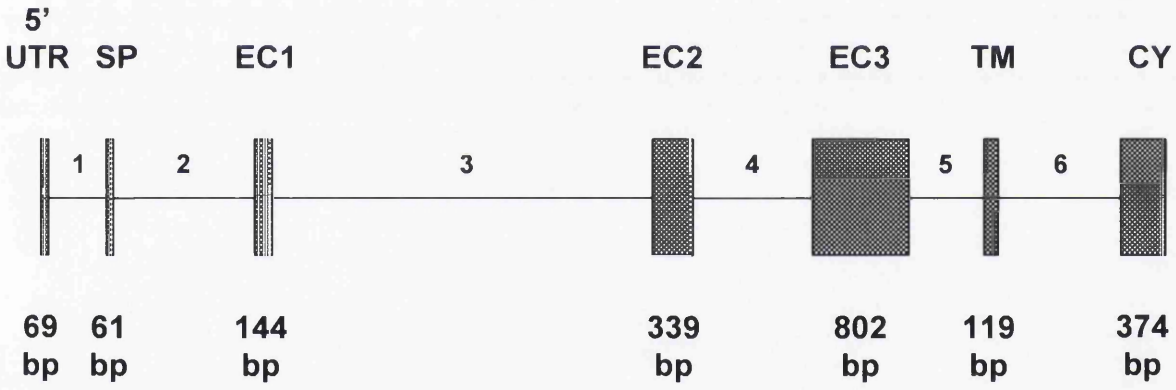


Figure 4.5. Predicted amino acid sequence of human Fcα/μR

The amino acid sequence of human Fcα/μR was derived by translation of the nucleotide sequence cloned from a human mesangial cell line. The predicted signal peptide and transmembrane regions are shown in bold. Cysteine residues contributing to the immunoglobulin domain (underlined) are emboldened (**C**) as are two potential sites for *N*-glycosylation (**N**).

```
1    MPLFLILCLL QGSSFALPQK RPHPRWLWEG SLPSRTHLRA
41   MGTLRPSSPL CWREESSFAA PNSLKGSRLV SGEPGGAVTI
81   QCHYAPSSVN RHQRKYWCRL GPPRWICQTI VSTNQYTHHR
121  YRDRVALTDF PQRGLFVVRL SQLSPDDIGC YLCGIGSENN
161  MLFLSMNLTI SAGPASTLPT ATPAAGELTM RSYGTASPVA
201  NRWTPGTTQT LGQGTAWDTV ASTPGTSKTT ASAEGRRTPG
241  ATRPAAPGTG SWAEGSVKAP APIPESPPSK SRSMSNTTEG
281  VWEGTRSSVT NRARASKDRR EMTTTKADRP REDIEGVRIA
321  LDAAKKVLGT IGPPALVSET LAWEILPQAT PVSKQQSQGS
361  IGETTPAAGM WTLGTPAADV WILGTPAADV WTSMEAASGE
401  GSAAGDLDAA TGDRGPQATL SQTPAVGPWG PPGKESSVKR
441  TFPEDESSSR TLAPVSTMLA LFMLMALVLL QRKLWRRRTS
481  QEAERVTLIQ MTHFLEVNPQ ADQLPHVERK MLQDDSLPAG
521  ASLTAPERNP GP
```

Figure 4.6. Fcα/μR expression in transfected COS-7 cells

Immunoblots of transfected COS-7 cell lysates were performed using an antibody specific for the V5 epitope tag. (A) Cells were transfected with either V5-tagged Fcα/μR cDNA (+) or with irrelevant DNA (-) in the same expression vector. Lysates were separated under reducing and non-reducing conditions. (B) A lysate from COS-7 cells transfected with Fcα/μR cDNA (+) was treated for 24 hours with *N*-glycanase (*). The figures to the left represent molecular weight in kDa.

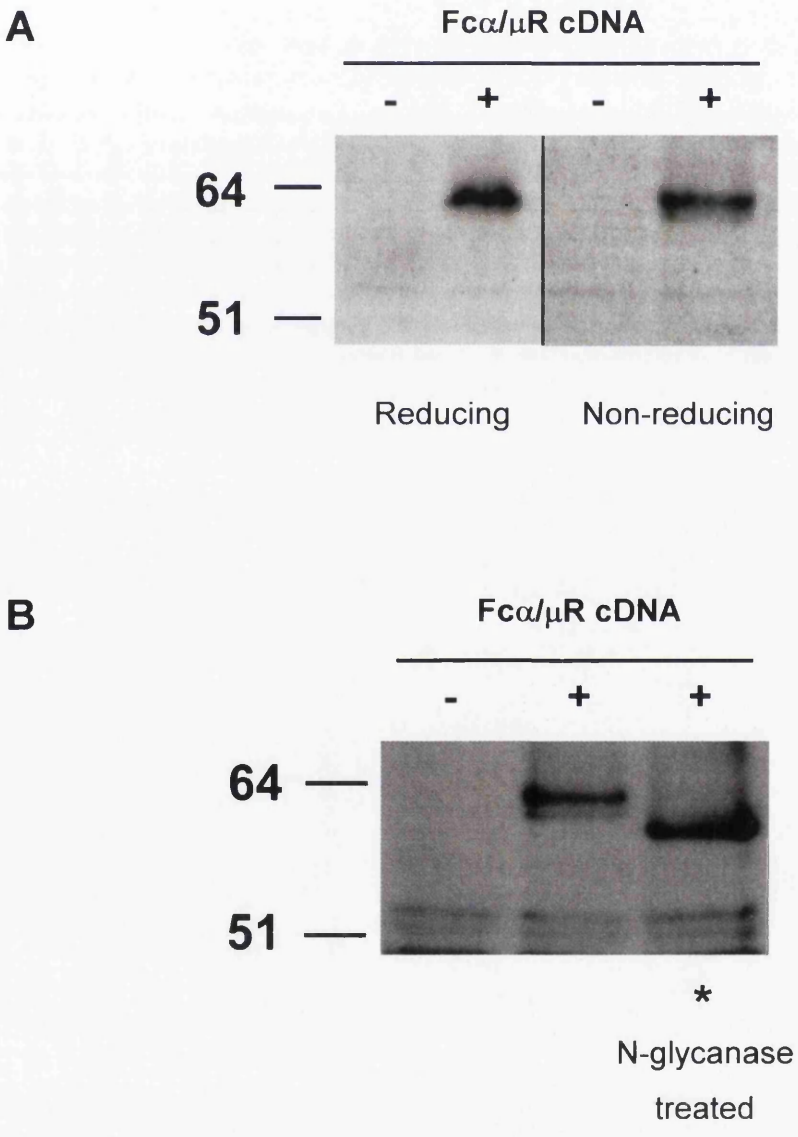
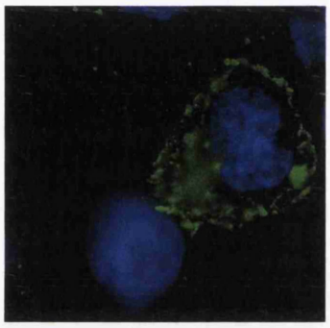
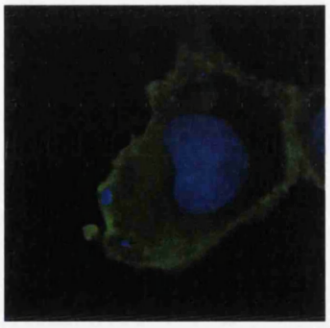


Figure 4.7. Fluorescence micrographs showing antibody binding to COS-7 cells transfected with Fcα/μR cDNA

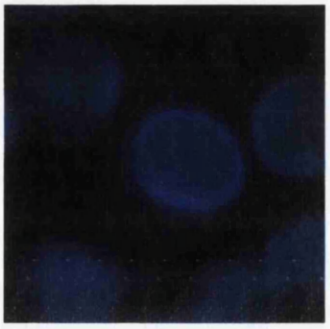
COS-7 cells transfected with human Fcα/μR cDNA were incubated for 45 minutes at 4°C with fluorescein-conjugated IgA, IgM or IgG each at a concentration of 10μg/ml and examined using fluorescence microscopy. Green staining represents antibody binding and cell nuclei are stained blue.



IgA



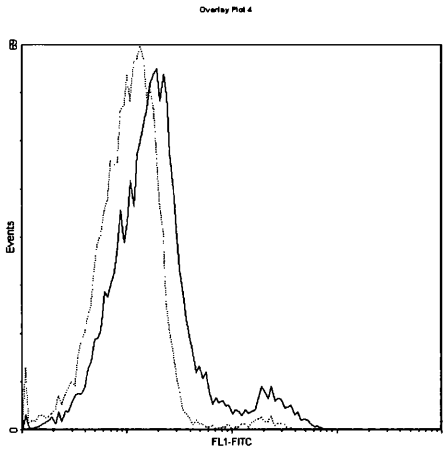
IgM



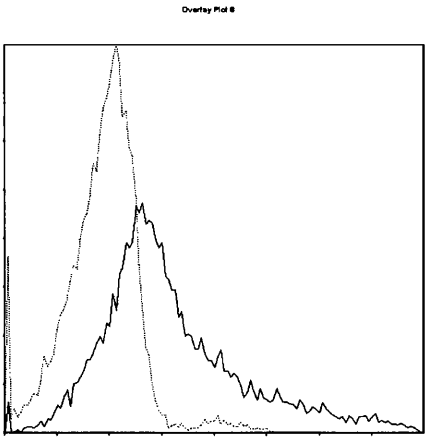
IgG

Figure 4.8. Flow cytometry showing antibody binding to CHO-K1 cells transfected with Fcα/μR cDNA

CHO-K1 cells transfected with either human Fcα/μR cDNA (bold line) or with irrelevant DNA as a control (light line) were incubated with 375μg/ml IgA or 250μg/ml IgM as indicated and antibody binding assessed using flow cytometry. Fluorescence intensity is shown on the *x*-axis and the number of events is on the *y*-axis. Assessment of antibody binding in this population of transiently transfected cells was demonstrated more clearly using fluorescence microscopy.



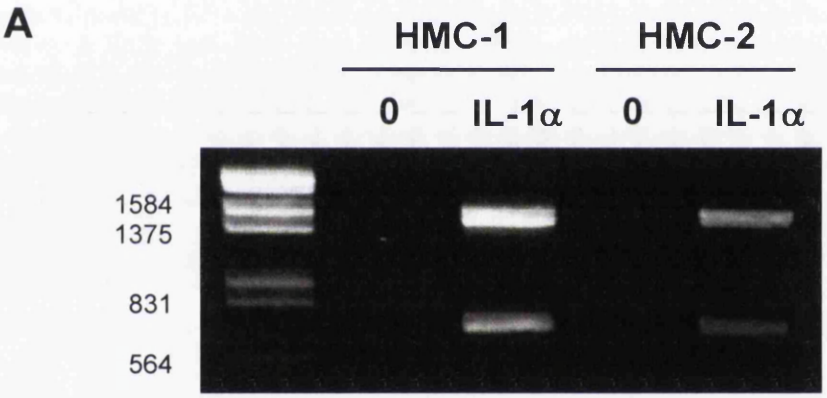
IgA



IgM

Figure 4.9. PCR for Fcα/μR showing expression of an alternatively spliced transcript by human mesangial cells

(A) RT-PCR for Fcα/μR using a primer set annealing at the "5'UTR" and "TM" exons was performed for two HMC lines. Some cells were stimulated with 50ng/ml IL-1α for 24 hours. The predicted size for wildtype Fcα/μR is 1463 base pairs. The figures to the left represent molecular size in base pairs. (B) Diagrammatic representation of the Fcα/μR ΔEC3 splice variant showing exon usage.



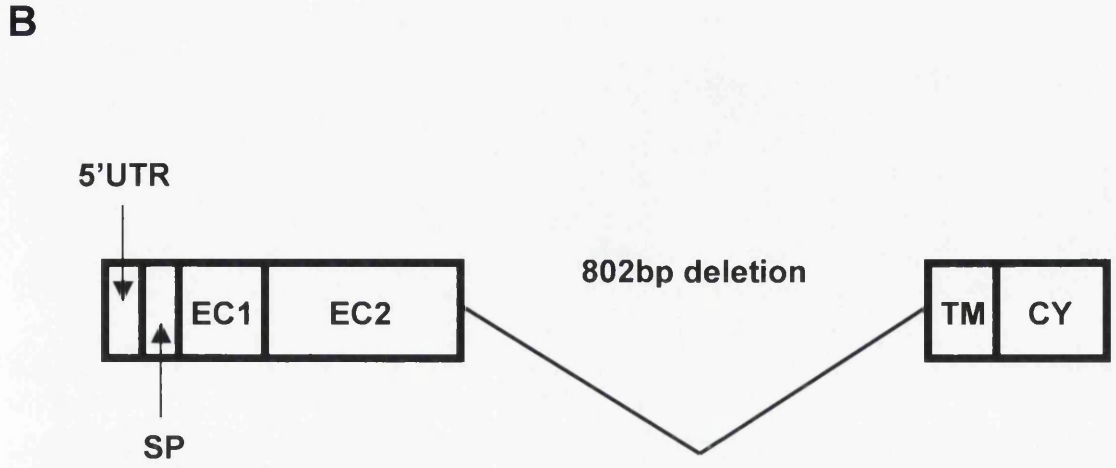


Figure 4.10. Predicted amino acid sequence of Fcα/μR ΔEC3 variant

The amino acid sequence encoded by the Fcα/μR ΔEC3 variant was elucidated by translation of the nucleotide sequence detected in a human mesangial cell line. The predicted signal peptide and transmembrane regions are shown in bold. Cysteine residues contributing to the immunoglobulin domain (underlined) are emboldened (**C**) as is a single potential site for *N*-glycosylation (**N**). The amino acid sequence diverges from that of the wildtype receptor at position 173 and this region is italicised.

1 **MPFFLILCLL QGSSFA**LPQK RPHPRWLWEG SLPSRTHLRA

41 MGTLRPSSPL CWREESSFAA PNSLKGSRLV SGEPGGAVTI

81 Q**C**HYAPSSVN RHQRKYWCRL GPPRWICQTI VSTNQYTHHR

121 YRDRVALTDF PQRGLFVVRL SQLSPDDIGC YL**C**GIGSENN

161 MLFLSM**N**LTI SA*VLFQKMKA ALG**PWLLSLP CWPCLCLWLW***

201 ***FY**CKGSSGEG GPLRRQKGSP*

Figure 4.11. Fcα/μR ΔEC3 protein expression in transfected CHO-K1 cells

Immunoblots of transfected CHO-K1 cell lysates were performed using an antibody specific for the V5 epitope tag. Cells were transiently transfected with either irrelevant control DNA, wildtype Fcα/μR cDNA or Fcα/μR ΔEC3 cDNA in the pcDNA3.1/V5/His/TOPO vector.

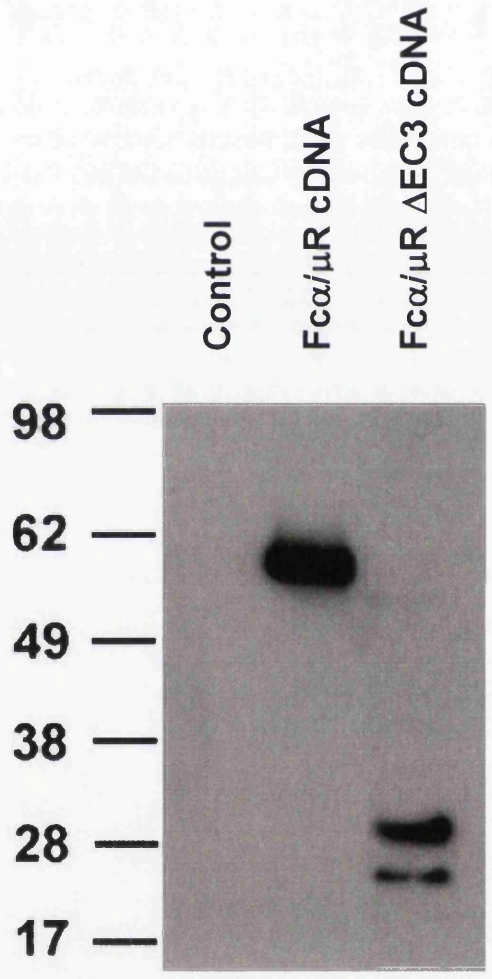


Figure 4.12. A fraction of Fcα/μR ΔEC3 protein is *N*-glycosylated in transfected cells

Immunoblotting of transfected CHO-K1 cell lysates was performed using an antibody specific for the V5 epitope tag. Cells were transfected with either V5-tagged Fcα/μR ΔEC3 cDNA or with irrelevant control DNA (CON) in the same expression vector. A lysate from cells transfected with Fcα/μR ΔEC3 cDNA was treated for 24 hours with *N*-glycanase (*). The figures to the left represent molecular weight in kDa.

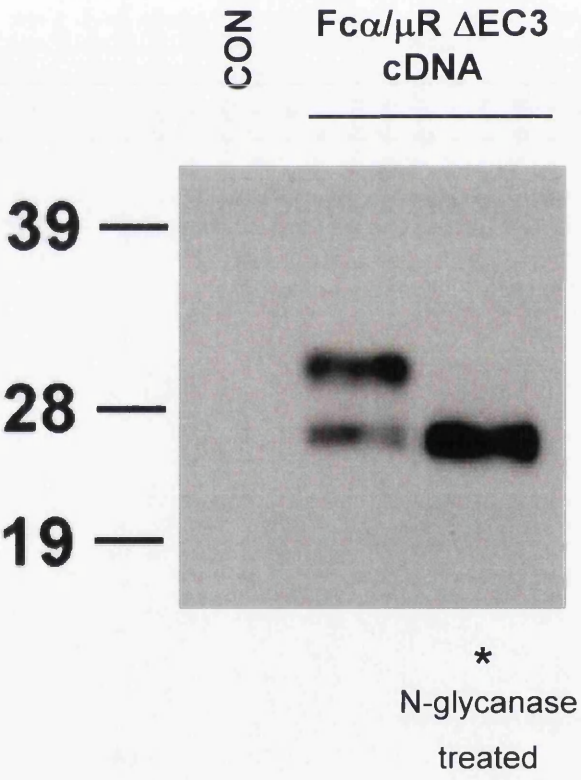


Figure 4.13. Fcα/μR ΔEC3 protein is expressed in a membrane-associated form

CHO-K1 cells were transfected with either wildtype Fcα/μR cDNA (lanes 1 and 3) or with Fcα/μR ΔEC3 cDNA (lanes 2 and 4). Membrane fractions were separated using ultracentrifugation and these preparations were immunoblotted with the anti-V5 antibody together with cytosolic fractions from the same lysates. The figures to the left represent molecular weight in kDa.

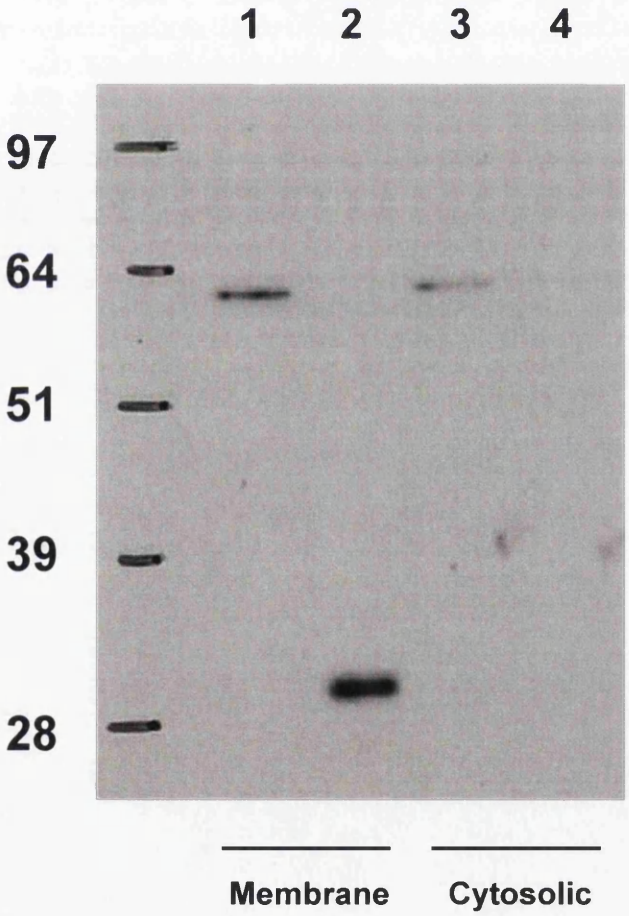
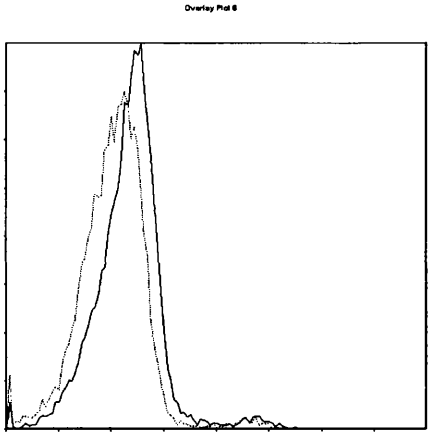
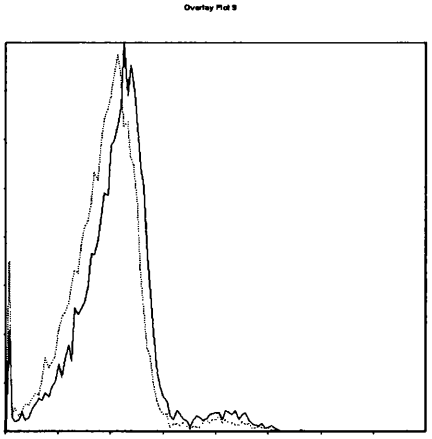


Figure 4.14. Flow cytometry showing absence of IgA and IgM binding to CHO-K1 cells transfected with Fcα/μR ΔEC3 cDNA

CHO-K1 cells transfected with either human Fcα/μR ΔEC3 cDNA (bold line) or with irrelevant DNA as a control (light line) were incubated with 375μg/ml IgA or 250μg/ml IgM as indicated and antibody binding assessed using flow cytometry. Fluorescence intensity is shown on the *x*-axis and the number of events is on the *y*-axis.



IgA



IgM

Figure 4.15. Fluorescence micrographs showing intracellular localisation of transfected Fcα/μR and Fcα/μR ΔEC3 proteins

(A) CHO-K1 cells were transiently transfected with green fluorescent protein (GFP)-labelled Fcα/μR or GFP-labelled Fcα/μR ΔEC3 cDNA. The transfected proteins were detected as green fluorescence on fluorescence microscopy and cell nuclei appear blue. (B) CHO-K1 cells transfected with GFP-Fcα/μR or GFP-Fcα/μR ΔEC3 constructs were incubated with 50μg/ml of polymeric IgA1. Red colour represents antibody binding.

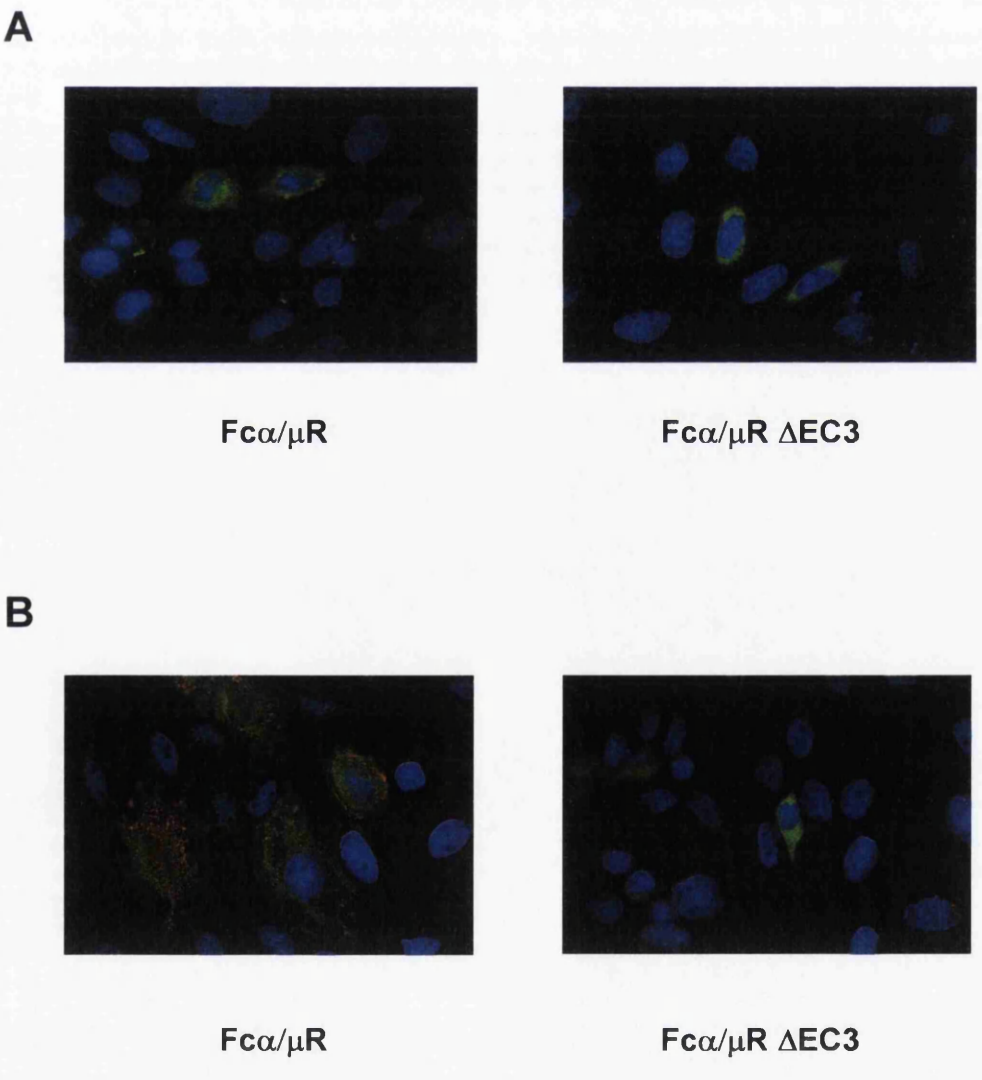


Figure 4.16. Fluorescence micrographs showing intracellular localisation of transfected Fcα/μR and Fcα/μR ΔEC3 proteins with calnexin

CHO-K1 cells were transiently transfected with green fluorescent protein (GFP)-labelled Fcα/μR or GFP-labelled Fcα/μR ΔEC3 cDNA. Cells were then stained with an antibody specific for calnexin which was detected with a fluorescently labelled secondary antibody. The variability observed in calnexin staining requires further validation with other ER markers.

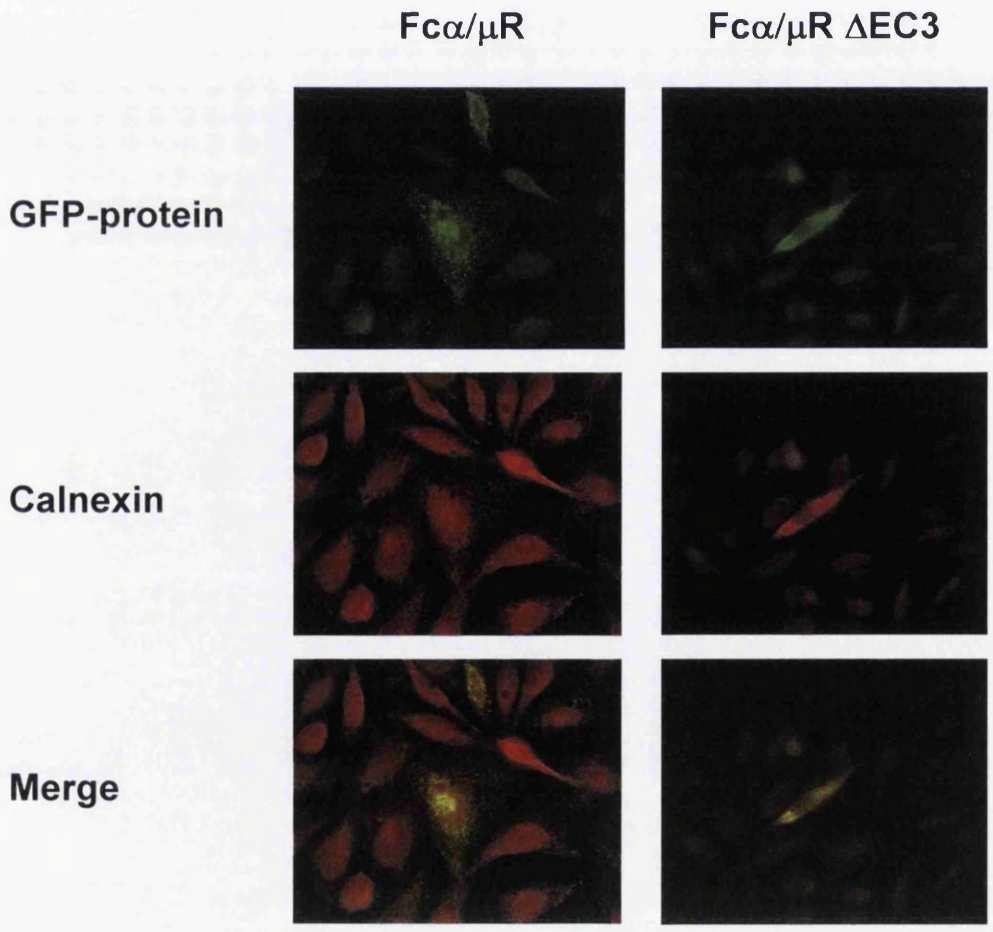


Figure 4.17. Fluorescence micrographs showing intracellular localisation of transfected Fcα/μR and Fcα/μR ΔEC3 proteins with caveolin

CHO-K1 cells were transiently transfected with green fluorescent protein (GFP)-labelled Fcα/μR or GFP-labelled Fcα/μR ΔEC3 cDNA. Cells were then stained with an antibody specific for caveolin which was detected with a fluorescently labelled secondary antibody.

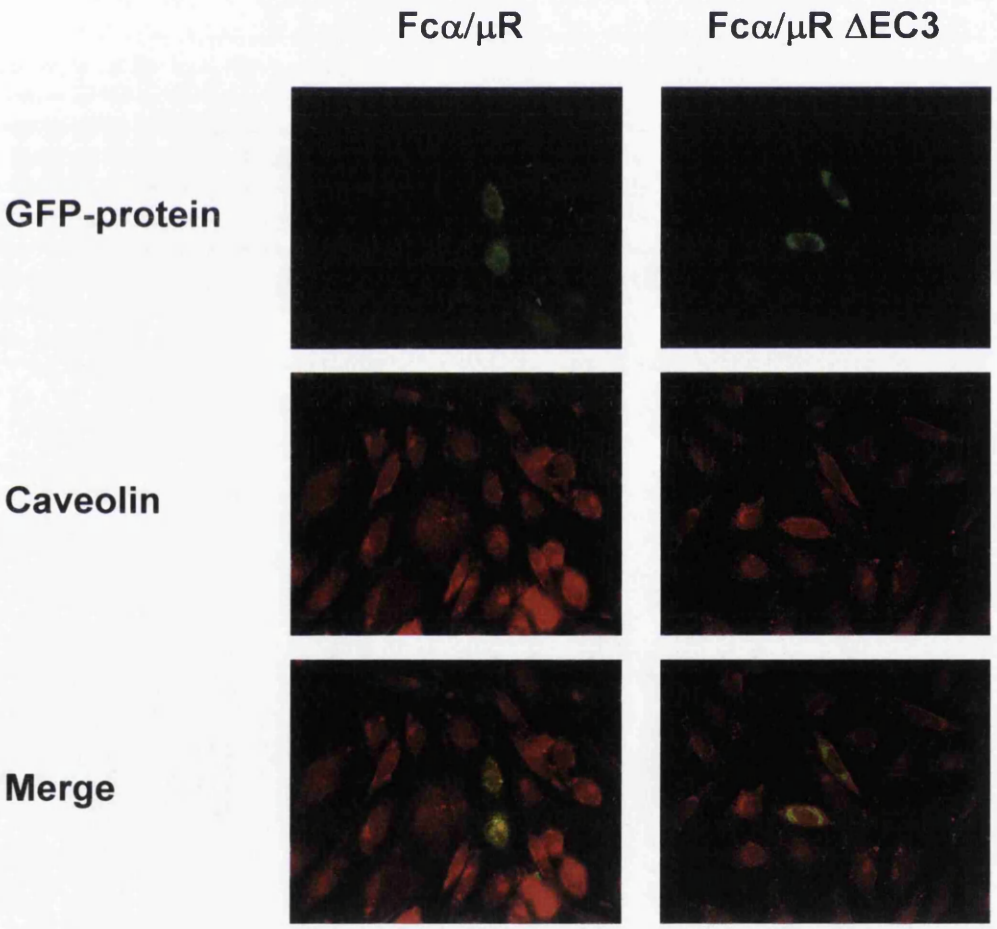


Table 4.1. Real time PCR for Fcα/μR

Real time PCR for Fcα/μR was performed using cDNA from U937 cells, COS-7 cells and four separate HMC cell lines in parallel with the appropriate reverse transcription controls (RT-CON). Reactions were performed in triplicate and mean threshold cycles (C_t) are shown along with the difference in mean C_t between samples and controls (ΔC_t).

	cDNA	RT-CON	ΔC_t
U937	31.6	36.7	5.1
COS-7	33.8	33.1	-0.7
HMC-1	28.3	33.8	5.5
HMC-2	31.1	34.2	3.1
HMC-3	28.0	31.8	3.8
HMC-4	27.5	32.3	4.8

Table 4.2. Real time PCR for Fcα/μR following stimulation of U937 cells and HMCs

Real time PCR for Fcα/μR was performed using cDNA from U937 cells and from an HMC line following 24 hours stimulation with either 1mM dibutyryl cyclic AMP (dbcAMP) or 50ng/ml IL-1α. Reactions were performed in parallel with the appropriate reverse transcription controls (RT-CON) and in triplicate. Mean threshold cycles (C_t) are shown along with the difference in mean C_t between samples and controls (ΔC_t).

		cDNA	RT-CON	ΔC_t
U937	Unstimulated	35.0	38.7	3.7
	dbcAMP	33.6	36.2	2.6
	IL-1α	33.9	38.1	4.2
HMC	Unstimulated	31.9	32.7	0.8
	dbcAMP	31.1	33.3	2.2
	IL-1α	25.1	33.4	8.3

Table 4.3. Human Fcα/μR intron-exon boundaries

The intron-exon boundaries of human Fcα/μR were derived by comparison of the cloned receptor with the human genome sequence. The splice site consensus elements are italicised. The initial 6 nucleotides of the adjacent 3' exon are shown for each intron

			Adjacent 3' exon	
Intron	**5' splice donor**	**3' splice acceptor**	**sequence**	**name**
1	*GT*AAGT	TTGC*AG*	GTCACC	SP
2	*GT*GAGT	CTGC*AG*	GTTCTT	EC1
3	*GT*ACAA	CTGC*AG*	CTCCAA	EC2
4	*GT*ATGA	ACAC*AG*	GTCCCG	EC3
5	*GT*AACC	CTGT*AG*	TACTTT	TM
6	*GT*GAGT	CTGC*AG*	CTCAGG	CY

Chapter 5

Immunoglobulin receptor expression by renal proximal tubular cells

5.1. Introduction

In common with other renal pathologies, progressive IgA nephropathy is associated with histological changes of tubular and interstitial injury which lead eventually to end stage renal damage. The severity of tubulointerstitial fibrosis and scarring can be correlated with the rate of loss of renal function (Nath, 1992). In proteinuric glomerular diseases, several mechanisms leading to tubulointerstitial damage are believed to operate. These include a direct toxic effect of filtered proteins on tubular cells, overload of the normal tubular endocytic protein reabsorption processes and the effect of locally produced mediators of inflammation from either tubular cells or glomerular cells on cells of the tubulointerstitial compartment. The latter mechanism has been demonstrated with cultured HMCs exposed to sera from IgA nephropathy patients. These HMC supernatants subsequently activated cultured proximal tubular cells and this effect was mediated by TNF-α (Chan *et al*, 2005). A final common feature of tubulointerstitial damage is infiltration of the region with immune cells such as monocytes and lymphocytes which in turn release further inflammatory mediators into the local microenvironment.

An additional feature of tubulointerstitial fibrosis is the accumulation of extracellular matrix proteins such as fibronectin, laminin and various forms of collagen that replace the normal tissue architecture. Transforming growth factor-beta 1 (TGF-β1) production by tubular cells and interleukin-1 (IL-1) released by infiltrating mononuclear cells have been implicated in the processes leading to matrix deposition (Yamamoto *et al*, 1996). IL-1β led to enhanced fibronectin production by cultured tubular cells and this was accompanied by increases in α-smooth muscle actin (α-

SMA) expression, TGF-β1 release and nitric oxide production. Fibronectin production induced by IL-1β appeared to be dependent on TGF-β1, and IL-1β had the additional effect of reducing proliferation of tubular cells. Induction of α-SMA expression by tubular cells is thought to be associated with "transdifferentiation" of these cells to a fibrotic myofibroblast phenotype (Ng *et al*, 1998). In this process, tubular cells progressively elongate, lose polarity, detach from their basement membranes and ultimately migrate through the damaged tubular basement membrane into the interstitium. In the rat model studied there was a significant association between the number of α-SMA positive tubular cells, the presence of α-SMA positive myofibroblasts and the degree of tubulointerstitial fibrosis.

Disruption of the normal glomerular filtration barrier in proteinuric nephropathies permits the abnormal passage of intact immunoglobulin molecules to the glomerular filtrate. As a consequence, proximal tubular cells can then be exposed to these molecules in potentially high concentrations. Levels of urinary IgG and IgA have been examined in patients with IgA nephropathy and with Henoch-Schönlein nephritis (Galla *et al*, 1985). Urinary concentrations of each immunoglobulin were higher in patients in comparison with controls and levels also correlated positively with serum creatinine concentration and the severity of proteinuria. Interestingly, urinary IgA from patients was almost exclusively in the monomeric form as opposed to the polymeric form which predominated in urine from healthy controls. A later study confirmed the finding of IgA and IgG in urine from patients with IgA nephropathy (Takahashi *et al*, 2004). These authors found that the cationic portion of IgG was absent from the urine of patients with proteinuric glomerulopathies and suggested that this was due to preferential reabsorption of this IgG fraction by proximal tubular cells. It was postulated that this was the consequence of a specific receptor-mediated reabsorption mechanism.

Specific activating responses by renal proximal tubular cells following stimulation by proteins present in the glomerular filtrate have been described. Production of the chemokine RANTES (regulated upon activation, normal T cell expressed and

secreted) was induced by cultured tubular cells in response to albumin and to IgG (Zoja *et al*, 1998). Release of RANTES from the cells was predominantly basolateral. Therefore it was concluded that release of this chemokine into the renal interstitium might contribute to the inflammatory cell infiltrate seen in chronic renal damage. Exposure of cultured proximal tubular cells to albumin also led to cellular activation via the Janus kinase (JAK) and the signal transducer and activator of transcription (STAT) signalling pathway (Nakajima *et al*, 2004). Activation of the JAK/STAT pathway appeared to be mediated by production of reactive oxygen species by the cells in response to albumin overload. Activation of proximal tubular cells has also been demonstrated following exposure to transferrin (Tang *et al*, 2002). In this study, increased production of several chemokines including monocyte chemoattractant peptide-1, interleukin-8, macrophage migration inhibitory factor but not RANTES was observed when tubular cells were stimulated with transferrin. This evidence supports the hypothesis that proximal tubular cells respond to the presence of excess or abnormal protein in glomerular filtrate by activating specific cellular signal transduction pathways that in turn lead to the local production of inflammatory mediators.

The neonatal Fc receptor (FcRn) was cloned from a placental cDNA library and is believed to mediate transplacental transfer of IgG from mother to foetus and the systemic absorption across the intestinal epithelium of maternal IgG from breast milk (Story *et al*, 1994). FcRn is structurally homologous to the MHC class I molecule and is associated with β_2-microglobulin. Receptor binding to IgG is pH-dependent with higher affinity binding at an acidic pH. Preliminary data had shown that mRNA for FcRn was detectable in human kidney and more precise localisation to glomerular epithelial cells and the brush border of proximal tubular cells was subsequently confirmed (Haymann *et al*, 2000). A functional role for FcRn was demonstrated in human renal tubular cells where the receptor mediated transcytosis of IgG (Kobayashi *et al*, 2002). This suggested that FcRn played a role in the reabsorption of IgG from the glomerular filtrate by cells of the proximal tubule.

As renal tubular cells can be exposed to immunoglobulins and immune complexes in proteinuric glomerulopathies such as IgA nephropathy we investigated whether these cells expressed any of the known immunoglobulin receptors. Signal transduction via such receptors could subsequently lead to cell activation, production of inflammatory mediators and initiation of processes leading to tubulointerstitial fibrosis.

5.2. Results

5.2.1. Human PTCs express mRNA for PIGR and FcRn

In order to examine expression of immunoglobulin receptors by renal tubular cells, primary human proximal tubular cells (PTCs) in culture were stimulated for 24 hours with 50ng/ml IL-1α, 50ng/ml IL-6, 50ng/ml TNF-α, 10^{-7}M phorbol 12-myristate 13-acetate (PMA) or 1mM dibutyryl cyclic AMP (dbcAMP). RNA was extracted from these cells along with RNA from unstimulated PTCs. cDNA was generated by reverse transcription and PCR performed using specific primers. PCR demonstrated equal expression of β actin for all samples (data not shown).

PCR for the polymeric immunoglobulin receptor (PIGR) was performed using the primers "PIGR-for" and "PIGR-rev" (table 2.2.). Primers "FcRn-for" and "FcRn-rev" were used to detect FcRn. It was anticipated that in common with other epithelial cells PTCs would express the PIGR. Similarly, immunohistochemistry demonstrating expression of the neonatal Fc receptor (FcRn) at the brush border of human PTCs had been previously reported (Haymann *et al*, 2000).

Expression of the PIGR and FcRn by unstimulated PTCs was confirmed by the presence of bands corresponding to predicted sizes of 694 and 359 base pairs for each receptor respectively (figure 5.1.). Stimulation of PTCs with the panel of biological mediators did not significantly affect expression of FcRn in this system. However, both PMA and dbcAMP appeared to reduce expression of the PIGR transcript.

5.2.2. Human PTCs do not express mRNA for FcγRs

To determine whether human PTCs expressed Fc gamma receptors, PCRs for FcγRI, FcγRIIb and FcγRIII were performed using cDNA from stimulated and unstimulated PTCs as described in section 5.2.1. The primer combinations used were "I-for" and "I-rev" for FcγRI, "II-for" and "IIb-rev" for FcγRIIb and "III-for" and "III-rev" for

FcγRIII. Primer sequences are shown in table 2.2. U937 cells are known to express FcγRI and FcγRIIb so cDNA from these cells was used as a positive control. Neutrophil cDNA was used as a positive control for FcγRIII expression and this cDNA was a generous gift from Dr Lorna Murray (MRC Centre for Inflammation Research, University of Edinburgh, UK). As RT-PCR for FcγRIIa yielded technically unclear results (data not shown), lysates from unstimulated PTCs and cells stimulated with 50ng/ml IL-1α or 50ng/ml TNF-α were immunoblotted with an antibody specific for this receptor. The anti-FcγRII antibody (IV3 clone) used (Medarex, Princeton, NJ, USA) has been reported to recognise only the FcγRIIa isoform of the receptor (Maresco *et al*, 1999). A lysate prepared from a culture of U937 cells was used as a positive control for FcγRIIa expression.

The results of these assays for FcγR expression by PTCs are shown in figure 5.2. In each case expression of the appropriate receptor with a band of expected size was confirmed for the positive control sample. Multiple transcripts of FcγRI have been reported for cells expressing this receptor and these can be seen in the appropriate positive control lane (Ernst *et al*, 1992). The predicted size of the band representing wildtype FcγRI is 876 base pairs. No transcripts for FcγRI, FcγRIIb or FcγRIII were detected in either stimulated or unstimulated PTCs. Similarly, no expression of FcγRIIa protein was found in these cells.

5.2.3. Human PTCs express mRNA for Fcα/μR but not for FcαRI or ASGPR

PCR for three IgA receptors was performed using cDNA prepared from unstimulated PTCs together with cells that had been stimulated with mediators as described in section 5.2.1. The primer combinations used were "89-F2" and "89-R2" for FcαRI, "H2-for" and "H2-rev" for the H2 subunit of the asialoglycoprotein receptor (ASGPR) and "amr-for1" and "amr-rev" for the Fcα/μR. These primer sequences are shown in table 2.2. cDNA derived from a culture of U937 cells was used as a positive control

for FcαRI expression and cDNA from HepG2 cells provided the positive control for ASGPR expression.

The results of these experiments are shown in figure 5.3. No expression of either FcαRI or ASGPR was detected in these primary PTCs. Each positive control cDNA yielded a band of the appropriate size for FcαRI and ASGPR. Expression of Fcα/μR transcript was demonstrated in PTCs. This result was confirmed using a separate primary PTC culture and with the immortalised human PTC line, HK-2 (Ryan *et al*, 1994)(data not shown). Stimulation of PTCs with IL-1α for 24 hours was associated with enhanced Fcα/μR mRNA expression. Incubation of PTCs with IL-6 and with TNF-α did not significantly alter Fcα/μR expression in this system. In contrast, both PMA and dbcAMP treatment of cells appeared to reduce levels of Fcα/μR transcript in PTCs.

5.2.4. Fcα/μR protein is expressed by renal tubular cells

In order to establish whether Fcα/μR protein was present on renal tubular cells, immunohistochemistry using a specific antibody was performed. The murine monoclonal IgG antibody was a generous gift from Dr Wei Zhang (Institute of Basic Medical Sciences, Beijing, China). This antibody was generated from recombinant human Fcα/μR protein encoded by our receptor clone described in section 4.2.3. Immunohistochemical staining of normal human tonsillar and renal sections was performed by Dr Barbara Young (Department of Pathology, Western Infirmary, Glasgow) and is shown in figure 5.4. Staining of lymphoid tissue was undertaken in order to test antibody reactivity with B lymphocytes. Expression of Fcα/μR by these cells was predicted based on the reported cellular distribution of the receptor by murine B lymphocytes (Shibuya *et al*, 2000).

Figure 5.4.A shows predominant staining of the mantle zone of a secondary tonsillar lymphoid follicle. This region contains B lymphocytes and the staining pattern would be consistent with expression of Fcα/μR by human B lymphocytes. Figure 5.4.B

confirms expression of Fcα/μR protein by human renal proximal tubular cells. Staining of glomerular podocytes was also observed but no other glomerular structures including mesangial areas were positive for Fcα/μR expression.

5.3. Discussion

These data describe expression and regulation of the Fcα/μR by human proximal tubular cells. Confirmation of neonatal Fc receptor expression was also documented but no evidence of the classical Fc receptors for IgG and IgA was found in these cells.

In these experiments we used primary cultures of proximal renal tubular cells derived form normal renal tissue. In order to induce expression of potential immunoglobulin receptors the cells were stimulated with a variety of biological mediators. Exposure of tubular cells to these mediators was an attempt to mimic the local microenvironment found in the inflamed and activated tubulointerstitium *in vivo*. These cultured primary PTCs expressed mRNA for the PIGR as anticipated by virtue of their epithelial origin (Abramowsky & Swinehart, 1986). Whether binding of ligand to this receptor has any pro-inflammatory consequences or whether the PIGR fulfils a simple transporting function at this site is not clear. None of the biological mediators tested appeared to increase expression of the PIGR in these cells. In contrast, both PMA and dbcAMP led to reduced expression of PIGR transcript in PTCs whereas each of these mediators can induce expression of other immunoglobulin receptors in other cell types. These observations would favour a non-inflammatory function of the PIGR in these cells.

The FcRn is responsible for the transplacental transfer of IgG in addition to mediating transport of IgG present in breast milk to the circulation across the infant gut. Receptor affinity for IgG is enhanced under acidic conditions and this property may underlie the relatively long half-life of IgG in the circulation by promoting ligand dissociation from receptor within neutral intracellular compartments and subsequent recycling of IgG to the bloodstream (Ghetie & Ward, 2000). The presence of FcRn on renal tubular epithelial cells has recently been described and a functional role for the receptor in the reabsorption of IgG from tubular filtrate has been suggested (Kobayashi *et al*, 2002). FcRn expression by our primary PTCs was confirmed but the inflammatory mediators examined did not enhance levels of receptor transcript. Once

again, this suggests a non-inflammatory transport and recycling function for FcRn in the proximal tubule.

No evidence for expression by PTCs of the classical Fc receptors for IgG was found using both stimulated and unstimulated cells. These cells did not express the activating receptors FcγRI, FcγRIIa and FcγRIII or the inhibitory FcγRIIb receptor. This is consistent with the reported distribution of these receptors on cells of haemopoietic origin only. Similarly, PTCs did not express either the classical IgA receptor, FcαRI or the H2 subunit of the ASGPR. However, these human renal proximal tubular cells did express transcript for the Fcα/μR and this expression was upregulated by treatment with IL-1α in a manner analogous to that observed in HMCs (see section 4.2.2.). Enhanced Fcα/μR expression in the renal tubule in response to IL-1α or other inflammatory mediators may be of significance in the pathogenesis of proteinuric IgA nephropathy where these cells are also exposed to filtered IgA. PMA and dbcAMP appeared to reduce Fcα/μR transcript levels in PTCs. Fcα/μR expression in the human renal proximal tubule was confirmed using immunohistochemistry with a specific monoclonal antibody.

A recent study by other investigators also examined expression of IgA receptors by primary human PTCs (Chan *et al*, 2005). In common with our results, these authors did not detect expression of FcαRI or the H2 subunit of ASGPR by PTCs. However, they also reported that PTCs did not express the PIGR or the Fcα/μR which is in contrast to our data. This difference is most likely explained by variations in cell harvesting or cell culture conditions. The most apparent differences are that these other cells were cultured in the presence of hydrocortisone and were "growth arrested" in medium containing 0.5% serum for 48 hours before use in experiments. The absence of PIGR expression by these epithelial cells is unexpected and may represent de-differentiation induced by culture conditions. Similarly, the failure to detect Fcα/μR expression by these cells may be due to altered cell phenotype. Our PTCs expressed the PIGR and therefore may represent the phenotype of PTCs *in vivo* more

closely. In addition, we documented expression of Fcα/μR protein by renal tubular cells in normal kidney sections by immunohistochemistry.

Therefore, we have demonstrated expression by human PTCs of the FcRn, the PIGR and the Fcα/μR. The most likely function of the FcRn at this site is the recycling of filtered IgG either across the epithelium to the circulation or back via the apical aspect of the cell to the tubular fluid. Based on the known functional characteristics of the PIGR it is likely that this receptor is expressed only at the basolateral surface of the tubular epithelium where it mediates transfer of dimeric IgA and IgM from the circulation to the tubular fluid. An integral part of this process involves proteolytic cleavage of the receptor so it is unlikely that intact PIGR is present on the apical cellular surface to permit interaction with immunoglobulins in the tubular fluid. The function of Fcα/μR expressed by renal proximal tubular cells is unknown. However, it is possible that this receptor is available to interact with filtered IgA and IgM in proteinuric glomerulopathies and receptor levels may be increased in the presence of inflammatory mediators. Under certain conditions this interaction may be a component of a clearance or recycling pathway for immunoglobulin but internalisation of excess ligand may induce cellular activation and production of cytokines or extracellular matrix contributing to the process of tubulointerstitial damage. Evidence of such a tubulopathic effect of IgG has been demonstrated in cultured rabbit renal tubular cells where production of the mediator endothelin-1 was induced in a dose-dependent fashion (Zoja *et al*, 1995).

Figure 5.1. RT-PCR for PIGR and FcRn in human proximal tubular cells

RT-PCRs for the polymeric immunoglobulin receptor (PIGR) and the neonatal Fc receptor (FcRn) were performed using cDNA from a primary human proximal tubular cell culture. Cells were either unstimulated (0) or had been stimulated for 24 hours with 50ng/ml IL-1α, 50ng/ml IL-6, 50ng/ml TNF-α, 10^{-7}M phorbol 12-myristate 13-acetate (PMA) or 1mM dibutyryl cyclic AMP (dbcAMP). The figures to the left represent molecular size in base pairs.

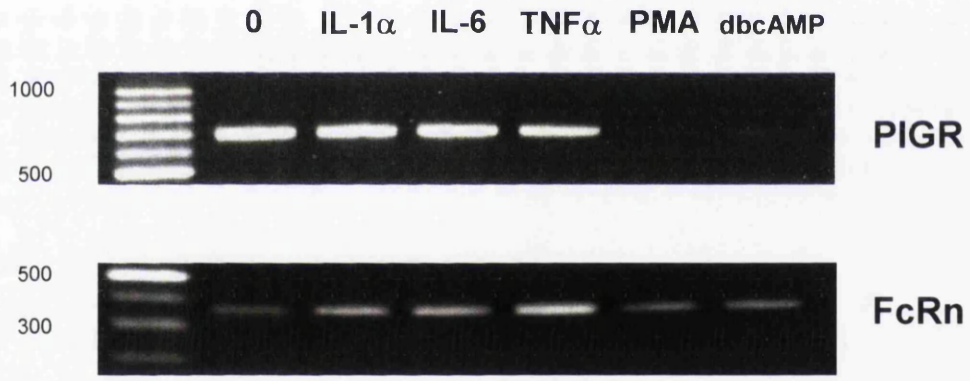


Figure 5.2. Human proximal tubular cells do not express FcγRs

RT-PCRs for FcγRI, FcγRIIb and FcγRIII were performed using cDNA from a primary human proximal tubular cell culture (A). Lysates from the same cells were also immunoblotted for FcγRIIa (B). Cells were either unstimulated (0) or had been stimulated for 24 hours with 50ng/ml IL-1α, 50ng/ml IL-6, 50ng/ml TNF-α, 10^{-7}M phorbol 12-myristate 13-acetate (PMA) or 1mM dibutyryl cyclic AMP (dbcAMP). Material from U937 cells was used as positive controls (+) when assessing expression of FcγRI, FcγRIIa and FcγRIIb. Neutrophil cDNA was used as the positive control for the FcγRIII PCR. The figures to the left represent molecular size in base pairs for the PCR images and molecular weight in kDa for the FcγRIIa immunoblot.

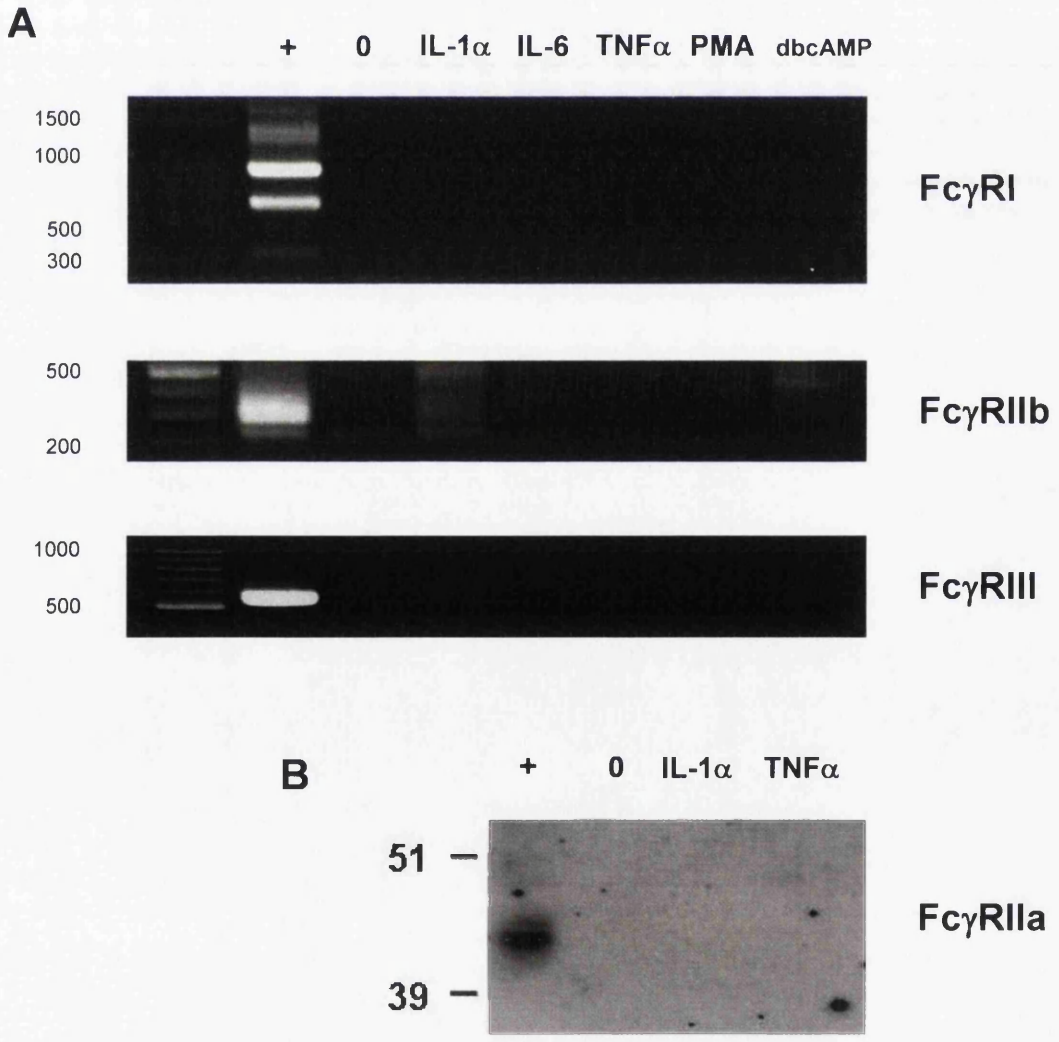


Figure 5.3. RT-PCR for IgA receptors in human proximal tubular cells

RT-PCRs for FcαRI, the asialoglycoprotein receptor (ASGPR) and the Fcα/μR were performed using cDNA from a primary human proximal tubular cell culture. Cells were either unstimulated (0) or had been stimulated for 24 hours with 50ng/ml IL-1α, 50ng/ml IL-6, 50ng/ml TNF-α, 10^{-7}M phorbol 12-myristate 13-acetate (PMA) or 1mM dibutyryl cyclic AMP (dbcAMP). The figures to the left represent molecular size in base pairs.

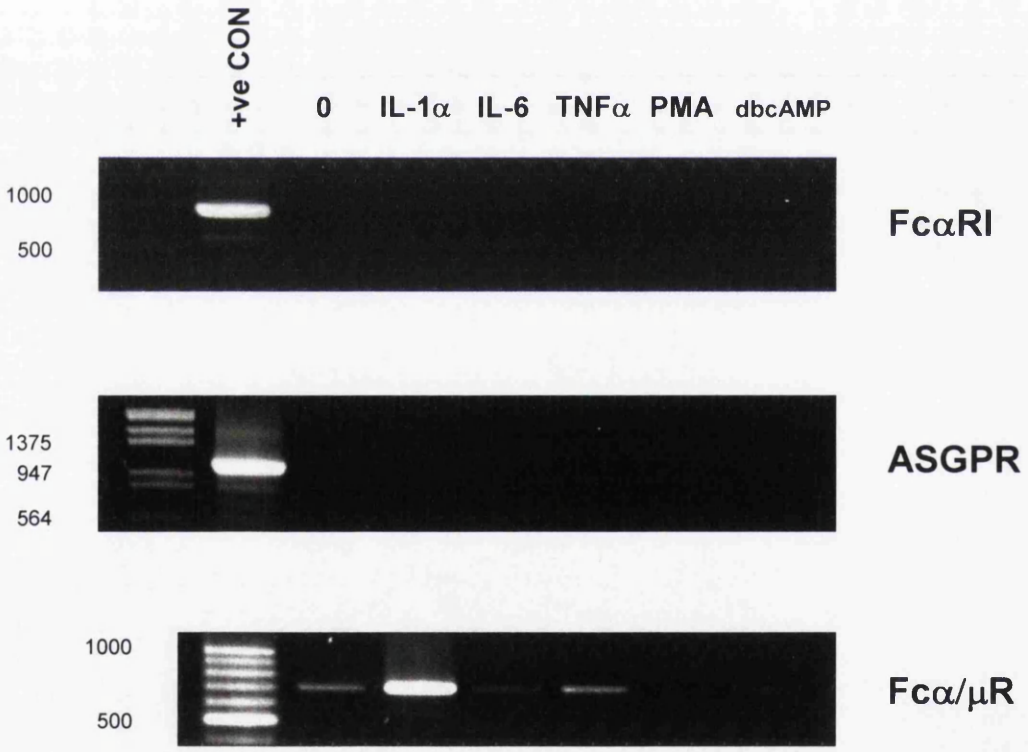
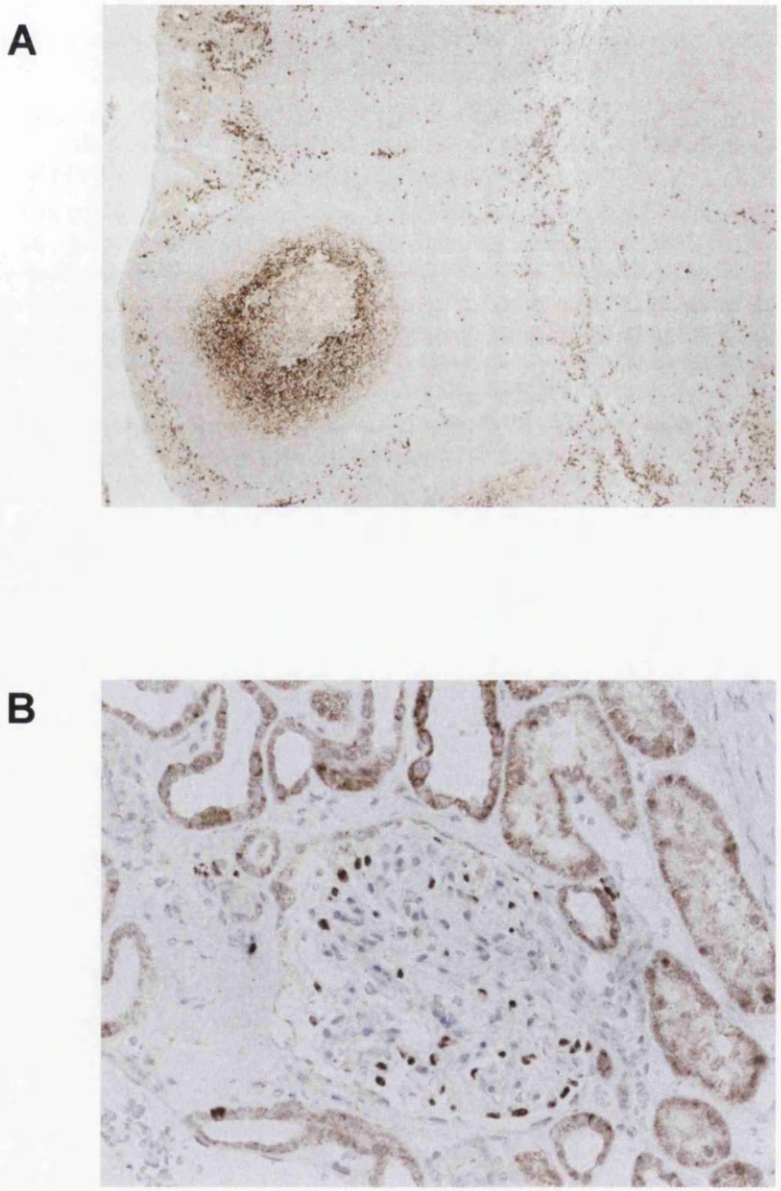


Figure 5.4. Immunohistochemical staining of tonsil and kidney for Fcα/μR

Immunohistochemistry of human tonsillar (A; x100 magnification) and renal (B; x150 magnification) tissues using a murine monoclonal antibody specific for human Fcα/μR. Positive staining is shown in brown.



Chapter 6

General discussion

In the four decades since IgA nephropathy was first described many clinical and epidemiological characteristics of the condition have been documented. Valuable data have emerged relating to aspects such as geographical distribution, clinical features, determinants of progression and the phenomenon of recurrent disease in renal allografts. In contrast, relatively little progress has been made toward an understanding of the molecular pathogenesis of IgA nephropathy. This difficulty reflects the complexity of what is undoubtedly a multifactorial and heterogeneous disease process.

There are several fundamental aspects of disease pathogenesis that remain unclear. IgA nephropathy is defined by the presence of IgA deposits in the glomerular mesangium and these deposits are associated with mesangial expansion and progressive tubulointerstitial damage leading to end stage renal disease in some patients but not in others. Although a causal relationship between glomerular IgA deposition and subsequent renal damage is widely assumed, the evidence for even this basic tenet of disease pathophysiology is at best circumstantial. Another significant area of uncertainty lies in the origin of mesangial IgA. Evidence of altered regulation of elements within the systemic immune system has been interpreted as supporting this limb of the immune response as the source of mesangial IgA. However, whether this association is causal or is alternatively a consequence of abnormalities in, or responses by, the mucosal immune system is debatable. The classical association of macroscopic haematuria with upper respiratory tract infection in IgA nephropathy intuitively implicates the mucosal immune system and the recent observation of cultured mesangial cell responses to secretory IgA and the detection of secretory IgA

in a transplanted kidney with recurrent disease should lead to close re-examination of the origins of mesangial IgA (Oortwijn *et al*, 2006).

Most progress in unravelling the pathogenesis of IgA nephropathy in recent years has been made in characterising the associated glycosylation abnormalities of the IgA1 molecule. Hypo-galactosylation of the *O*-linked glycans at the IgA1 hinge region promotes formation of multimeric IgA immune complexes, increases IgA binding to mesangial cells and mesangial matrix and induces production of biological mediators by mesangial cells. Patients with IgA nephropathy have higher proportions of serum IgA with reduced *O*-linked glycosylation and the same glycosylation abnormality has been described in IgA recovered from nephrectomy specimens. However, abnormal IgA glycosylation alone is unlikely to be sufficient to explain disease pathogenesis but may be necessary in combination with other factors for IgA nephropathy to emerge. The abnormality is not absolute and a fraction of IgA in normal individuals will be relatively under-glycosylated, although a critical threshold may exist at which point the amount or proportion of the abnormally glycosylated species exerts a pathological action. Another piece of evidence against abnormal IgA glycosylation being an independent determining factor for disease is the absence of recurrent IgA deposition in approximately half of transplanted kidneys of patients with IgA nephropathy. In this situation mesangial IgA deposition does not occur despite the persistent abnormality in circulating IgA glycosylation suggesting that some other factor or factors are required to permit expression of the disease phenotype.

Ultimately the clinical definition of IgA nephropathy is dictated by a combination of diagnostic convenience and limited understanding of the pathophysiology. It would not be unreasonable to propose that the condition represented a common channel for several discrete pathological processes each with a distinct array of initiating, susceptibility and response characteristics. The presence of mesangial IgA deposits would then represent a common pathway of expression for several molecularly distinct renal pathologies in a manner analogous to tubulointerstitial fibrosis. Although this scenario would be likely to further complicate the process of understanding and

investigating disease pathology, such a concept may help explain for instance, why the presence of glomerular IgA deposits is associated with renal damage only in certain patients.

An aspect of disease pathophysiology that has received more recent attention is that of putative receptors for IgA in the glomerular mesangium. Such receptors could be of central importance to the pathological mechanism of IgA nephropathy, as they would facilitate a link between mesangial IgA deposits and activation and proliferation of mesangial cells leading thereafter to renal damage. Antagonism of a receptor operating in this way would be a potential strategy for therapeutic intervention. It is also possible that IgA immune complexes are localised to the mesangium by non-receptor mediated mechanisms such as interaction with components of the extracellular matrix or by an electrostatic interaction with cells. Such associations could be promoted by the abnormal glycosylation profile of circulating IgA1. However, the interaction reported between IgA and cultured human mesangial cells suggests that these cells express a population of specific receptors for IgA as opposed to IgA association by a purely non-receptor mediated mechanism (Diven *et al*, 1998; Barratt *et al*, 2000). Initial reports suggested that human mesangial cells in culture expressed the classical IgA receptor, FcαRI. However later studies contradicted this finding and our results confirmed and extended these reports using a variety of approaches.

We demonstrated mRNA expression of the novel Fcα/μR by cultured human mesangial cells. This finding is of significance as any IgA receptor expressed by mesangial cells would be a candidate for mediating the deposition of IgA in IgA nephropathy and could potentially underlie mesangial cell activation by IgA immune complexes. Fcα/μR transcript expression was enhanced by the inflammatory cytokine IL-1α and this finding could be of relevance when considering the array of inflammatory mediators present within the kidney in IgA nephropathy (Yoshioka *et al*, 1993; Yano *et al*, 1997). We also showed that human Fcα/μR was able to bind IgA and IgM in a similar manner to that reported for the mouse receptor and identified two

variant transcripts of the Fcα/μR gene. Differential expression of an Fcα/μR splice variant by mesangial cells may be a basis for diversification of receptor function due to distinct properties of the variant form. Such a mechanism may have implications for mesangial cell responses to IgA. The function of the ΔEC3 transcript was not determined but it is possible that it represents a component part of a larger multimeric complex with a function distinct from, but related to, that of the wildtype receptor. Such a distinct function could be variable affinity for different species or molecular forms of IgA or specificity for either IgA or IgM alone. Based on differences between cytoplasmic sequences, it is possible that these receptors could be components of different multimeric complexes and would activate distinct signal transduction pathways. We hypothesised that the di-arginine motif in the cytoplasmic tail of the Fcα/μR ΔEC3 variant acted as a retention/retrieval motif that confined the protein to the endoplasmic reticulum in the absence of an additional specific signal or association with another molecule. The nature of this signal was undetermined but association with an additional molecule may only occur in a specific native cell type or in response to a particular intracellular signalling event.

A limitation to these studies was the absence of a specific antibody for the human Fcα/μR that meant we were unable to determine whether the receptor was expressed at the cell surface in cultured mesangial cells. However, such an antibody became available more recently and immunohistochemistry of normal kidney showed Fcα/μR expression by renal tubular cells but not by mesangial cells. Receptor expression by tubular cells has important implications for the pathogenesis of IgA nephropathy as IgA present in the glomerular filtrate could then interact with and activate tubular cells contributing to the development of renal injury. The failure to confirm Fcα/μR protein expression by mesangial cells can be interpreted in different ways. Firstly, mesangial cells may not actually express Fcα/μR transcript at all and the expression detected in primary cultures may be due to contamination by surviving tubular or glomerular epithelial cells. Secondly, mesangial cells may express only Fcα/μR mRNA and not protein under normal circumstances. However, Fcα/μR expression could then be induced in IgA nephropathy either directly by abnormally glycosylated

IgA-containing immune complexes or by components of the general inflammatory environment. It could be hypothesised that functional receptor expression occurs in only a subset of patients determined by genetic or other factors and that this may define disease susceptibility or determine those who subsequently develop progressive disease. Alternatively, cell surface expression of a splice variant protein may occur or be induced under certain conditions and this protein may not be detected by the monoclonal antibody.

There is strong evidence to support a role for the transferrin receptor (TfR) in binding of polymeric IgA1 to mesangial cells. TfR binding of IgA1 was modulated by IgA1 glycosylation, IgA immune complexes from IgA nephropathy patients were bound more avidly and the receptor mediated IgA1-induced cytokine production by mesangial cells (Moura *et al*, 2004; Moura *et al*, 2005). Furthermore, mesangial TfR expression was enhanced in renal biopsies from patients with IgA nephropathy and receptor distribution was coincident with that of mesangial IgA deposits (Haddad *et al*, 2003). However, other data suggest that IgA binding to mesangial cells is probably not exclusively via the TfR and that alternative receptor mechanisms are likely to be involved. Use of exogenous transferrin as a competitive inhibitor reduced IgA1 binding to HMCs by a maximum of only 50%, implying the existence of other IgA receptors on these cells (Moura *et al*, 2004). Additionally, binding of monomeric IgA1 and IgA2 to HMCs has been reported (Diven *et al*, 1998; Barratt *et al*, 2000) but TfR has no affinity for these molecular forms of IgA. Similarly, TfR does not bind secretory IgA (Moura *et al*, 2004) so presumably an alternative mechanism must be responsible for the secretory IgA deposition described recently in a transplanted kidney with recurrent IgA nephropathy (Oortwijn *et al*, 2006). Other reports have also documented binding of secretory IgA by cultured HMCs (Barratt *et al*, 2000; Leung *et al*, 2000).

It has been reported that HMCs in culture do not bind IgM and that IgM does not inhibit binding of IgA to these cells (Barratt *et al*, 2000; Leung *et al*, 2000). This would not be consistent with a significant contribution to IgA binding by wildtype

Fcα/μR in quiescent, cultured HMCs. However, this does not exclude the possibility of Fcα/μR-mediated binding of IgA by activated mesangial cells *in vivo*. It is possible that restricted expression of such a receptor under specific conditions is associated with a specific cellular response, perhaps underlying signalling pathways leading to renal damage or disease progression. Alternatively, expression of a variant form of Fcα/μR by HMCs may permit IgA binding while precluding binding of IgM.

Any current model of the molecular pathogenesis of IgA nephropathy is complicated by significant knowledge gaps in several areas and must therefore be speculative. An overview of such a model is illustrated in figure 6.1. One constant feature of the disease phenotype is the presence of increased levels of circulating immune complexes containing IgA1 with reduced *O*-linked glycosylation in the hinge region. Whether these complexes develop spontaneously in genetically susceptible subjects or whether they are produced in response to an environmental antigen encountered by either the systemic or mucosal immune system in susceptible individuals is unknown. It is possible that a range of different stimuli or initiating factors lead to the development of IgA1-containing immune complexes thus contributing to the heterogeneity of the condition. Other components of these immune complexes such as soluble FcαRI, anti-glycan IgG, or fibronectin may also be variables in the manifestation of IgA nephropathy.

The interaction between circulating IgA and the renal mesangium must be central to the development of IgA nephropathy as the condition is defined by the presence of IgA at this site. Good evidence exists to support the notion of an interaction based on specific IgA receptors although this does not preclude non-receptor mediated mechanisms operating in parallel and contributing to IgA deposition. The factors determining whether IgA becomes deposited within the mesangium at this point are unclear but most attention has been traditionally focussed on characteristics of circulating IgA such as molecular form and glycosylation profile. Consequently, the model of the kidney as an "innocent bystander" has arisen whereby the crucial determinants of mesangial IgA deposition reside with the IgA molecule. However it is

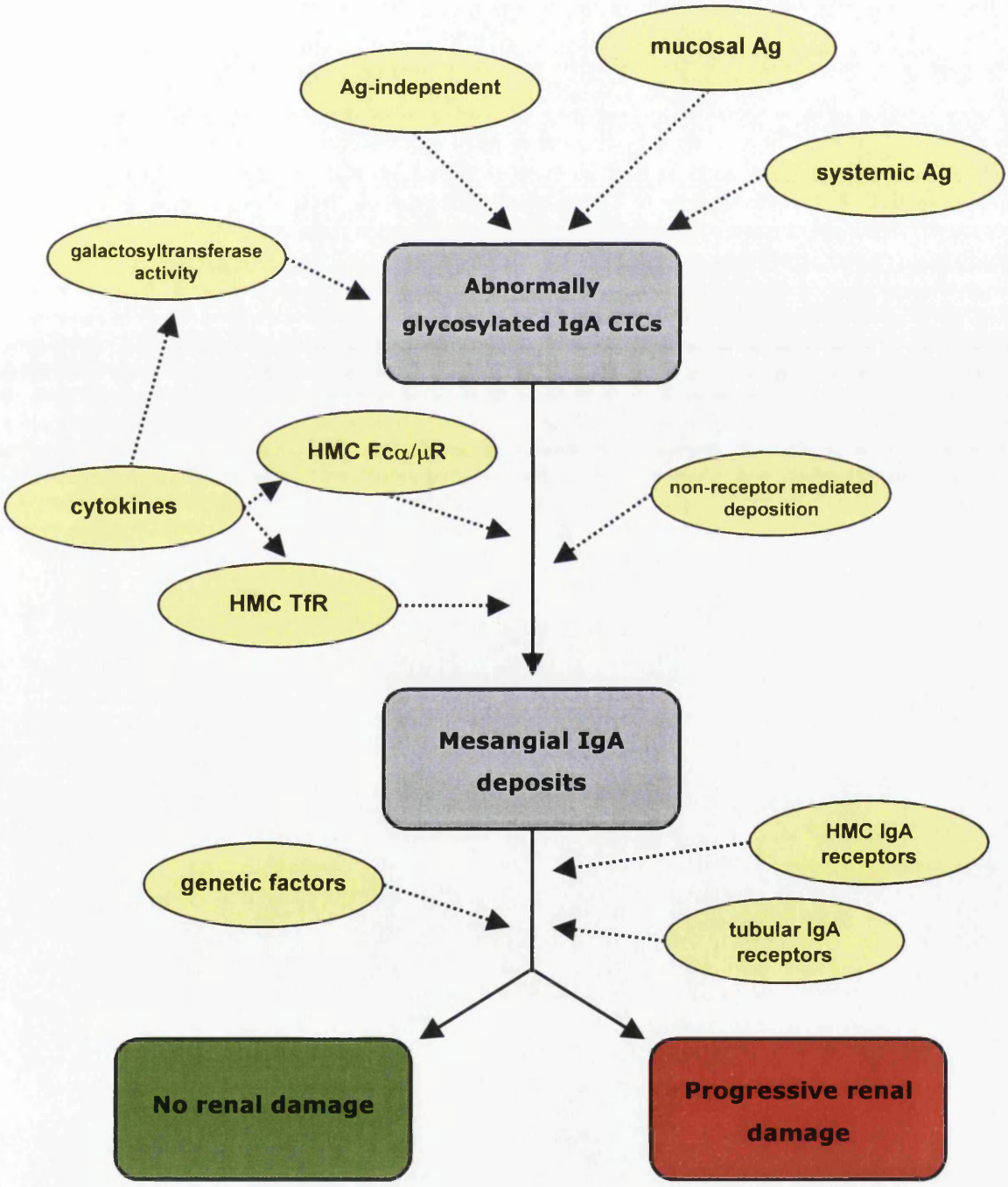
equally feasible that mesangial factors and characteristics also influence whether or not IgA becomes deposited. Evidence to support this concept comes from the observation that only around half of allograft kidneys transplanted into patients with IgA nephropathy as their primary cause of renal failure subsequently succumb to recurrent deposition of mesangial IgA. Thus, variability at the level of mesangial cell receptors or of mesangial cell responses may facilitate or prevent IgA deposition in the setting of abnormal IgA immune complexes. Therefore for mesangial IgA deposition to occur, and for IgA nephropathy to develop, two conditions must be fulfilled – the presence of appropriately abnormal IgA-containing immune complexes and a permissive array or state of mesangial receptors. A variety of mesangial cell IgA receptors could potentially be involved. Some, such as the TfR, may be constitutively expressed by all cells whereas others such as Fcα/μR or a variant form may only be expressed in particular inflammatory environments or by genetically defined modulators. This variability could determine either a harmful or protective mesangial cell response depending on whether an activating signal transduction pathway was initiated or immune complexes were effectively processed and cleared by the cell.

In the proportion of patients destined to have progressive IgA nephropathy, large proteins including immunoglobulins will eventually breach the glomerular filtration barrier. Consequently, both normal and abnormally glycosylated species of circulating IgA will emerge in the tubular filtrate and be available to interact directly with renal tubular cells via both non-selective and specific receptor-mediated mechanisms. Fcα/μR expressed at this site could mediate the response of tubular cells to IgA. Tubular cell activation may occur leading to local cytokine production and tubulointerstitial fibrosis or alternatively renal damage may be determined instead by failure of a receptor-mediated clearance mechanism for IgA.

The molecular pathogenesis of IgA nephropathy remains incompletely defined. Identification of Fcα/μR expression by mesangial cells and renal tubular cells should contribute to new hypotheses in relation to physiological and pathological renal responses to IgA in IgA nephropathy.

Figure 6.1. Model for the pathogenesis of IgA nephropathy

The cardinal features and possible outcomes of IgA nephropathy are shown in rectangular boxes. Potential determining and modifying factors are shown at various points.



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Appendix

Figure A.1. pCR4 vector map

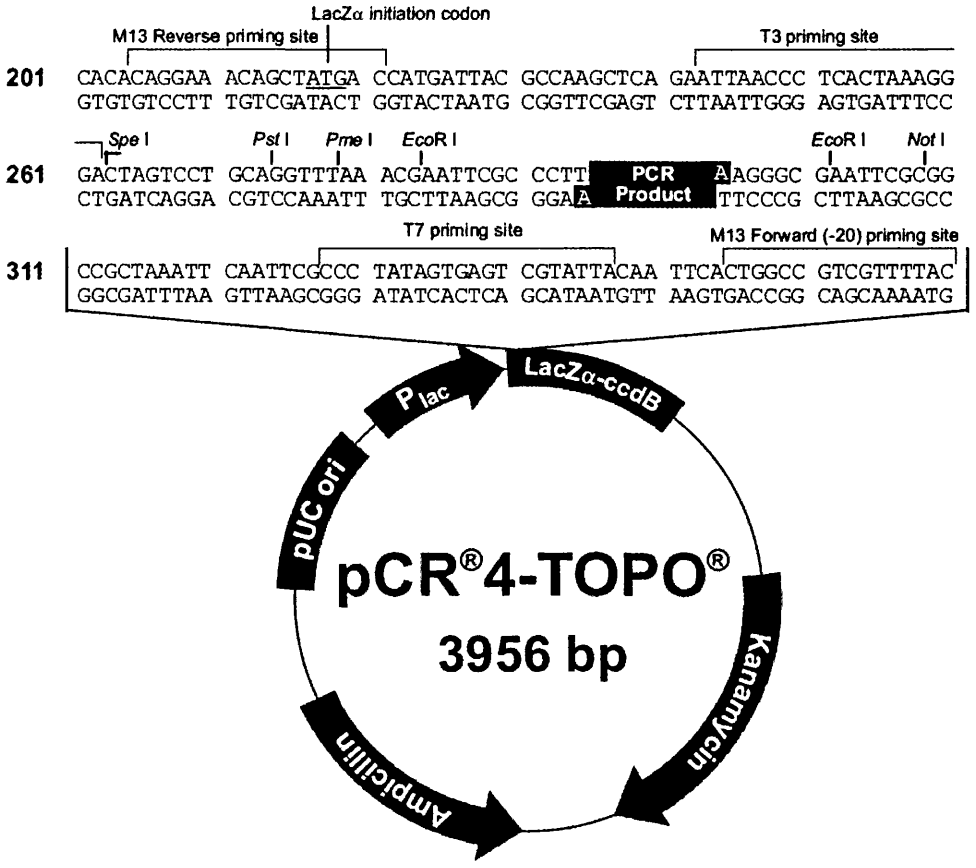

Figure A.2. pcDNA3.1/V5/His/TOPO vector map

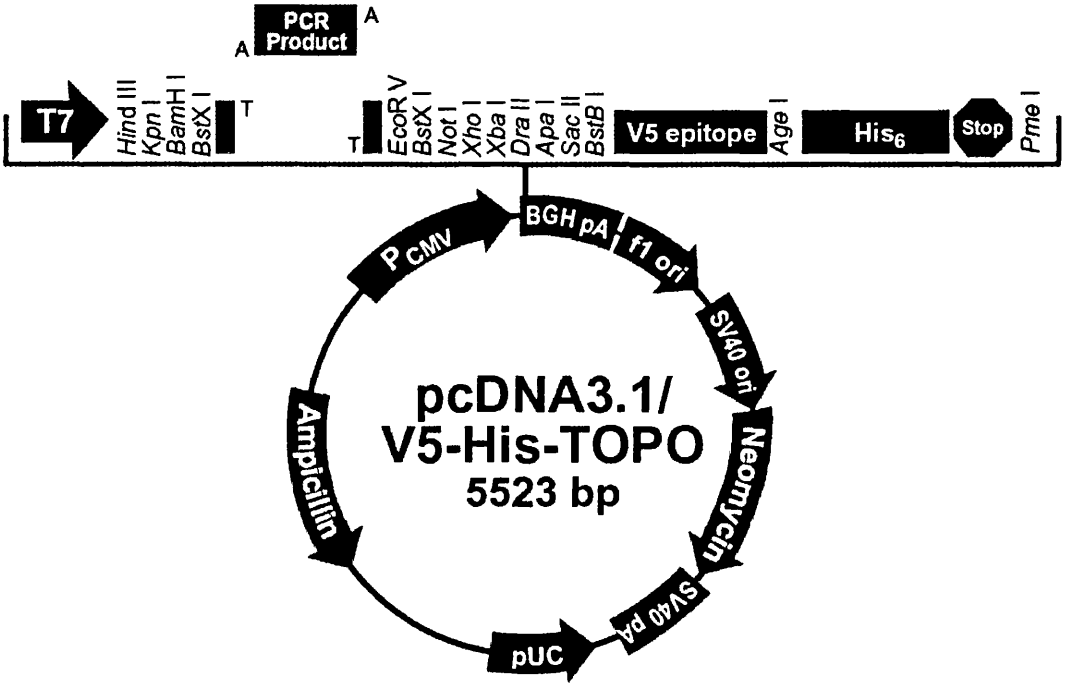

Figure A.3. pcDNA3.1/CT-GFP-TOPO vector map