The Proteoglycans of Metastatic and Non-Metastatic B16 Murine Melanomas

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Dedicated to my father

John Moran Hamilton (1933-1991)

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CONTENTS:

Page 1: SECTION 1: INTRODUCTION
Page 19: SECTION 2: THE EXTRACELLULAR MATRIX PROTEINS LAMININ, FIBRONECTIN AND COLLAGEN
Page 49: SECTION 3: PROTEOGLYCANS AND GLYCOSAMINOGLYCANS
Page 59: SECTION 4: MATRIX DISSOLUTION
Page 67: SECTION 5: MATERIALS & METHODS
Page 83: SECTION 6: RESULTS
Page 131: SECTION 7: DISCUSSION
Page 161: SECTION 8: REFERENCES

FIGURES & ILLUSTRATIONS:

Fig.1: Tumour cell metastasis
Fig.2a: The structure of laminin
Fig.2b: The functional domains of laminin
Fig.3a: The functional domains of fibronectin
Fig.3b: The cell-binding sites of fibronectin
Fig.4: Models of collagen IV intermolecular association
Fig.5: The structure and distribution of glycosaminoglycans

B16 Murine Melanoma Growth characteristics in vitro

Fig.6a: B16 melanoma growth rate in vitro: B16F1 and B16F1M3±0.5% DMF
Fig.6b: B16 melanoma growth rate in vitro: B16F1 and B16F1M5±0.5% DMF
Fig.6c: B16F1M3 melanoma cell growth response to varying concentrations of DMF
Fig.6d: B16 melanoma growth rate in vitro: B16F1H5 and B16F1D2

Figs.7A-7H: B16 melanoma cell morphology in vitro:
A; B16F1 cells
B; B16F1M3 cells
C; B16F1+0.5% DMF
D; B16F1M3+0.5% DMF
E; B16F1H5 cells
F; B16F1D2 cells
G; B16F1D2 control cells (+DMSO)
H; B16F1D2+0.5mM ß-D-xyloside

Figs.7I-7P: B16 melanoma cells as 24 hour spheroid cultures:
I; B16F1 cells
J; B16F1M3 cells
K; B16F1+0.5% DMF
L; B16F1M3+DMF
M; B16F1H5 cells
N; B16F1D2 cells

Figs.7I-7P: B16 melanoma cells as 24 hour spheroid cultures (continued)
O; B16F1D2 control cells (+DMSO)
P; B16F1D2+0.5mM ß-D-xyloside

Fig.8a: The effect of DMF on tyrosinase activity
Fig.8b: The effect of DMF on intracellular melanin content
Fig.9: Cell spread area in vitro

Cell Adhesion Assays:

Cell attachment to BSA
Fig.10: B16F1 versus B16F1M3±0.5% DMF
Fig.11: B16F1D2 versus B16F1H5
Fig.12: B16F1 versus B16F1M5

Cell attachment to collagen type I
Fig.13: B16F1 versus B16F1M3±0.5% DMF
Fig.14: B16F1D2 versus B16F1H5
Fig.15: B16F1D2±0.5mM ß-D-xyloside
Fig.16: B16F1 versus B16F1M5

Cell attachment to fibronectin
Fig.17: B16F1 versus B16F1M3±0.5% DMF
Fig.18: B16F1D2 versus B16F1H5
Fig.19: B16F1D2±0.5mM ß-D-xyloside
Fig.20: B16F1 versus B16F1M5

Cell attachment to laminin/nidogen
Fig.21: B16F1 versus B16F1M3±0.5% DMF
Fig.22: B16F1 versus B16F1M5
Fig.23: B16F1D2 versus B16F1H5
Fig.24: B16F1D2±0.5mM ß-D-xyloside

Cell attachment to collagen type IV
Fig.25: B16F1 versus B16F1M3±0.5% DMF
Fig.26: B16F1 versus B16F1M5
Fig.27: B16F1D2 versus B16F1H5
Fig.28: B16F1D2±0.5mM ß-D-xyloside

Cell attachment to CPAE monolayers
Fig.29: B16F1 versus B16F1M3±0.5% DMF
Fig.30: B16F1D2 versus B16F1H5

Cell attachment to CPAE subendothelial matrix
Fig.31: B16F1 versus B16F1M3±0.5% DMF
Fig.32: B16F1D2 versus B16F1H5

Homotypic ell attachment to B16 melanoma monolayers
Fig.33: B16F1 versus B16F1M3±0.5% DMF

Degradation of Metabolically-Labelled Subendothelial Extracellular Matrix:

Release of ^{3}H-Proline from CPAE subendothelial matrices
Fig.34a: B16F1 versus B16F1M3±0.5% DMF
Fig.34b: B16F1D2 versus B16F1H5

Release of ^{35}Sulphate from CPAE subendothelial matrices
Fig.35a: B16F1 versus B16F1M3±0.5% DMF
Fig.35b: B16F1D2 versus B16F1H5

Qualitative Glycosaminoglycan Analyses:

Chromatography elution standards
Fig.36a: Initial DEAE-Cellulose IEC separation
Fig.36b: Sephadex G50 GPC/Chondroitinase ABC digestion eltion profile
Fig.36c: Sephadex G50 GPC/Nitrous acid deamination elution profile
Fig.36d: Sephadex GPC/Nitrous acid deamination of HS-GAG elution profile

B16F1 versus B16F1M3: DEAE-Cellulose IEC
Fig.37: Medium fraction CS-GAG
Fig.38: Medium fraction HS-GAG
Fig.39: Trypsin fraction CS-GAG
Fig.40: Trypsin fraction HS-GAG
Fig.41: Cell fraction HS-GAG
Fig.42: Cell fraction HS-GAG

B16F1 versus B16F1M3: Mono-Q-Sepharose IEC
Fig.43: Medium fraction CS-GAG
Fig.44: Trypsin fraction CS-GAG
Fig.45: Trypsin fraction HS-GAG
Fig.46: Medium fraction HS-GAG

B16F1M3±0.5% DMF: DEAE-Cellulose IEC
Fig.47: Medium fraction CS-GAG
Fig.48: Trypsin fraction CS-GAG
Fig.49: Cell fraction CS-GAG
Fig.50: Medium fraction HS-GAG
Fig.51: Trypsin fraction HS-GAG
Fig.52: Cell fraction HS-GAG

B16F1M3±0.5% DMF: Mono-Q-Sepharose IEC
Fig.53: Medium fraction CS-GAG
Fig.54: Trypsin fraction CS-GAG
Fig.55: Medium fraction HS-GAG
Fig.56: Trypsin fraction HS-GAG

B16F1 versus B16F1M5: DEAE-Cellulose IEC
Fig.57: Medium fraction HS-GAG
Fig.58: Trypsin fraction HS-GAG

B16F1D2 versus B16F1H5: DEAE-Cellulose IEC
Fig.59: Medium fraction CS-GAG
Fig.60: Trypsin fraction CS-GAG
Fig.61: Trypsin fraction HS-GAG
Fig.62: Medium fraction HS-GAG

B16F1D2 versus B16F1H5: Mono-Q-Sepharose IEC
Fig.63: Medium fraction CS-GAG
Fig.64: Trypsin fraction HS-GAG
Fig.65: Trypsin fraction CS-GAG
Fig.66: Medium fraction HS-GAG

B16F1M3±50mM sodium chlorate: DEAE-Cellulose IEC
Fig.67: Medium fraction HS-GAG
Fig.68: Cell-Lysate fraction HS-GAG
Fig.69: Medium fraction CS-GAG

B16F1M3±20µg/ml heparin: Mono-Q-Sepharose IEC
Fig.70: Medium fraction HS-GAG

B16F1D2±ß-D-xyloside: DEAE-cellulose IEC
Fig.71: Cell-lysate fraction CS-GAG
Fig.72: Medium fraction HS-GAG
Fig.73: Medium fraction CS-GAG
Fig.74: Cell-lysate fraction HS-GAG

Qualitative Proteoglycan Analyses:

B16F1H5 versus B16F1D2: Sepharose CL4B GPC

Fig.75: Medium fraction PG/GAG
Fig.76: Cell-lysate fraction PG/GAG
Fig.77: Medium fraction CSPG/GAG/Pronase
Fig.78: Medium fraction HSPG/HS-GAG/Pronase
Fig.79: Cell-lysate fraction CSPG/CS-GAG/Pronase
Fig.80: Cell-lysate fraction HSPG/HS-GAG/Pronase
Fig.81: Cell-lysate fraction HSPG/GAG
Fig.82: Cell-lysate fraction CSPG/GAG
Fig.83: Medium fraction HSPG/GAG
Fig.84: Medium fraction CSPG/GAG

B16F1D2±ß-D-xyloside: Sepharose CL4B

Fig.85: Medium fraction PG/GAG
Fig.86: Cell-lysate fraction PG/GAG
Fig.87: Medium fraction PG/GAG/Pronase
Fig.88: Cell-lysate fraction PG/GAG/Pronase
Fig.89: Medium fraction CSPG/GAG
Fig.90: Medium fraction HSPG/GAG
Fig.91: Cell-lysate fraction CSPG/GAG
Fig.92: Cell-lysate fraction HSPG/GAG

TABLES & ILLUSTRATIONS:

Table.1 Lung colonizing potentials

Quantitative Glycosaminoglycan Analyses:

B16F1 versus B16F1M3±0.5% DMF
Table.2: % Glycosaminoglycan composition
Table.3a: Quantity of glycosaminoglycan as measured by ^{3}H-GlcN incorporation
Table.3b: Total glycosaminoglycan as measured by ^{3}H-GlcN incorporation
Table.3c: $^{35}SO_4$-incorporation into glycosaminoglycan

B16F1D2 versus B16F1H5
Table.4: % Glycosaminoglycan composition
Table.5a: Quantity of glycosaminoglycan as measured by ^{3}H-GlcN incorporation
Table.5b: Total glycosaminoglycan as measured by ^{3}H-GlcN incorporation
Table.5c: $^{35}SO_4$-incorporation into glycosaminoglycan
Table.5d: $^{3}H/^{35}S$ ratio
Table.5e: HS-GAG N-sulphate: O-sulphate ratio

B16F1D2±0.5mM ß-D-xyloside
Table.6: % Glycosaminoglycan composition
Table.7a: Quantity of glycosaminoglycan as measured by ^{3}H-GlcN incorporation
Table.7b: Total glycosaminoglycan as measured by ^{3}H-GlcN incorporation
Table.7c: $^{35}SO_4$-incorporation into glycosaminoglycan
Table.7d: $^{3}H/^{35}S$ ratio
Table.7e: HS-GAG N-sulphate: O-sulphate ratio

ABBREVIATIONS.

ACA: Aminocaproic acid
aFGF: Acidic fibroblast growth factor
α-MSH: α-Melanocyte stimulatory hormone
bFGF: Basic fibroblast growth factor
ß-APN: ß-aminoproprionitrile fumarate
ß-D-xyloside: p-nitrophenyl-ß-D-xylopyranoside
BM: Basement membrane
BSA: Bovine serum albumin
^{14}C-GlcN: Carbon^{14}-glucosamine
C-4-S (CS-A): Chondroitin-4-sulphate
CPAE: Calf Pulmonary Arterial Endothelium
^{51}Cr: Chromium^{51} (as Cr_2O_7)
CS (CS-GAG): Chondroitin sulphate
C-6-S (CS-C): Chondroitin-6-sulphate
CSPG: Chondroitin sulphate proteoglycan
DEAE-Cellulose: Diethylaminoethyl Acetate Cellulose
DMF: N,N-dimethylformamide
DMEM: Dulbecco's Minimal Essential Medium
DMSO: Dimethyl sulphoxide
dpm: decays per minute
DS (CS-B): Dermatan sulphate
DSPG: Dermatan sulphate proteoglycan
DTT: Dithiothreitol
ECM: Extracellular matrix
EDTA: Di-sodium ethylenediamine tetra-acetate
EGF: Epidermal growth factor
EHS: Engelbreth-Holm Swarm sarcoma
FCS: Foetal calf serum
GAG: Glycosaminoglycan
GalNac: N-acetylgalactosamine
GlcN: Glucosamine
GlcNAc: N-acetylglucosamine
GlcU: Glucuronic acid
^{3}H-GlcN: Tritiated glucosamine
GPC: Gel Permeation Chromatography
HA: Hyaluronic acid
HS (HS-GAG): Heparan sulphate
HSPG: Heparan sulphate proteoglycan
HUdR/KL103: 5'-hexyl-2-deoxyuridine
IdUA: Iduronic acid
IEC: Ion Exchange Chromatography

K_{av}: the ratio of [elution volume for a molecular species] to $[V_o - V_i]$

KS: Keratan sulphate

L-DOPA: L-beta-3,4-dihydroxyphenylalanine

MEM: Eagle's Minimal Essential Medium

MMP: Matrix metalloproteinase

M_r: Molecular mass

NCS: Newborn calf serum

N-EM: N-ethylmaleimide

N-MF: N-methylformamide

PA: Plasminogen activator

PAI: Plasminogen activator inhibitor

PAPS: Phosphoadenosylphosphosulphate

PBS: Phosphate buffered saline

PG: Proteoglycan

PMSF: Phenylmethylsulphonylfluoride

^{35}S: Sulphur^{35} (as H_2SO_4)

s.d.: Standard deviation

SEM: Standard error of the mean

SDS: Sodium dodecyl sulphate

TCA: Trichloroacetic acid

TCSF: Tumour collagenase stimulatory factor

TGF-ß1: Transforming growth factor ß1

TIMP: Tissue inhibitor of metalloproteinase

Tris: 2-amino-2-(hydroxymethyl)-1,3-propandiol

Triton X-100: Octylphenoxypolyethoxyethanol

UA: Uronic acid

uPA: Urokinase-type plasminogen activator

VGP: Vertical growth phase

V_i: Included volume

V_o: Void volume

V_t: Total volume

Summary:

Proteoglycans and glycosaminoglycans are implicated in a wide range of biological processes and phenomenom. Perhaps most importantly, the are attributed with a role in the modulation and maintenance of the physiochemical properties and integrity of the extracellular matrix, and in mediating cell adhesion and growth.

Our understanding of the role of proteoglycans and glycosaminoglycans in tumorigenesis and metastatic spread is limited. Few proteoglycans have been determined to possess specific biological functions. Other studies have tried to correlate changes in the synthesis and properties of proteoglycans and glycosaminoglycans with metastatic potential and progression, but many of the observations have proved to be somewhat conflicting. Therefore the effects and relevance of changes in the properties of and alterations in the biosynthesis of these molecules have not been fully elucidated.

In this study, the proteoglycans and glycosaminoglycans isolated from a series of B16 melanoma cell lines that differed in metastatic potential (as measured by lung colonization) were analyzed, and their chemical properties determined using ion exchange and size exclusion chromatography. In addition, the cell phenotypes of the cell lines were partially characterized, with cell adhesive and proteolytic responses being measured. Chemical agents, acknowledged as being capable of modifying glycosaminoglycan biosynthesis, were employed to modify the chemical properties of these molecules. The aim was to determine whether changes in the charge properties and the size of the GAG chains influence the ability of these cells to form lung metastases after intravenous inoculation, and whether a change or consistant alteration in

the properties of the proteoglycans and glycosaminoglycans could be identified as being related with metastatic potential.

Cell adhesion is a central process in many phases of the metastatic cascade. Many tumour cell types can be demonstrated as possessing adhesive preferences for extracellular matrices or isolated matrix proteins in vitro. The B16 cell lines were therefore assayed for such preferences and whether or not chemical modulation of the biochemical properties of the proteoglycans and glycosaminoglycans synthesized influenced their adhesive responses.

No consistent adhesive preference could be identified that appeared to be related to metastatic potential for all of the cell lines tested. Modulation of the chemical properties of the glycosaminoglycans synthesized by the cell lines also appeared not to correlate with changes in metastatic potential. Differences in the composition and properties of the glycosaminoglycans synthesized did not appear to influence the level of cell adhesion either. In conclusion, preferential adhesion to complex matrices and individual matrix proteins did not appear to be related to the metastatic potentials of the B16 melanoma cell lines used in this study, and alterations in the chemical properties of the glycosaminoglycans synthesized also did not appear to influence lung colonization.

Two consistant relationships were however identified. The ability to release ^{35}S-isotope from metabolically labelled subendothelial matrix and an increased expression of cell surface glycosaminoglycans correlated with high levels of lung colonization. Increased cell surface glycosaminoglycans may be related to an increase in the number or size of the of the glycosaminoglycan chains polymerized onto proteoglycan core

protein, whereas increased ^{35}S-releasing activity may represent an increase in the ability of more metastatic cell lines to penetrate basement membrane-like structures, a rate regulating step in the process of metastasis. The relevence of these two parameters is discussed in more detail within the text.

SECTION 1. INTRODUCTION

Page
1: 1.1.1. Neoplasia and metastatic disease
3: 1.1.2. Tumour induction
4: 1.1.3. The origin and nature of metastasis
6: 1.2.1. The three-step theory of tumour cell invasion
7: 1.2.2. Tumour cell adhesion to the extracellular matrix
7: 1.2.3. Matrix degradation
9: 1.2.4. Tumour cell locomotion
10: 1.3.1. Organ colonization
11: 1.3.2. Angiogenesis and intravasation
12: 1.3.3. Extravasation and cell killing
15: 1.4.1. The extracellular matrix
16: 1.4.2. The basement membrane
17: 1.5.1. Project details

FIGURES & ILLUSTRATIONS:

Page number or adjacent page number in parentheses:

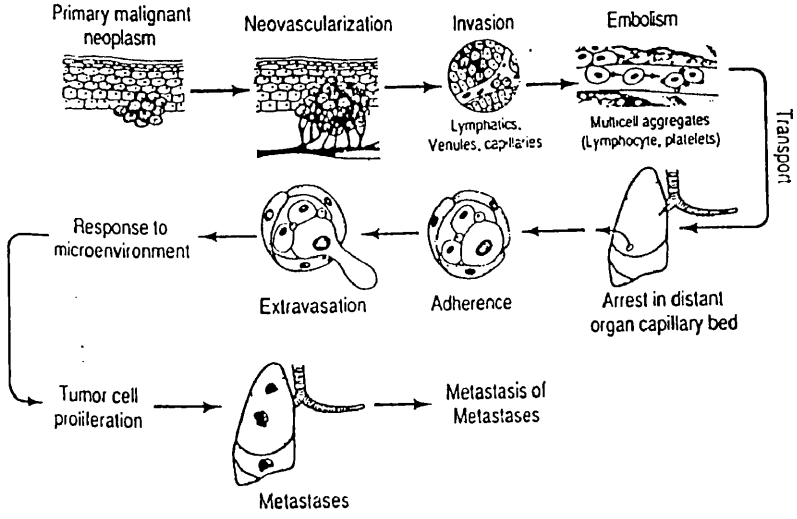
(1): Fig.1: Tumour cell metastasis

The Proteoglycans of Metastatic and Non-Metastatic B16 Murine Melanomas

1.1.1. Neoplasia and Metastatic Disease

The development of secondary neoplastic disease rather than the primary neoplastic lesion is often the major cause of cancer morbidity. Secondary disease originates through the dissemination of malignant tumour cells resulting in the establishment of cancerous deposits in dispersed anatomic locations. This process of metastasis is a complex, multi-step process involving an interplay between the biological properties of tumour cell and host (Fig.1): malignant tumour cells detach from the primary tumour and invade adjacent tissue, and may also disseminate by intravasation into the microvasculature or lymphatics. The interactions between tumour cells within the vasculature or lymphatics and the constituents of blood and lymph influence the outcome of the metastatic event. Upon lodgement in an organ, tumour cells extravasate from the microvasculature and gain access to the organ parenchyma which, if suitable, supports tumour cell proliferation and the establishment of secondary tumours.

Fig.1: Tumour Cell Metastasis (reproduced from Fidler, 1990)



Conventional therapies have failed to overcome or limit the problems associated with metastatic disease. The dispersed distribution of metastatic lesions, the heterogeneity of cell phenotype within a lesion, and the common biological properties shared by both normal and neoplastic cells contribute to this failure. Attention has focussed upon determining, early on in the course of the disease, the aggressiveness and metastatic potential of individual tumours thereby permitting the appropriate stage-specific therapies to be devised. Advances in the identification and characterization of the biochemical markers and factors specific to tumour cells, and in particular to malignant cells, have been made with the aim of providing improved strategies for the earlier identification and treatment of tumours and their metastases.

Melanoma, a malignant tumour of melanocytes, is the leading cause of death among diseases of the skin and its incidence continues to rise (Liotta *et al.*, 1987; Herlyn, 1990). Melanocytes are responsible for skin pigmentation, and are derived from neural crest cells (Herlyn, 1990) that migrate during embryogenesis into the embryonic skin and organs. In normal skin, melanocytes are found distributed amongst the basal keratinocytes in the epidermis and are separated from the dermis by the epithelial basement membrane.

The prognosis of melanoma has been correlated with clinical and histological features, the most important being tumour thickness (Breslow, 1970). Primary malignant melanomas develop fundamentally as a single pathological process but with different clinical, histological, and biological expressions (Ackerman & Su, 1979; Herlyn, 1990). The current criteria used for the prognosis of melanoma are useful in predicting the

aggressiveness of a melanoma in a population but cannot accurately predict the aggressiveness of an individual's melanoma. There is therefore a need for highly accurate biochemical markers.

1.1.2. Tumour Induction

The failure of cells to respond to those signals that regulate cell growth and phenotype can result in neoplasia. Neoplasms are believed to originate as focal and probably clonal proliferative lesions (Fidler & Talmadge, 1986). Initially, primary tumours are most probably benign, and this stage tends not to be life-threatening, with many such lesions regressing spontaneously. The loss of growth regulation results in tumorigenesis and the development of the primary lesion. Subsequent lesions with abnormal form and cytology and the biological properties of cancer appear later.

The development of a malignant, life-threatening, phenotype is separate and distinct from tumorigenesis, and gives rise to metastasis. Tumorigenesis is an early event in cell transformation and is a prerequisite for metastasis (Newbold *et al.* 1982). These two events are controlled by separate genetic elements. Cell hybrids generated from fusions between (metastatic x non-metastatic cells) and (metastatic x normal cells) separated tumorigenic ability from metastatic competence. Tumorigenic, non-metastatic but not non-tumorigenic, metastatic hybrids could be isolated. Metastatic revertants which arose from non-metastatic hybrids possessed karyotypic alterations including the loss of genetic elements or whole chromosomes (Weiner *et al.*, 1974; Stanbridge *et al.*, 1981). Chromosomal abnormalities have been detected in chromosomes 1, 6, and 7 of malignant melanomas and in vertical growth phase (VGP) melanomas which are predisposed to metastasize (Herlyn *et al.*,1985), and alterations in oncogene status, including the overexpression of *N-myc*

(Bauer *et al.*, 1990), the deletion or translocation of *c-myb* (Meese *et al.*, 1989), and mutations in *K-ras* (Shukla *et al.*, 1989) have all been detected in human melanomas.

The genetic instability of hybrids has minimised their potential in analysing the genetic events underlying the metastatic process. This problem has been circumvented by the cloning of cell lines differing in metastatic potential from primary tumours, metastases and established tumour cell lines. (Fidler, 1973a; Fidler & Hart, 1982; Poste *et al.*, 1982a). Tumour cells of increasing metastatic capacity have been isolated from successive metastases after intravenous or subcutaneous inoculation (Fidler, 1973a; Poste *et al.*, 1982a), and cell lines isolated in this manner have provided greater population stability for the analysis of the events underlying and contributing to the metastatic process. Various selection procedures have demonstrated the heterogeneity in cell phenotype that exists in primary and secondary tumours and in cultured tumour cell populations (Fidler, 1973a; Frost & Kerbel, 1981; Poste *et al.*, 1982b; Garbisa *et al.*, 1988).

1.1.3. The origin and nature of metastasis

Tumour populations are heterogeneous, containing cells of differing metastatic potentials. In addition to *in vivo* and *in vitro* selection procedures, tumour cell subpopulations can be selected on the basis of particular cellular characteristics which may be important in the metastatic process. These include lectin-binding (Schirrmacher *et al.*, 1982; Lotan *et al.*, 1989), resistance to natural killer (NK) cells and macrophages (Hanna, 1985; Mantovani, 1990), interactions with blood components and the microvasculature (Gasic, 1984; Weiss *et al.*, 1989), adhesion to extracellular matrices and their components (Liotta *et al.*,

1986b; Juliano, 1987), and drug resistance (Bradley *et al.*, 1988). Each of these factors constitutes a selective pressure, implying that metastasis is a non-random process.

Metastasis is a selective event, dependent upon specific cellular properties which enable the cell to successfully complete each stage of the process (Nicolson, 1982a, 1988a; Nicolson *et al.*, 1982). Poorly metastatic cells have little probability of successful metastasis, whereas highly metastatic cells have a high probability. Even when highly metastatic cells are injected intravenously, not every cell survives to proliferate at a distant site, indicating that all metastatic cells do not have an equal chance to produce a metastasis (Price *et al.*, 1986; Fidler, 1989).

Malignant cells constitute small subpopulations within the primary tumour but become dominant in metastases through the actions of selective pressures. Although metastasis formation represents a cloning process (Talmadge *et al.*, 1982; Fidler & Talmadge, 1986; Price *et al.*, 1986), the cell population within a metastasis is susceptible to phenotypic drift. Primary neoplasms are often assumed to be monoclonal in origin but the generation of different cell subpopulations within the tumour occurs through time. Cell heterogeneity develops in rapidly expanding clonal populations of tumour cells (Poste & Fidler, 1980; Poste *et al.*, 1982b; Raz, 1982). Cells isolated from metastases and clonal populations do not possess equal metastatic ability. The majority of spontaneous metastases that originate are clonal in origin (Poste *et al.*, 1982b; Talmadge *et al.*, 1982; Nicolson, 1984b; Hu *et al.*, 1987), and within individual metastases, variant clones arise which further diversify the phenotype (Raz, 1982; Chambers *et al.*, 1984).

Metastasis appears to be a transient phenomenon, possibly in response to changes in intratumour environment dependent upon both tumour and host factors. Alterman *et al.* (1989) described a B16 melanoma which was non-metastatic in young mice but spontaneously metastasize in aged or hyperimmunized young mice. Similar altered cellular responses to intratumour environment are also observed when tumour cells are exposed to hypoxic conditions. The resultant increase in metastatic potential may be a consequence of an increase in the ploidy of the affected cells (Young *et al.*, 1988; Hill, 1990; Young & Hill, 1990). Hypoxic conditions arise in poorly vascularized tumours and metastasis may therefore be a response to such stress conditions (e.g. hypoxia, nutrient depletion, immune cell infiltrates, and necrosis) which develop within growing tumours. Once cells metastasize and the conditions of stress have diminished, they may lose their acquired metastatic potential which may only be reacquired when conditions of stress develop once more. The development of cell heterogeneity within a tumour therefore appears to be modulated by environmental factors and conditions.

1.2.1. The Three-Step Theory of Tumour Cell Invasion

Tumour cell invasion of the extracellular matrix (ECM) is not a consequence of the mechanical aspects of uncontrolled cell growth forcing cells into adjacent tissue. Cell invasion can occur without cell proliferation and is an active process requiring ongoing protein synthesis and proteolysis. Tumour cell invasion and metastasis can be blocked or inhibited by both natural and synthetic inhibitors of cell adhesion, protein synthesis and proteolysis (Thorgeirsson *et al.*, 1982; Iwamoto *et al.*, 1987; Humphries *et al.*, 1988b; McCarthy *et al.*, 1988; Reich *et al.*, 1988a; Hoyhtya *et al.*, 1990).

In malignant tumours, cell proliferation is followed by local invasion of the adjacent host tissues. Invasion is preceded by local degradation and remodelling of the ECM surrounding the tumour cells. Three sequential biochemical events appear to underlie tumour cell invasion of the tissue matrix (Liotta, 1986): cell adhesion, matrix degradation, and cell locomotion .

1.2.2. Tumour Cell Adhesion to the ECM

The ECM places both physical and biochemical constraints upon cells by providing positional and behavioural information and by influencing cell phenotype (Liotta, 1986; Patel & Lodish, 1987; Hay, 1989). The response of tumour cells within a matrix often differs to the response exhibited by normal or benign tumour cells which may shift into a resting or differentiated state. ECM alterations can therefore induce alterations in both cellular characteristics and behaviour (Fairbairn *et al.*, 1985; Iozzo, 1985; Boyd *et al.*, 1988; Noel *et al.*, 1988; Starkey, 1990).

Malignant cells invade local tissue either as individual cells or as small cell aggregates. This requires that the invading tumour cells adhere to some component(s) of the ECM. Cell attachment to a substrate is a necessary step for cell-mediated matrix degradation. Increased adherence to specific matrix components, and to naturally occurring and reconstituted matrices, can correlate with the metastatic behaviour of some tumour systems (Terranova *et al.*, 1984; Liotta, 1986; Chung *et al.*, 1988; Hunt, 1989; Hutchinson *et al.*, 1989; Lichtner *et al.*, 1989; Tullberg *et al.*, 1989). The ECM also influences and modulates the secretion and activities of the enzymes responsible for its degradation (Turpeenniemi-Hujanen *et al.*, 1986; Nabeshima *et al.*, 1986; Emonard *et*

al., 1990).

1.2.3. Matrix Degradation

Matrix dissolution can occur through several mechanisms. Tumour cells can directly secrete specific proteinases into the local environment or utilize surface- or matrix-bound proteinases (Moscatelli & Rifkin, 1988; Zucker, 1988). Proteinases can then directly degrade the matrix or alternatively, activate other proteinases present within the matrix. Matrix lysis most likely occurs at the region close to the tumour cell surface where the concentration of protease can outweigh the natural protease inhibitors present in matrix and in serum (Beckerle *et al.*, 1987; Pollanen *et al.*, 1988; Estreicher *et al.*, 1990), and ECM destruction and the presence of elevated levels of proteinases can correlate with metastatic potential (Liotta *et al.*, 1977; Sloane *et al.*, 1981, 1982).

Liotta *et al.* (1980, 1982) proposed that invading tumour cells secreted matrix degrading enzymes. Collagens are the major structural component of ECMs and tumour cell-derived collagenases have in some instances correlated with both tumour aggressiveness and metastatic potential (Salo et al., 1982; Turpeenniemmi-Hujanen *et al.*, 1985, 1986; Emonard *et al.*, 1990). Additionally, tumour cells can degrade the non-collagenous components of the ECM. The production of heparanase (Ricoveri & Cappalletti, 1986; Vlodavsky *et al.*, 1988), plasminogen activators (Wang *et al.*, 1980; Borgenmann & Jones, 1983), cathepsins (Sloane *et al.*, 1981, 1982), and the ability of cells to utilise matrix-bound proteases has also been demonstrated to correlate with metastatic potential.

Traversal of the vascular basement membrane (BM) is a vital step for the haematogeneous dissemination of tumour cells, an important route for

metastatic spread. Several studies have described the loss or disruption of host BM adjacent to the invasive front of *in situ* tumours, but the significance of these abnormalities in the pathology of the disease remains unclear, and may be one consequence of the process of dedifferentiation that many tumour cells undergo (Hayman *et al.*, 1982; Barsky *et al.*,1983; Gabbert, 1985; Forster *et al.*, 1986; Gusterson *et al.*, 1988). Local modification of the membrane may be necessary for the migration of tumour cells to occur through this structure, and it is possible that tumour cells that intravasate are those subpopulations of cells selected for their ability to degrade vascular BMs.

1.2.4. Tumour Cell Locomotion

Tumour growth and invasion stimulates both angiogenic and immune responses, allowing tumours to recruit endothelial cells and lymphocytes to their vicinity (Gabbert, 1985). The generation of a capillary network and cellular immune response are natural, invasive mechanisms. Factors produced by tumour cells and by invading lymphocytes and endothelial cells may facilitate the spread of malignant cells by remodelling the matrix and by stimulating cell growth and directed locomotion (Gasic, 1984; Shing *et al.*, 1984; Hanna, 1985; Nabeshima *et al.*, 1986; Gospodarowicz *et al.*, 1987; Savion *et al.*, 1987; Terranova *et al.*, 1989; Weiss *et al.*, 1989; Blood & Zetter, 1990). Matrix dissolution destabilizes the physical constraints on malignant cells permitting cell migration. The final step in the invasion process is tumour cell locomotion into the region of proteolytically modified matrix.

Tissue invasion continues by repetition of the adhesion, degradation and locomotion steps. Tumour cells may also migrate in response to ECM and BM components and their degradation products (Fligiel *et al.*, 1986; Furcht

et al., 1986; Liotta, 1986; Nabeshima *et al.*, 1986; Blood *et al.*, 1988; Humphries *et al.*, 1989; Hunt, 1989) and to motility factors (Liotta & Schiffman, 1988; Kohn *et al.*, 1990), and these may interact to direct cell locomotion and influence the specificity of metastases.

Many tumours have associated oedema, necrosis and immune cell infiltrates (Gabbert, 1985). Oedema causes distension and loss of the regular structure in the ECM, increasing the intercellular spaces and producing a more porous tissue which may more readily permit tumour cell invasion. Tumours themselves have no lymphatics but lymph has been detected to flow from the centre of the tumour towards its periphery and invasion front. Invading cells at the invasion front of tumours tend to exist as single cells or as small cell clusters, and this flow of lymph may passively assist the invasive process by physically directing cells towards the lymphatics.

1.3.1. Organ Colonization

Certain tumour types appear to preferentially metastasize to specific body sites (Nicolson, 1988a; Fidler, 1989). The patterning is often determined by the tumour site and can be influenced by the mechanical and physical forces acting on the tissue. However, this does not explain all patterns of metastasis. Even when tissue fragments are transplanted to ectopic sites, tumour cells exhibiting an organ preference still preferentially colonize the displaced tissue (Poste & Fidler, 1980; Hart & Fidler, 1981). This *homing* response is not due to a preferential accumulation of cells within the tissue. The intravenous administration of radiolabelled tumour cells into animals demonstrated that there was no bias in the distribution of tumour cells to specific organs or tissues (Fidler, 1989). Tumour cells were detectable in all organs tested, even

those in which the tumours rarely or never occurred. It can be concluded that the concept of tumour cell homing is two-step: tumour cells must initially lodge and subsequently grow at specific organ sites. The predictability of the pattern of metastasis for tumours of defined histological type indicates that metastasis contains non-random elements.

The pattern of metastasis distribution cannot be predicted from the pattern of initial tumour cell arrest. Similar numbers of cells from a K-1735 murine melanoma cell lines were found to initially lodge in the lungs of experimental animals immediately after intravenous inoculation, irrespective of the metastatic potential (Price *et al.*, 1986). Rather, the tumour cell survival rate appears to be a major determinant of colonization pattern. At 24 hours, non-metastatic cells had a lower survival rate than metastatic cells, and after 3 days only metastatic cells were still viable. Paget (1889) suggested that certain tumour cells might possess an affinity for the environment provided by certain organs, and this might account for the non-random distribution of tumour metastases. The organ-specificity colonization exhibited by various tumour cell lines may be dependent upon factors produced within the organs which may induce homing responses (Liotta & Schiffman, 1988; Kohn *et al.*, 1990) and stimulate tumour cell growth (Cavanaugh &Nicolson, 1989). In conclusion, both host tissue and tumour cell properties are important in dictating the patterns of tumour metastasis.

1.3.2. Angiogenesis and Intravasation

The development of a blood supply within a solid tumour mass is critical for its survival. Tumour cells can release factors from the ECM which stimulate endothelial cell growth and direct their migration towards the

tumour mass (Shing *et al.*, 1984; Vlodavsky *et al.*, 1982, 1988; Blood & Zetter, 1990; Mahadevan & Hart, 1990). These factors include the heparin-binding growth factors and endothelial cell growth factors. The capillary networks within tumours are defective compared to those from normal tissues (Blood & Zetter, 1990; Bouck, 1990). These defects include abnormal capillary endothelial cell morphology and subendothelial matrix and increased permeability, which may result in leaky vascular capillary networks. Defective capillaries may not be as an efficient barrier to invasion and therefore allow tumour cells to gain easier access to the general circulation. The abnormal microvasculature may be the consequence of the tumour cell-induced alterations in the matrix or caused by the factors they secrete or release from the matrix.

Tumour cells within vascularized tumours are found as cuffs of cells around new microvessels (Blood & Zetter, 1990; Bouck, 1990). Those malignant cells that penetrate the endothelial barrier may be transported to distant tissues, or proliferate at the site of penetration and directly shed cells into the blood system for dissemination. Removal of the primary tumour results only in removal of the initial source of malignant cells, and since most tumours are detectable only at an advanced stage of development, metastasis will have most probably occurred if the phenotype has been generated. Cells which have metastasized and formed secondary lesions also possess the potential to become the source of new metastatic cells.

1.3.3. Extravasation and Cell Killing

Once in the circulatory or lymphatic systems, tumour cells must overcome a new series of problems. Most tumour cells lodge in the first organ capillary bed they encounter and it is estimated that only 0.1% of

circulating tumour cells succeed in forming a successful metastases (Poste & Fidler, 1980). Cell lodgement in a capillary bed can either be a specific or passive process. Specific lodgement involves adhesion to endothelial cells or subendothelial BM and most probably involves specific cell surface receptors on both the tumour and endothelial cell. Tumour cells within the circulatory system interact with blood components and other circulating cells, both normal and neoplastic, a process which may influence the cells' fate.

Most disseminated malignant cells are destroyed within the circulation, apparently by two distinct mechanisms of cell killing. The host immune system is acknowledged as being responsible for some of this killing (Fidler & Bucana 1977; Fidler & Kripke 1980). Macrophages, neutrophils, and natural killer cells constitute the non-specific immune surveillance mechanisms. Specific immunity is mediated by activated antigen-dependent cytotoxic T-lymphocytes and antibodies against tumour antigens. The rapid destruction of all circulating tumour cells cannot be accounted for by the immune system alone. Passive mechanisms also play a prominent role in tumour cell killing. Tumour cells encounter mechanical forces, including turbulence and impaction as they are transported rapidly within the general circulation. These forces probably account for the high death rate of circulating tumour cells. Other factors may include the reduced deformability of tumour cells compared with that of blood cells, and this would affect the tumour cells' ability to survive the high shear forces present within the microcirculation (Weiss *et al.*, 1989). The survival of circulating tumour cells may thus be related to an increase in tumour cell deformability or their ability to form emboli with other tumour cells (Nicolson 1982a; Weiss et al., 1989) or with other tumour cells or with circulating blood cells (Gasic *et al.*, 1986).

Embolus formation is advantageous for the tumour cell and may endow it with a distinct selective metastatic advantage (Fidler & Bucana 1977; Fidler & Kripke 1980; Gasic 1984). Embolus formation increases the probability of tumour cell lodgement in blood capillaries through physical entrapment (Nicolson, 1988a, Blood & Zetter, 1990), and is a potential means of masking the tumour cell from the hostile environment of the vasculature. Tumour cells may also utilize the biological properties of the other cells within the embolus to adhere to the capillary endothelium and initiate extravasation (Weiss *et al.*, 1989; Blood & Zetter, 1990).

The arrest of tumour cells in a capillary may physically disrupt the endothelial cell layer or cause its partial retraction exposing the underlying endothelial BM (Lapis *et al.*, 1988). Traversal of this matrix is the rate limiting step in extravasation. Tumour cells can adhere to the BM matrix and to the basolateral surface of the retracted endothelial cells. Once tumour cells have adhered, the endothelium re-extends over them. Endothelia derived from different origins responds differently to tumour cell attachment; arteriole endothelium does not retract but outgrows and encompasses attached tumour cells. As tumour cells proliferate they occlude the arteriole lumen and extravasate only when the original endothelium is mechanically disrupted (Lapis *et al.*, 1988).

Invasion of the endothelial BM completes the process of metastasis. Local dissolution of the matrix occurs just below the tumour cell after 8-24 hours followed by extension of tumour cell pseudopodia into this region (Poste & Fidler, 1980). Within 24-48 hours the tumour cell has fully extravasated and is in a position to initiate a secondary tumour by firstly proliferating around the capillary bed before migrating through the

perivascular interstitial matrix and into the organ parenchyma.

Metastasis is therefore a selective, sequential process. The probability of metastasis is never 100% and only a very small percentage of malignant cells survive to form metastases. Stochastic events influence the outcome (e.g. cell arrest in organs which will not support metastases or destruction in the circulation). Metastasis requires that tumour cells complete all of the individual processes that comprise the metastatic cascade. It is not sufficient for tumour cells to excel at any particular aspect(s) of the process, since failure to complete any of the other phases of the process inevitably results in tumour cell death.

1.4.1. The Extracellular Matrix

The extracellular matrix (ECM) is a complex 3-dimensional meshwork of collagen and elastin embedded in a viscoelastic ground substance of glycoproteins, glycosaminoglycans, and proteoglycans. The ECM represents a supportive scaffold that compartmentalizes tissues and acts as a mechanical barrier to invasion. It determines tissue architecture, mediates cell attachment, influences morphogenesis, differentiation and mitogenesis, and serves as a macromolecular filter. The ECM is believed to exert its influence through interactions with specific cell surface receptors. In neoplasia these interactions between cell and matrix may be altered and this in turn may influence cell behaviour. Different ECMs exist which are composed of tissue-specific collagens and glycoproteins, secreted and assembled by the constituent cells of the tissue, and which are responsible for the different structural and physical properties of the tissue (Junker & Heine, 1987; Campbell & Terranova, 1988; McDonald,

1988; Hay, 1989; Timpl, 1989).

1.4.2. The Basement Membrane

The basement membrane (BM) is a highly specialised ECM that further compartmentalizes tissues and is central to both the progression of invasive tumours and haematogenous dissemination (Kramer *et al.*, 1982; Liotta *et al.*, 1977, 1980, 1982; Vlodavsky *et al.*, 1982; Barsky *et al.*, 1983; Martinez-Hernandez & Amenta, 1983; Furcht *et al.*, 1986). The BM prevents the movement of cells between different tissue compartments, and acts as a molecular sieve in regulating the movement of macromolecules and ions (Kanwar *et al.*, 1981). It is primarily composed of BM-specific collagen IV, laminin-nidogen, and heparan sulphate proteoglycans (HSPG) (Leblond & Inoue, 1989; Timpl, 1989). Electron microscopy visualizes the BM as a 20-200nm thick region comprised of two discrete zones; a lamina densa (electron dense) and a lamina lucida (electron lucid). The BM is anchored to connective tissue (CT) via the pars fibroreticularis, a network of collagen VII microfibrils that extend from the lamina densa into the adjacent CT where they terminate in BM-like anchoring plaques interconnected by collagen VII microfibrils.

The cells that remain in close contact with the BM are normally responsible for its synthesis and deposition. Cell interactions with the BM are regulated by specific cellular receptors which bind defined BM components (Hynes, 1987; Lotz *et al.*, 1990), and the close contact between the BM and the cells responsible for its synthesis may represent a form of autoregulation. BM structural integrity is maintained through covalent cross-links and non-covalent interactions between the constituent proteins and proteoglycans resulting in a highly insoluble structure (Kleinman *et al.*, 1983; Martin *et al.*, 1984; Leblond & Inoue,

1989; Timpl, 1989).

1.5.1. Details of Project

The B16 murine melanoma is a standard model for metastasis studies. It has been maintained in culture since it was first isolated in 1954, and from this cell line many variants have arisen which differ in metastatic abilities and organ preference. The variants described in this series of experiments were derived from a poorly metastatic B16F1 melanoma as described by Fidler (1973a), and preferentially colonizes the lungs of syngeneic C57BL mice after intravenous inoculation. From this cell line, variants were derived using cell cloning techniques and serial lung colonization selection methods. From these cell lines the glycosaminoglycans (GAGs), and in some instances proteoglycans (PGs), were partially characterized in order to determine whether qualitative or quantitative differences existed between different cell lines, and furthermore, whether they correlated with metastatic potential. The charge density of the various GAGs was of particular interest since this could influence various cell properties including cell adhesion and ligand binding.

Due to the location of the proteoglycans and GAGs at the cell surface, and their potential to interact with other cell and matrix structures, the effects of agents which altered GAG polymer modifications were used to investigate whether alterations in GAG biosynthesis influenced other cellular characteristics, in particular cell-cell and cell-matrix interactions, and more importantly, metastatic potential. Metastatic cells often exhibit a higher affinity for the adhesive glycoproteins and collagens of the ECM, and an ability to synthesize or utilise matrix degrading enzymes; the B16 melanomas were therefore assayed for these

properties.

The evidence concerning the relationship between cell GAGs and metastatic potential is often conflicting. Different cell types exhibit widely differing GAG profiles and thus no clear picture has been established. It was intended that by characterizing some of the properties of the GAGs synthesized by a series of related B16 melanomas that we might identify and gain an insight into those properties which may be critical for or advantageous to the metastatic phenotype.

2.1.1. Cell adhesion and the extracellular matrix

Cell adhesion to the extracellular matrix (ECM) and other cells is a necessary and critical step for several biological functions. Many normal cell functions including cell spreading, growth and division, migration, proteolysis, differentiation, and morphogenesis rely upon adhesive interactions during their progression. Many of these events are paralleled in the processes of tumour cell invasion and metastasis and specific steps within the metastatic cascade can be identified where cell adhesions are involved. Consequently, tumour cell adhesive interactions with the ECM and other cells are critical to the successful completion of metastasis.

The ECM provides functional and positional information which mediates, regulates, and co-ordinates cell function and behaviour. This requires intimate contact between cells and the matrix, which is mediated via distinct, functional cell surface receptors for matrix proteins. Responses are therefore governed by the cells' ability or inability to adhere to the constituents of the matrix. The ECM is generated by the secretion of glycoproteins, proteoglycans and glycosaminoglycans (GAG) which are assembled locally into organized networks by cells. The composition and organization of the ECM differ between tissues; connective tissue fibroblasts and muscle cells are surrounded by their matrix whereas epithelial cells are in contact with the matrix at one cell surface only.

Tumour cell metastasis involves multiple cell-cell and cell-matrix adhesion events, initiating at the detachment of cells from the primary tumour and terminating upon the formation of metastatic foci in secondary organs. Throughout the metastatic process, tumour cells must also evade the host immune system. Many of these multiple cell-cell and cell-matrix interactions that occur may represent transient phases which facilitate

other processes deemed necessary for metastasis. For example, matrix dissolution requires the initial attachment of a cell to a matrix component, while tumour cell emboli formation via cell-cell interactions is believed to afford protection and improve tumour cell survival in the haematogenous phase of metastasis.

Several antimetastatic therapies have concentrated on trying to block the adhesive interactions formed by tumour cells and some success has been recorded. Intact cell surface bound ECM components can influence artificial metastasis (Terranova *et al.* 1984), and antibodies and peptides which block in vitro cell adhesion can completely inhibit experimental metastasis (Humphries *et al.* 1986b, Iwamoto *et al.* 1987, Vollmers & Birchmeier, 1983a, 1983b). Such results emphasize the importance of cell adhesion and its possible correlation with metastatic potential.

The level of cell-matrix and cell-cell adhesion has been demonstrated to have both positive and negative correlations with the metastatic potential of various tumour cell lines. Organ-derived ECMs enhance the attachment of murine neuroblastoma cells compared to controls (Hutchinson *et al.*, 1989) with enhanced attachment correlating with increased metastatic potential in vivo. Conversely, cells selected in vivo for increased metastatic potential exhibited an enhanced attachment to organ-derived matrices and target organ endothelia (Nicolson, 1982b). This correlation is not universal however. Zoller and Matzko (1989) reported that a reduction in the adherence of fibrosarcoma cells did not alter their metastatic potential, whereas Benke *et al.* (1988) described a murine lymphoma adhesion variant with similar in vitro metastatic potential as its parental, non-adherent cell line but differed in its in vivo growth and dissemination characteristics.

Cell adhesion to target organ endothelium and its underlying basement membrane are amongst the earliest adhesion events in the experimental metastasis model. Tumour cells can exhibit adhesion preferences to these structures with cells being able to distinguish between capillary and lymphatic endothelial cells and subendothelial matrices from different sources and can correlate with metastatic potential (Lichtner *et al.*, 1989; Bevacqua *et al.*, 1990). Alby & Auerbach (1984) reported that tumour cells exhibited higher adhesion to endothelial cells of similar histologic derivation, and Nicolson and Winkelhake (1975) demonstrated that B16 murine melanoma cells exhibit the same heterotypic aggregation preference with partially purified target organ cells as they do in organ colonization in vivo.

In some instances, altered tumour cell adhesion is concomitant with the expression of antigens not present on the surface of normal cells and tissues (Vollmers & Birchmeier. 1983a, 1983b). Since monoclonal antibodies directed against these antigens have no effect upon the attachment of normal or differentiated cells, it would appear that under certain circumstances tumour-associated antigens may contribute to the altered adhesive response.

The ECM exerts profound effects upon cell phenotype. ECM synthesized by differentiated colon carcinoma cells induces poorly differentiated, highly metastatic carcinoma cells to acquire a more benign phenotype (Boyd *et al.*, 1988). The matrix and its components can modulate cell shape and morphology with subsequent changes in gene expression. In the presence of plasma fibronectin, chondrocytes have a polygonal morphology but develop an elongated, fibroblastic morphology in response to cellular fibronectin

(reviewed by Watt, 1986). The influence of ECM on cell shape is therefore dependent not only upon its composition and organization, but also the origin of the cell.

A common approach in the examination of cell-matrix interactions is to study the response of cells to individual ECM components. In this study, having isolated B16 melanoma cell variants of differing metastatic potentials, their abilities to attach to defined ECM components of stromal and BM localization was investigated. The proteins of major interest were fibronectin (reviewed by Humphries *et al.*, 1989) and laminin (reviewed by Hunt, 1989, Campbell & Terranova, 1988) whose biological functions and importance, especially in metastasis, are well documented. Adhesion to the major structural components of ECMs, interstitial collagens I/III and BM collagen IV was also investigated.

SECTION 2. THE EXTRACELLULAR MATRIX PROTEINS LAMININ, FIBRONECTIN AND COLLAGEN

Page
19: 2.1.1. Cell adhesion and the extracellular matrix
23: 2.1.2. Cell adhesion and tumour cell metastasis
24: 2.2.1 Laminin
24: 2.2.2. The structure of laminin
26: 2.2.3. Functional domains of laminin
28: 2.2.4. Laminin as an attachment factor
29: 2.2.5. Laminin and cell motility
30: 2.2.6. The laminin receptor
31: 2.2.7. Laminin and immunosurveillence
32: 2.2.8. Laminin and invasion
32: 2.2.9. Laminin and cell phenotype
33: 2.3.1. Fibronectin
33: 2.3.2. Fibronectin structure
35: 2.3.3. Functional domains of fibronectin
36: 2.3.4. Fibronectin and cell attachment
36: 2.3.5. Fibronectin and motility
37: 2.3.6. Fibronectin and metastasis
38: 2.3.7. The fibronectin receptor
39: 2.3.8. Fibronectin and cell migration
41: 2.4.1. Collagen
41: 2.4.2. Structure of interstitial collagens
42: 2.4.3. The structure of the interstitial collagens I and III
44: 2.4.4. Basement membrane collagen IV
45: 2.4.5. Structure of collagen IV
47: 2.4.6. Collagen IV networks
47: 2.4.7. Collagen IV and cell adhesion

FIGURES & ILLUSTRATIONS:

Page number or adjacent page number in parentheses:
(25): Fig.2a: The structure of laminin
(27): Fig.2b: The functional domains of laminin
(34): Fig.3a: The functional domains of fibronectin
(34): Fig.3b: The cell-binding sites of fibronectin
(45): Fig.4: Models of collagen IV intermolecular association

2.1.2. Cell adhesion and tumour cell metastasis

ECMs exert their biological influences through their constituent proteins, many of which are multifunctional. Matrix proteins can induce a more normal phenotype in transformed cells (Fairbairn *et al.*, 1985; Boyd *et al.*, 1988; Luikart *et al.*, 1990), the synthesis of proteolytic enzymes (Turpeenniemi-Hujanen *et al.*, 1986; Teale *et al.*, 1988), differentiation (Boucaut *et al.*, 1984; Bronner-Fraser, 1986; Foster *et al.*, 1987; Hay, 1989) and the synthesis of differentiation-associated proteins (Bissell *et al.*, 1987; Bloom & Wicha, 1988), in addition to influencing cell growth (Couchman *et al.*, 1982; Bitterman *et al.*, 1983). These cell responses are governed by the ability to adhere to constituents of the ECM via cell surface integrin and non-integrin receptors (Hynes, 1987; Juliano, 1987).

The adhesive proteins of the ECM actively contribute to the pathogenesis of diseases characterized by aberrant adhesion such as cancer. The acquisition of a metastatic phenotype probably involves alterations in the cell's adhesive properties, as observed during neoplastic transformation (Junker & Heine, 1987; Benke *et al.*, 1988). Metastasis involves a series of co-ordinate cell processes many of which depend upon cell adhesion, and agents which interfere with cell adhesion can also block tumour cell metastasis (Vollmers and Birchmeier, 1983a, 1983b, Vollmers *et al.*, 1984; Humphries *et al.*, 1988b; McCarthy *et al.*, 1988).

The ECM can exert profound effects upon cell behaviour. Many tumour cell lines demonstrate reproducible alterations in their adhesive interactions with other cells, matrices, and their individual components (Nicolson, 1988a; Noel *et al.*, 1988; Weiss *et al.*, 1989). In this study, the adhesive responses exhibited by B16 melanoma cells to individual matrix proteins, endothelial cell-derived BM, and to cell monolayers were determined in an

attempt to relate differences with changes in metastatic potential.

2.2.1. Laminin

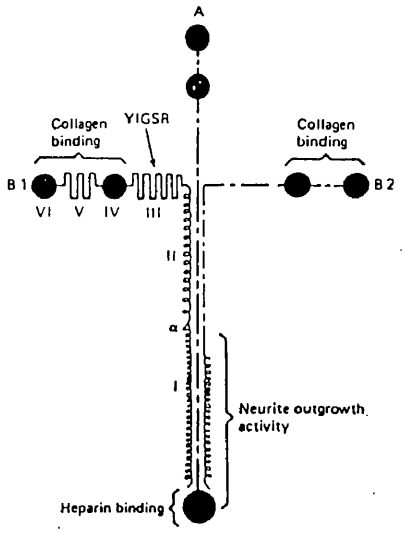
Laminin, initially isolated and characterized by Timpl*et al.*, (1979) from the Engelbrecht-Holm-Swarm (EHS) sarcoma, is a large, multifunctional component of many eukaryotic tissues and is the major adhesive glycoprotein of the basement membrane (BM). Laminin is synthesized by epithelial and endothelial cells, muscle cells and by some types of fibroblasts. The biological properties of laminin include direct effects on cell adhesion and morphology, growth and differentiation, and the migration of normal and neoplastic cells (Kleinman *et al.*, 1985; Hunt, 1989), in addition to a role in tumour cell-immune effector cell interactions (Huard *et al.*, 1985, 1986).

2.2.2. The structure of laminin

The multifunctional nature of laminin is a property related to its large size and multidomain structure (reviewed by Martin & Timpl, 1987)It has a M_r 850-1000KD and is composed of three polypeptide chains (Timpl *et al.*, 1979): one 440KD A-chain and two B-chains, B1 and B2, with M_r 220KD and 230KD respectively. The human laminin polypeptides are encoded on different chromosomes; the A-chain on chromosome 18, the B1-chain on chromosome 7, and the B2-chain on chromosome 1 (Pikkarainen *et al.*, 1987; Mattei *et al.*, 1988; Timpl, 1989). The B-chains are complexed with the A-chain via disulphide bonds. A fourth tissue specific component, the M-chain (230-240KD), has been isolated from placenta but has not been identified in all tissues (Dixit, 1985), and may be responsible for the distinct antigenic sites observed in laminins derived from the BMs of different tissues (Wewer *et al.*, 1986).

The B1-chain of laminin contains 7 distinct domains (Sasaki *et al.*, 1987) and these include a-helical, globular and cysteine-rich repeats with homology to epidermal growth factor (EGF). The B2-chain has homologous domains. The A-, B1- and B2-chains are connected by disulphide bonds to form a cross-like structure of one long and three short arms (fig.2a). The short arms extend 37nm from the centre of the cruciform structure and the long arm 77nm. Two globular domains are present in each of the short arms, but the long arm contains a single globular domain formed exclusively from the carboxy-terminal of the A-chain (Engel *et al.*, 1981). Laminin can self-assemble and polymerize in vitro in a concentration- and Ca^{2+}-dependent manner (Yurchenco *et al.*, 1985; Charonis *et al.*, 1986). Native laminin binds to collagen IV and its procollagen (Charonis *et al.*, 1985; Rao *et al.*, 1985), HSPG and heparin (Sakashita *et al.*, 1980; Laurie *et al.*, 1986), nidogen (Dziadek *et al.*, 1985; Paulsson *et al.*, 1987a; Mann *et al.*, 1989), and possesses at least two different binding sites for cellular laminin receptors (Goodman *et al.*, 1987; Kleinman *et al.*, 1989).

Fig.2a: The Structure of Laminin (reproduced from Sasaki *et al.*, 1987)



Native laminin is heavily N-glycosylated (approx. 12% of its mass) after post-translational assembly of the AB1B2 complex (Chung *et al.*, 1979; Morita *et al.*, 1985a). At least 43 potential N-linkage sites have been described in the A-chain, with a further 13 in the B1-chain and 14 in the B2 chains (Sasaki *et al.*, 1987, 1988). The glycosylation appears to be important for function since agents which interfere with this process result in an abnormally slow rate of laminin secretion (Morita *et al.*, 1985b). Additionally, at least one B16 melanoma cell spreading mechanism requires the interaction between the terminal GlcNAc of laminin oligosaccharide chains and cell surface ß-galactosyltransferase (Runyan *et al.*, 1988; Bouzon *et al.*, 1990).

2.2.3. Functional domains of laminin

Biologically functional fragments can be generated from laminin by limited proteolysis, and this has permitted the identification and characterization of its functional domains (fig.2b). In the central region of the cruciform are two cell-binding domains located in domain III of the B1-chain (Graf *et al.*, 1987; Kleinman *et al.*, 1989). The binding domain for nidogen is also localized to this region (Paulsson *et al.*, 1987a, Timpl, 1989). The long arm of laminin is also reported to support cell adhesion (Goodman *et al.*, 1987), and collagen IV-binding is mediated through the globular domains on the short arms (Rao *et al.*, 1982b, Terranova *et al.*, 1983).

Fig.2b: The functional domains of laminin (reproduced from Liotta, 1986)

Molecule or fragment		Structural features	Biological functions
Whole laminin		Short arms, 35 nm Long arms, 76 nm Rich in α-D-mannopyranosyl residues	Promotes cell attachment, spreading, migration, growth morphogenesis, and metastases
Short arm domain		Globular ends rich in α-D-galactosyl end groups Arms may be composed of more than one type of chain	Globular ends bind to type IV collagen and sulfatides Promotes cell attachment and spreading
Rod-shaped intersection of short arms		Disulfide-bonded "knot" Relatively protease resistant Contains mannose-terminated oligosaccharide units	Contains laminin receptor-binding domain Inhibits cell attachment and metastases
Long arm		May contain α-helix structure Protease labile	Binds heparin sulfate proteoglycan Promotes neurite outgrowth

The long arm globular domain contains a heparin- and possibly a collagen IV-binding domain (Charonis *et al.*, 1986), and the terminal region of the long arm also promotes neurite outgrowth (Edgar *et al.*, 1984; Engvall *et al.*, 1986). The inner rod-like segments from the short arms, which contain the cell-binding domains, consist of cysteine-rich EGF-like repeats that possess growth factor activity (Panayotou *et al.*, 1989). The presence of these multiple cell-binding sites within the laminin molecule suggests that multiple adhesion systems may be employed by cells in order to attach to laminin.

Proteolytic fragments of laminin can mimic the functional properties of the intact molecule. Fragments which contain both the cell-binding and collagen IV-binding domains stimulate experimental pulmonary metastasis by murine tumour cells, and this stimulation is dependent upon the collagen IV-binding domains. Fragments containing only the cell-binding domains inhibit experimental metastasis and cell attachment to BM in vitro (Barsky *et al.*, 1984; Furcht *et al.*, 1986; McCarthy *et al.*,

1988). These results indicate the importance of BM laminin and its interaction with collagen IV in experimental metastasis.

2.2.4. Laminin as an attachment factor

A diverse range of cell types uses laminin as an adhesive substrate, and laminin-binding can influence the rate and level of cell attachment to other matrix proteins including BM collagen IV. Intact laminin has been demonstrated to augment experimental metastasic potential (Terranova *et al.*, 1982, 1984), and in some instances a tumour cell's capacity to utilize laminin as an attachment factor for collagen IV is related to its metastatic potential. This correlation is however not universal (Giavazzi *et al.*, 1982; Terranova *et al.*, 1982; Varani *et al.*, 1987; Ormerod *et al.*, 1988; Hunt & Sherbet, 1989).

The use of cell surface laminin as an attachment factor for collagen IV is relevant to the haematogenous phase in metastasis (Liotta, 1986). Tumour cells may either bind directly to the BM via laminin or use cell surface bound laminin to interact with BM collagen IV. Cell surface laminin has significant consequences for a cell's metastatic potential, since non-metastatic cells which do not express cell surface laminin can acquire an increased metastatic potential through preincubation with soluble laminin prior to inoculation (Malinoff *et al.*, 1984).

The distinctive patterns of dissemination exhibited by many tumour types and the location and survival of metastases appears to be mediated by environmental factors and components of the cell surface of both tumour cells and their target organs (Nicolson, 1982a, 1988a; Fidler, 1989). Differences in the organ colonizing specificity may be related to specific cell adhesion preferences and differing distributions of adhesion proteins

in target organs. Laminin stimulates metastatic tumour cells to adhere and spread, in addition to stimulating cell motility (Junker & Heine, 1987; Fridman *et al.*, 1990). Such adhesion preferences may reflect organ-colonizing preferences: Lewis lung carcinoma cells which preferentially adhere to laminin preferentially colonize liver tissue whereas their lung-colonizing counterparts adhere preferentially to fibronectin (Chung *et al.*, 1988).

2.2.5. Laminin and cell motility

Laminin can influence cell motility, a necessary event for invasion and metastasis (Fligiel *et al.*, 1986). B16 mouse melanoma cells respond to chemotactic and haptotactic gradients of laminin in vitro (McCarthy & Furcht, 1984), and such mechanisms are believed to facilitate and direct tumour cell migration towards regions of matrix dissolution and invasion in vivo.

Motility and the synthesis and secretion of laminin appear to be linked in some tumour systems. Laminin stimulates the motility of B16 melanoma variants apparently through the interaction of laminin with the cell surface (Fligiel *et al.*, 1986). Increased cell motility may be a consequence of laminin synthesis since the levels of intrinsic motility correspond positively with the level of laminin production.

Adhesion to laminin is mediated through specific receptors and antibodies directed against theses receptor inhibit a wide range of processes including laminin-induced migration (Wewer *et al.*, 1987a), attachment to laminin and experimental metastasis.

2.2.6. The laminin receptor

Several cell adhesion mechanism are employed by cells in order to attach to laminin. The 67-70KD non-integrin cell surface laminin receptor has been identified on a wide range of normal and transformed cell types (Terranova *et al.*, 1983) and has a high affinity for its substrate [dissociation constant <2nM (von der Mark & Kuhl, 1985)]. Additional evidence indicates that integrin receptors also mediate attachment to laminin (Kramer *et al.*, 1989; Clyman *et al.*, 1990; Lotz *et al.*, 1990; Sonnenberg *et al.*, 1990). The identification of several distinct laminin-binding proteins may suggest that distinct biological functions may be mediated through the different receptor systems. Some of these laminin receptors possess receptor activity for other matrix proteins: the avian CSAT antigen binds both laminin and fibronectin (Knudsen *et al.*, 1985; Horwitz *et al.*, 1985). Other laminin-binding systems include the sulphated glycolipids (sulphatides) from erythrocytes (Roberts *et al.*, 1985; 1986).

Laminin receptor expression and distribution can be related to cell phenotype. Several malignant epithelial tumours stain more intensely for the receptor and have a more diffuse cell surface receptor distribution than normal tissues or dysplastic lesions (Hand *et al.*, 1985), a patterning which may facilitate migration. BM disruption is often associated with malignant progression (Barsky *et al.*, 1983; Wewer *et al.*, 1987b). Under normal circumstances. laminin receptors are fully saturated through their contact with the BM. However at regions of matrix disruption, receptors may become available and facilitate further interactions between invading tumour cells and the BM. The over-expression of the 67-70KD laminin receptor is a common characteristic in neoplasia (Hand *et al.*, 1985) and may exert some genetic control over the metastatic phenotype and account

for the enhanced cellular response to laminin. Conversely, a reduction in laminin receptors levels appears to impose a less invasive and less metastatic tumour phenotype.

2.2.7. Laminin and immunosurveillence

Successful metastasis requires that tumour cells evade the host immune system, and in particular the lymphocytic natural killer (NK) and natural cytotoxic (NC) cells (Hanna & Fidler, 1980; Hanna, 1985). Laminin affords tumour cells protection from immune effector cell-mediated cell killing by inhibiting their recognition by both NK-cells and NC-cells (Malinoff *et al.*, 1984; Hiserodt *et al.*, 1985a). This appears to be dependent upon the expression of tumour cell laminin receptors. Laminin-like molecules expressed on the surface of NK-cells may mediate their interaction with tumour cells (Hiserodt *et al.*, 1985b), and saturation of tumour cell laminin receptors would seem to protect against this recognition.

In contrast, laminin also appears to promote tumour cell recognition and killing by host macrophages which express both cell surface laminin and its receptor (Wicha & Huard, 1983; Huard *et al.*, 1985, 1986). The expression of cell surface laminin receptors may promote the recognition of and attachment to tumour cells which express cell surface laminin (Huard *et al.*, 1985). ThislLaminin-mediated tumouricidal activity is not universal: although laminin promotes the killing of human melanoma cells it does not promote the killing of carcinoma cells by monocytes and macrophages (Perri *et al.*, 1985). Other factors are evidently required for successful immunological recognition and killing.

2.2.8. Laminin and invasion

The interactions between the epithelium and BM maintains an intact covering for body surfaces, and subsequent cytoskeletal reorganization maintains cell polarity and optimal epithelial cell functioning. The absence of a BM or disruptions in its integrity are often observed in invasive or metastatic lesions (Barsky *et al.*, 1983; Charpin *et al.*, 1986). For some tumours the loss of BM laminin correlates with an increase in malignant potential (Meyer *et al.*, 1985; d'Ardenne *et al.*, 1986). Although BM loss is suggestive of invasion and malignancy it is not an absolute indicator. Rather, the relevence of alterations in BM composition and integrity are dependent upon tumour cell type: both non-invasive, malignant in situ carcinomas and locally invasive non-metastatic basal cell carcinomas possess an intact BM (Barsky *et al.*, 1983).

2.2.9. Laminin and cell phenotype

Cell shape can modulate cell phenotype (Raz & Ben Ze'ev, 1983; Ben Ze'ev, 1985), and laminin modulates both cell-cell and cell-matrix interactions. MCF-7 carcinoma cells exhibit increased cell-cell and decreased cell-matrix interactions when cultured on laminin substrates (Noel *et al.*, 1988). The laminin receptor influences cell shape through its interaction with cytoskeletal actin and the clustering of laminin receptors results in the reorganization of the cytoskeleton (Wicha, 1984; Cody & Wicha, 1986). Cytoskeletal changes influence gene expression (Bloom & Wicha, 1988) and attachment to laminin may induce the synthesis of proteins advantageous for metastasis: the interaction between laminin and its receptor induces the secretion of BM-degrading type IV collagenase in both human and murine cells (Turpeenniemi-Hujanen *et al.*, 1986).

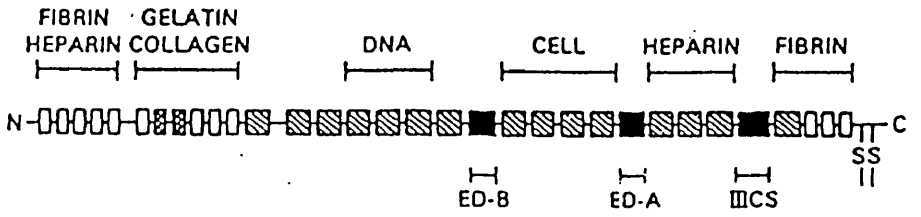
2.3.1. Fibronectin

The ubiquitous adhesive glycoprotein fibronectin is a prominent component of blood and the ECM and is associated with the BM. Fibronectin is synthesized in vitro by a variety of cells and is often incorporated into their pericellular matrix. It has a prominent role in tissue remodelling, cell migration, wound healing, and in embryonic development. Like laminin, fibronectin is a multifunctional protein, with implications for the pathogenesis of cancer (reviewed by Humphries *et al.*, 1989).

2.3.2. Fibronectin structure

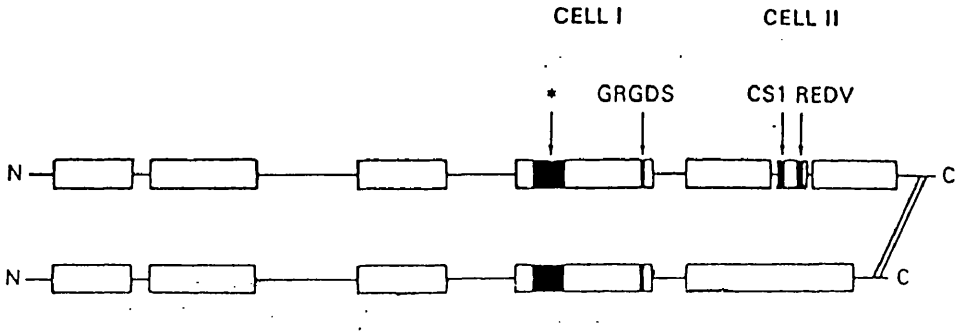
Fibronectin is dimeric, comprised of similar but non-identical subunits that are disulphide bond-linked close to their C-termini. The subunits (M_r 250-280KD) contain multiple but distinct globular, protease-resistant domains (Fig.3a) responsible for its multifunctional nature. These domains are arranged in tandem along each subunit and are connected by protease-sensitive polypeptide sequences (Alexander *et al.*, 1978; Colonna *et al.*, 1978). Fibronectin polymerizes into large disulphide-linked aggregates on the cell surface. The two fibronectin subunits differ in the presence or absence of an alternatively spliced IIICS domain that contains a cell-type specific cell-binding sequence (fig.3b). Each fibronectin subunit contains individual cell-, collagen-, and DNA-binding domains (fig.3a) in addition to two fibrin- and heparin-binding domains located at both the C- and N-terminals (Humphries *et al.*, 1989).

Fig.3a: The functional domains of fibronectin



Fibronectin consists of two similar subunits, which differ in the presence or absence of the IIICS region, linked by two disulphide bridges close to their C-termini. The diagram depicts the positioning of the functional domains of the molecule (reproduced from Humphries *et al.*, 1989).

Fig.3b: The cell-binding domains of fibronectin



The Cell I region, denoting the central cell-binding domain, contains the cell attachment peptide, GRGDS, and the accessory domain (*) necessary for optimal activity. Cell II, the alternatively spliced IICS region, contains the cell-type specific cell-binding domains CS1 and CS5 which contains the REDV peptide.

Fibronectin contains N-linked oligosaccharide chains which appear to function against proteolysis (Olden *et al.*, 1979) and modulate the biological functions of the molecule (Jones *et al.*, 1986). The loss of these carbohydrate chains increases its interaction with gelatin and enhances the attachment and spreading of fibroblasts. The complexing of fibronectin with collagen and proteoglycans is important in the formation of the ECM (Ruoslahti & Engvall, 1980; Hynes, 1985; Ruoslahti & Pierschbacher, 1986). Fibronectin can be used by cells as an attachment factor for other matrix proteins and its interactions with collagen and fibrin are thought to be important for cell migration through blood clots and connective tissues.

2.3.3. Functional domains of fibronectin

The structure and functional domains of fibronectin have been characterized via a combination of limited proteolysis and affinity chromatography. The cell adhesion activity of fibronectin has been restricted to a pentapeptide sequence, GRGDS, that resides within a hydrophobic region of the central cell-binding domain (fig.3b) (Pierschbacher & Ruoslahti, 1984a, 1987; Yamada & Kennedy, 1984, 1985). GRGDS peptides promote fibroblast adhesion and inhibit attachment to intact fibronectin (Akiyama & Yamada, 1985a). The GRGDS-motif interacts with the cell surface fibronectin receptor and the activity of the peptide is dependent upon the maintenance of the central RGD sequence with minor changes abolishing its activity (Pierschbacher & Ruoslahti, 1984a, 1984b, 1987; Hayman *et al.*, 1985; Yamada & Kennedy, 1985). A second active sequence within this domain is required for optimal activity of the GRGDS signal (Obara *et al.*, 1987; 1988).

2.4.4. Fibronectin and cell attachment

GRGDS-peptides exhibit a potent inhibitory effect on a variety of biological processes in vivo (Yamada & Kennedy, 1984). During embryonic development fibronectin stimulates migratory events including nural crest migration, gastrulation, and some aspects of immunological development, and injection of GRGDS-peptides can block these processes (Boucaut *et al.*, 1984; Naidet *et al.*, 1987; Savagner *et al.*, 1986). Fibronectin is also involved in intermolecular interactions with other matrix constituents, including collagen (Ruoslahti & Engvall, 1980; Nagata *et al.*, 1985) and proteoglycans (Laterra *et al.*, 1983; Lark & Culp, 1984).

The IIICS region exhibits a cell-type specific cell-binding activity, whereas the RGD dependent cell-binding activity appears to be specific for fibroblasts. The IIICS region contains two hydrophilic domains (CS1 and CS5) that mediate adhesion with CS1 being the most active. The adhesion promoting activity of CS5 has been attributed to the peptide REDV. The IIICS region is specific for various cell types including murine melanoma cells and peripheral neurons (Humphries *et al.*, 1986a, 1987, 1988a). It is suggested that changes in the splicing of the IIICs region may be important in the regulation of cell adhesion in vivo. The adhesion promoting activities of CS1 and CS5 are additive and may function separately or in tandem to mediate cell interactions with fibronectin.

2.3.5. Fibronectin and motility

Fibronectin is both chemotactic and haptotactic for many cell types (McCarthy & Furcht, 1984) and such responses can be blocked by antibodies directed against fibronectin or its receptor. Fibronectin and high molecular weight GRGDS-containing fragments stimulate cell motility (Ali & Hynes, 1978; Postlethwaite *et al.*, 1981; Seppa *et al.*, 1981; Rovasio

et al., 1983) suggesting that the cell-binding domain is involved in motility responses.

2.3.6. Fibronectin and metastasis

The loss of cell surface fibronectin is a common occurrence for tumour cells cultured in vitro (Hayman *et al.*, 1982), and its addition can restore cellular characteristics associated with a more mature phenotype (Boyd *et al.*, 1988; Noel *et al.*, 1988). Fibronectin will also induce cellular characteristics advantageous for metastatic spread including cell proliferation and motility (Couchman *et al.*, 1982; Bitterman *et al.*, 1983).

The experimental metastasis of murine melanoma cells can be inhibited by GRGDS-peptides (Humphries *et al.*, 1986b) even though such cells attach to fibronectin through the IIICS region (Humphries et al., 1986a, 1987). It would appear that although all cell types do not attach to fibronectin in a GRGDS-dependent manner, GRGDS-dependent adhesion systems are a principal feature of experimental metastasis. The ability of GRGDS-based peptides to inhibit artificial metastasis closely parallels their anti-adhesive activities in vitro (Humphries *et al.*, 1988b), and the anti-metastatic activity, at least, appears to be dependent upon the preservation of the RGD motif.

GRGDS-peptides inhibit the lodgement of B16 melanoma cells in the lungs of experimental animals and promotes their rapid loss from this organ (Humphries *et al.*, 1986b, 1988b). In addition the GRGDS-peptides prolong the survival time of animals inoculated with tumour cells. These observations suggest that fibronectin is involved in the early phases of cell lodgement and extravasation.

GRGDS may influence other important aspects of metastasis. Embolus formation increases the probability of metastasis formation (Fidler, 1973b; Liotta *et al.*, 1976). GRGDS inhibits fibronectin-induced platelet aggregation and subsequently might interfere with the interactions between tumour cells and the constituents of blood in the early stages of organ colonization. GRGDS retains its anti-metastatic activity in animals deficient in platelets or NK-cells (Humphries et al., 1988b), suggesting that these blood components are of little significant importance in GRGDS-mediated inhibition of experimental metastasis. Inhibition of tumour cell arrest and promotion of cell loss suggests that GRGDS interferes with the adhesive interactions that occur between tumour cells and the target endothelium or exposed matrices (Humphries *et al.*, 1986b), or with the subsequent migratory and chemotactic events after extravasation.

2.3.7. The fibronectin receptor

Attachment to and motile responses to fibronectin substrates is mediated through multiple fibronectin receptor species, and antibodies directed against these receptor inhibit such responses (Greve & Gottlieb, 1982; Decker *et al.*, 1984).

The fibronectin receptor is a member of the integrin family of matrix receptors (Buck & Horwitz, 1987; Juliano, 1987), and is a heterodimer comprised of a large ligand-specific α-subunit (140-210KD) non-covalently linked to a smaller ß-subunit (115-135KD). The receptor recognizes and binds to the central cell-binding domain of fibronectin and is sensitive to GRGDS-peptides. The second cell-type specific cell-binding region, IIICS, is also recognized by an integrin (Mould *et al.*, 1990). In

addition to mediating cell adhesion and subsequent adhesion-dependent responses, the fibronectin receptor provides a means of linking the ECM to the cell cytoskeleton through its ability to interact with actin microfilaments (Hynes & Yamada, 1982; Burridge *et al.*, 1988). Several different integrin species have been demonstrated to bind to fibronectin, possibly indicate that this promiscuity may reflect or mediate functional heterogeneity in response to fibronectin. The cellular modulation of the integrin expression and promiscuity, and the affinity of the integrin for its ligand(s) and second messenger systems used to convey the integrin-ligand interaction would permit multiple and varied (and possibly graded) responses to be evoked.

2.3.8. Fibronectin and cell migration

Differences in the distribution and expression of the fibronectin receptor have been observed in different cell populations. Elevated levels of receptor with a diffuse cell surface distribution are often associated with migrating cells, whereas stationary cells express lower levels of receptor found in prominent linear arrays that co-distribute with extracellular fibronectin and intracellular α-actinin (Chen *et al.*, 1986; Duband *et al.*, 1986). Since migration on fibronectin substrates requires the interaction between fibronectin and its receptor, the diffuse receptor distribution and lack of stable cell-substratum contacts on motile cells suggest that highly motile cells exhibit weak and transient interactions with fibronectin that would facilitate movement. The number and cellular distribution of these receptors may be important in assessing the ability of cells to migrate and invade. In addition, cytoskeletal reorganization is a consequence of the interaction between fibronectin and its receptor (Woods *et al.*, 1986) and has the potential to effect cell behaviour.

Higher levels of fibronectin receptor are expressed by both motile normal and neoplastic cells than by their non-motile counterparts (Chen *et al.*, 1986; Duband *et al.*, 1986; Krotoski *et al.*, 1986; Saga *et al.*, 1988), and this may contribute to the invasive ability of these cells. The cell-binding motif GRGDS is not unique for fibronectins. It is present in other matrix proteins including vitronectin, von Willebrand factor, thrombospondin, laminin, and collagens. Since RGD-peptides can interfere with cell attachment to other adhesion proteins (Humphries *et al.*, 1986b), it suggests that such peptides interfere with the potential interactions between other RGDX-dependent receptors and their respective ligands. It may be therefore that GRGDS represents a common adhesion signal in a series of adhesive proteins rather than being exclusive for fibronectin.

2.4.1. Collagen

The collagens constitute the major structural and mechanical components of connective tissues and are a major physical constraint on cell movement. These proteins are classified as a single class of structural proteins on the basis of common properties, and in common with other matrix proteins, they possess the ability to modulate cell phenotype. Subsequently, cell activities which interfere with the physical properties and integrity of collagen matrices may contribute to the process of metastasis.

2.4.2. Structure of interstitial collagens

All collagens are composed of three α-polypeptides which associate to form a polymer containing a distinct triple α-helical region which, in many instances, is responsible for the functional properties of the molecule. Collagens are synthesized as procollagens that, in addition to the triple-helical segments, contain globular domains (10-150KD), many of which are removed during processing and matrix formation. Specific newly synthesized procollagens (e.g. types I-III, V, VII, and XI) also undergo processing to remove portions of α-helix and/or globular domains prior to their incorporation into the ECM (reviewed by Burgeson, 1988). Twelve collagen types have already been identified and are encoded on multiple genes. Within various collagens there also exist small, protease-susceptible, non-triple-helical regions which coincide with regions of increased helical flexibility.

Collagen molecules self-assemble in vivo and in vitro into microfibrils and fibrils of variable diameters and length. Several collagens form banded fibres which differ between tissues in length, diameter, and orientation, parameters that partly determine the physical properties of

tissues. The characteristic banding patterns are reflective of the molecular arrangement of collagen molecules within the fibrils. Most banded collagen fibres contain more than one collagen type (Fleischmajer *et al.*, 1988; Keene *et al.*, 1987), e.g. fibrils of human tendon collagen contain both collagens I and III (Keene *et al.*, 1987). Collagen fibril copolymers have been demonstrated in human skin, aorta, and amnion, whereas other distinct extracellular networks such as BM contain unique collagens.

2.4.3. The structure of the interstitial collagens I and III

Collagen I is the main constituent of skin, bone, tendon, and blood vessels and is synthesized by a variety of cells, including fibroblasts, osteoblasts and smooth muscle cells. Both collagens I and III are similar in structure, being rod-like with a molecular weight of 280KD and with a length and diameter of approximately 295nm and 1.4nm respectively. Each α-chain of collagens I and III contains two functionally and structurally distinct regions; a central triple-helical domain and globular domains at both the carboxy- (C-) and amino- (N-) terminals (Fietzek & Kuhn, 1976). The C-terminal region globular domains of collagens I and III are similar and contain interchain disulphide bonds necessary for proper helix formation. The triple-helical region controls the formation and stabilization of the triple-helix and is responsible for the fibril self-assembly.

Collagen I is composed of two genetically distinct a-chains, α1(I) and α2(I) most commonly polymerized as a hetropolymer, $\alpha1(I)_2\alpha2(I)$, but the homopolymer, $[\alpha1(I)]_3$, has also been detected. Collagens II and III are homopolymers of $\alpha1(II)_3$ and $\alpha1(III)_3$ respectively. The structure of the

triple-helical domain of collagen is based upon a repeated tripeptide motif, Gly-X-Y, which is essential for helix formation. The assembly of collagen fibrils is dependent upon the non-uniform distribution of polar and hydrophobic amino acid residues into separate polar and hydrophobic clusters along the peptide chains of the α-helix, and the characteristic cross-striated patterning of collagen reflects the distribution of the polar residues (von der Mark *et al.*, 1970; Fietzek & Kuhn, 1976; Kuhn & Glanville, 1980).

Fibrillogenesis occurs by a process of self-assembly in the extracellular space and is influenced by other ECM components: collagens I and III contain GAG-binding sites (Koda & Bernfield, 1984), and decorin, the small proteoglycan from tendon, has been demonstrated to influence the formation of collagen I and collagen III fibres (Vogel *et al.*, 1984).

Collagen microfibrils are generated by the aggregation of four collagen monomers in a quarter-staggered array, producing a characteristic 67nm periodicity representative of the banded collagens. The 67nm overlap of collagen molecules in each microfibril permits the best interaction between the polar and hydrophobic domains of neighbouring molecules (Hofmann *et al.*, 1978; Piez & Trus, 1978). The parallel alignment of collagen microfibrils generates collagen fibrils (10-100nm) which also show 67nm periodicity in cross-striations.

The level of collagen fibril organization is refelctive of tissue type and structural properties. In skin, parrallel, mechanically stable networks of collagen I fibrils exist, but in bone fibrils are organized into more irregularly packed 3-dimensional networks. The ability to form different

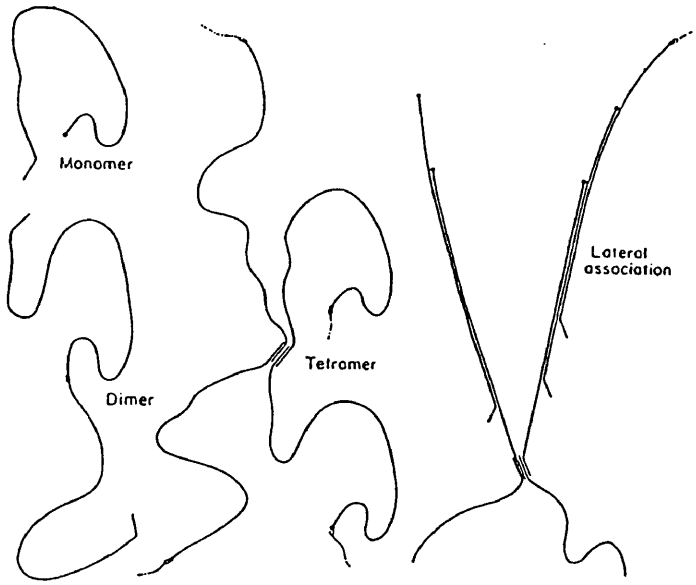
macromolecular structures indicates that this level of fibril organization is regulated by mechanisms other than only amino acid sequence, and interactions with other connective tissue components may be involved in this process. The ratio of collagen I to collagen III appears to influence the biomechanical properties of tissues; collagen I appears to contribute to mechanical stability and collagen III to the elasticity and flexibility of connective tissues. Alterations in these ratios has pathological consequences in various connective tissue diseases including Marfan's syndrome (Krieg & Muller, 1977) and Ehlers-Danlos syndrome type IV (Pope *et al.*, 1975).

2.4.4. Basement membrane collagen IV

The BM is a thin, specialized ECM that surrounds epithelial tissues, nerves, adipocytes, and muscle, and contains a common set of components: type IV collagen, laminin/nidogen and HSPG. It is the first ECM to appear during embryogenesis and is believed to create a barrier to control embryonic cell segregation and differentiation. In the adult, the BM serves as a molecular filter in capillaries and glomeruli and as a scaffolding to maintain normal tissue form during regeneration and growth.

The BM is composed of a dense network of fine cords (Inoue *et al.*, 1983). Highly cross-linked collagen IV provides the basic scaffolding for this dense network upon which the other BM components are assembled. The collagen IV network exists as open, planar or condensed polygonal structures (Yurchenco *et a*l., 1986) which form by the end-to-end and lateral alignment of collagen IV monomers (fig.4).

Fig.4: Models of collagen IV intermolecular association



Collagen IV monomers (upper left) may associate end-to-end at their C-terminal globular domain to form dimers or at their N-terminal 7S domain to form tetramers via a parallel/anti-parallel association of the 7S domains. Tetramers are capable of lateral associations, with such associations being usually limited to short segments of the associating molecules. Utilization of these three means of intermolecular associations generates the observed collagen networks described (reproduced from LeBlond & Inoue,1989).

2.4.5. Structure of collagen IV

Collagen IV does not form banded collagen fibres. The collagen IV monomer is a heteropolymer, $\alpha1(IV)_2\alpha2(IV)$, with Mr 540KD (Mayne & Zettergren, 1980; Trueb *et al.*, 1982) and is organized into a 390nm long triple-helical rod terminating at the C-terminal in the globular domain, NC1, and at the N-terminal in a 30nm long triple-helical domain, 7S. Each of these structures is involved in oligomerization (Timpl *et al.*, 1981). The NC1 domain contains minor amounts of two other components, M2 and M3, which appear to correspond to analogous segments of two other collagen

polypeptides, α3(IV) and α4(IV). The M2 component contains the Goodpasture epitope, a cryptic epitope for some human autoantibodies. In human collagen IV the α1(IV) and α2(IV) chains respectively contain 21 and 23 interruptions in their Gly-X-Y triplets which in most cases match each other in position along the peptides, and give rise to a total of 25 imperfections distributed along the length of the triple-helix (Babel & Glanville, 1984; Brazel *et al.*, 1987, 1988; Killen *et al.*, 1987). These interruptions are believed to be of functional importance by increasing the flexibility of the collagen IV helix (Hofmann *et al.*, 1984). Disulphide linked intra- and inter-molecular links occur along the length of the triple-helix and 7S domain. Intramolecular cross-links are mainly formed in the more central region close to the site of action for type IV collagenase. The α2(IV) chain contains a disulphide-bonded loop of 22 amino acids not present in the α1(IV) chains.

The 7S and NC1 domains of the collagen IV molecule are both critical for its polymerization (Timpl *et al.*, 1981). The interactions between NC1 domains generate dimers, and 7S domains tetramers (fig.4), which are rapidly stabilized in situ by covalent cross-links (reviewed by Leblond & Inoue, 1989, Timpl, 1989), and inhibition of the cross-linking enzyme, lysyl oxidase, by lathyritic agents, e.g. ß-APN, increases the solubility of collagen IV (Kleinman *et al.*, 1983).

The folding of the triple-helix appears to start from the C-terminal and therefore NC1 domains may be involved in the selection and alignment of newly synthesized α1(IV) and α2(IV) chains. Interactions between 7S domains produce dimers which can interact with similar dimers in an anti-parallel/parallel manner to generate tetramers. Collagen fibrils are

stabilized by lysine-derived intermolecular cross-links which generate the very high tensile strength necessary for it to function as a major structural element in connective tissues (reviewed by Ricard-Blum & Ville, 1988).

2.4.6. Collagen IV networks

Collagen IV in situ is assembled with other BM proteins into highly insoluble supramolecular aggregates, and partially extracted BM is a complex, 3-dimensional network of highly branched filaments. Dimers and tetramers formed by NC1 and 7S domains have the potential to generate open but unlimited network (Timpl *et al.*, 1981; Tsilibary & Charonis, 1986). The triple-helix contains several NC1 recognition sites,and lateral associations can occur along the major portion of the triple-helix. A maximum of four collagen IV molecules can intersect the triple-helix through their NC1 domains at a distance of 100-200nm to generate irregular polygonal networks. Isolated NC1 disturb network formation by binding to the triple-helix at a regular distance of 100nm (Tsilibary & Charonis, 1986).

Type IV collagen is incorporated directly into the BM without enzymatic processing to a less soluble form as observed with other collagen types. In addition to providing the large, supportive structures for the BM and its components, two cell-binding domains have been identified in addition to binding domains for laminin and HSPG.

2.4.7. Collagen IV and cell adhesion

Laminin is recognized as being the major cell adhesive component of the BM but a comparable role has been indicated for collagen IV. Cell

attachment requires an intact triple-helix and two potential cell-binding sites have been suggested at the C- and N-terminals (reviewed by Timpl, 1989). The triple-helix also contains many RGD sequences (Brazel *et al.*, 1987, 1988) but their involvement in cell adhesion is unconfirmed, although such sequences in gelatin can be utilized by integrins.

In addition to promoting tumour cell adhesion and spreading, collagen IV also stimulates cell growth (Junker & Heine, 1987). However many cell types utilize other matrix proteins in order to bind to collagen IV. Cells which when presorbed with laminin exhibit an increased binding to collagen IV in vitro and increased organ colonization in vivo, whereas other cell types exhibit an increase in binding to collagen IV in the presence of fibronectin (Hunt & Sherbet, 1989).

SECTION 3. PROTEOGLYCANS AND GLYCOSAMINOGLYCANS

Page
49: 3.1.1. Glycosaminoglycans and proteoglycans
49: 3.1.2. Structure of proteoglycans
53: 3.1.3. Biological functions of proteoglycans
54: 3.1.4. Proteoglycans and cell growth
55: 3.1.5. Proteoglycans and the extracellular matrix
57: 3.1.6. Glycosaminoglycans and cell transformation

FIGURES & ILLUSTRATIONS:

Page number or adjacent page number in parentheses:

(50): Fig.5: The structure and distribution of glycosaminoglycans

3.1.1. Glycosaminoglycans and proteoglycans

Proteoglycans and glycosaminoglycans are constituents of the ECM and the cell surface and have important implications in a wide range of cellular and physiological processes ranging from cell-cell and cell-matrix interactions, growth and motility to matrix organization and stabilization. The importance of normal proteoglycan biosynthesis and functioning is further emphasized since few naturally occurring mutations affecting proteoglycan biosynthesis have been recorded.

3.1.2. Structure of proteoglycans

Proteoglycans are composed of a protein core with one or more covalently attached glycosaminoglycan (GAG) chains. This somewhat loose biochemical classification groups together a wide range of molecules which differ in both functions and properties. The GAG chains are linear, polyanionic polysaccharides composed of repeating disaccharide subunits. These are negatively charged at physiological pH due to the presence of sulphate- and carboxyl-groups within the disaccharide subunits (reviewed by Gallagher *et al.*, 1986; Ruoslahti, 1988a).

Four glycosaminoglycan species have been identified: .1. dermatan sulphate (DS), chondroitin-4-sulphate (C4S), and chondroitin-6-sulphate (C6S), .2. heparin and heparan sulphate, .3. keratan sulphate (KS) and .4. hyaluronic acid (HA). GAG species differ in the composition of their constituent dissacharides which are composed of alternating uronic acid and N-acetylated amino-sugar moieties (fig.5). Chondroitin sulphate proteoglycans (CSPG) are constituents of both the ECM and the cell surface although they tend to be distributed primarily in connective tissues. The heparan sulphate proteoglycans (HSPG) are most commonly found as constituents of the cell surface and the basement membrane. The only

non-sulphated GAG species, hyaluronic acid, is a major component of the ECM and is found as a free GAG not associated with core protein.

Fig.5. The structure and distribution of glycosaminoglycans (reproduced from Molecular Biology of the Cell. Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J. D. Garland Publishing, Inc. New York, 1989)

Glycosamino-glycan	Molecular Weight	Repeating Disaccharide $(A\text{-}B)_n$		Sulfates per Disaccha-ride Unit	Linked to Protein	Other Sugar Compo-nents	Tissue Distribution
		Monosaccharide A	Monosaccharide B				
Hyaluronic acid	4,000 to 8×10^6	D-glucuronic acid	*N*-acetyl-D-glucosamine	0	–	0	various connective tissues, skin, vitreous body, cartilage, synovial fluid
Chondroitin sulfate	5,000–50,000	D-glucuronic acid	*N*-acetyl-D-galactosamine	0.2–2.3	+	D-galactose D-xylose	cartilage, cornea, bone, skin, arteries
Dermatan sulfate	15,000–40,000	D-glucuronic acid or L-iduronic acid	*N*-acetyl-D-galactosamine	1.0–2.0	+	D-galactose D-xylose	skin, blood vessels, heart, heart valves
Heparan sulfate	5,000–12,000	D-glucuronic acid or L-iduronic acid	*N*-acetyl-D-glucosamine	0.2–2.0	+	D-galactose D-xylose	lung, arteries, cell surfaces, basal laminae
Heparin	6,000–25,000	D-glucuronic acid or L-iduronic acid	*N*-acetyl-D-glucosamine	2.0–3.0	+	D-galactose D-xylose	lung, liver, skin, mast cells
Keratan sulfate	4,000–19,000	D-galactose	*N*-acetyl-D-glucosamine	0.9–1.8	+	D-galactos-amine D-mannose L-fucose, sialic acid	cartilage, cornea, intervertebral disc

Proteoglycan display considerable chemical and functional heterogeneity. GAGs are linked to core proteins through the neutral trisaccharide sequence Xylosyl-Galactosyl-Galactose (Xyl-Gal-Gal) which functions as a primer for the biosynthesis of the GAG chains. Synthesis is initiated by the covalent linkage of xylose to a core protein serine residue which then act as an acceptor for the successive transfer of a further two galactose units. Subsequent polymer formation involves the transfer of alternating residues of uronic acid and amino-sugar onto the non-reducing end of the growing GAG chain.

Proteoglycan biosynthesis is readily modulated in vitro. ß-D-xylosides substitute for Xyl-Gal-Gal primers and disrupt proteoglycan biosynthesis (Galligani *et al.*, 1975, Robinson *et al.*, 1975). The presence of ß-D-xylosides within cell cultures increases the number of available primers and results in an increase in the amount of total GAG synthesized with a concomitant decrease in the molecular size of the GAG chains (Schwartz *et al.*, 1974; Schwartz, 1979). Inhibition of protein synthesis which reduces the number of available core proteins results in an increase in the molecular size of the GAG chains synthesized (Kimura *et al.*, 1981). The mechanisms that govern chain length are however not fully understood but it is evident that the availability of Xyl-Gal-Gal primer is critical.

Following GAG chain elongation, chemical heterogeneity is introduced into newly polymerized chains via a series of polymer modifications which confer biochemical heterogeneity. Many of the biological properties of GAGs are related to their charge density and a major determinant of this is the degree of polymer sulphation. The level of polymer sulphation varies between and within each GAG species (fig.5). In general, chondroitin sulphates (CS-GAG) usually possess only one sulphate group per repeating disaccharide but "oversulphated" (e.g. DS-GAG) and "undersulphated" CS-GAG do occur (Razin *et al.*, 1982; Ito *et al.*, 1982; Kolset *et al.*, 1983). Heparan sulphates (HS-GAG) and heparin tend to be more sulphated than CS-GAG. Heparin and HS-GAG undergo similar polymer modifications that are often extensive and highly characteristic: N-acetylglucosamine (GlcNAc) can be O-sulphated on C3 and deacetylated on C2. The subsequent N-sulphation of C2 is exclusive to heparin and HS-GAG and generates a highly sulphated disaccharide. In some instances the positions and levels of sulphation are identified with particular biological functions; the

O-sulphation of glucosamine (GlcN) at C3 appears to be essential for a pentasaccharide sequence from heparin and HS-GAG with affinity for the protease inhibitor anti-thrombin III (Atha *et al.*, 1984; Pejler *et al.*, 1987). The factors that control the processes of polymer modification are unknown, but the extent of sulphation may be dependent upon the substrate specificity of the particular sulphotransferases involved in the reactions (Nakanishi et al., 1981). In heparin biosynthesis, the enzyme responsible for N-deacetylation may have a role in regulating the overall degree of sulphation (Riesenfeld *et al.*, 1982). With the possible exception of N-sulphation, polymer modifications are incomplete; all potential sites will not undergo modification thereby contributing to the structural heterogeneity of GAG chains.

Heparins are believed to be synthesized exclusively by mast cells but other cell types synthesize heparin-like molecules (Marcum *et al.*, 1986). Heparin is synthesized as a unique proteoglycan consisting of more than ten 100KD polysaccharide chains attached to a core protein that is predominantly composed of glycine and serine residues (Hook *et al.*, 1984). Heparan sulphate proteoglycan (HSPG) has a ubiquitous distribution and is present in the BM and as a component of the cell surface. It contains the same structural units as those found in heparin but is generally less sulphated.

Proteoglycans are associated with the plasma membrane via several mechanisms. They have been identified as integral membrane proteins intercalated into the membrane via hydrophobic core protein domains. Peripherally-associated proteoglycans interact with other plasma membrane constituents through the core protein or GAG chains via

salt-labile interactions (Fransson, 1987). Peripheral proteoglycans may be derived from intercalated proteoglycans via proteolytic processing of the core protein. Thirdly, proteoglycans can be covalently linked to plasma membrane inositol-phospholipids (Fransson, 1987).

3.1.3. Biological functions of proteoglycans

Extracellular proteoglycans and GAG interact with ECM proteins to not only generate the matrix, but also to function in its stablization and organization. The majority of proteoglycans and GAG in the ECM probably do not interact directly with cells, but those in close proximity may interact with the cell surface. Consequently cell surface proteoglycans have the potential to interact with components of the ECM and therefore act as receptors for various ECM molecules (Maciag *et al.*, 1984; Gordon *et al.*, 1987; Saunders & Bernfield, 1987), thereby contributing to the ECM-dependent regulation of cellular function.

Proteoglycans are potential candidates in connecting the ECM to the cytoskeleton (reviewed by Rapraeger *et al.*, 1987). Murine mammary epithelial cell HSPG has been shown to interact with polymerized actin microfilaments (Rapraeger *et al.*, 1986), and actin and HSPG co-distribute in rat fibroblast cultures with the HSPG being enriched within focal adhesions (Woods *et al.*, 1984).

The biological functions of many proteoglycans have yet to be identified. A fibroblast HSPG species is identical to or structurally related to the transferrin receptor (Fransson *et al.*, 1984; Coster *et al.*, 1986). The receptor for TGF-ß1 has been identified as a HSPG (Segarini & Seyedin, 1988) and the TGF-ß type III receptor (betaglycan) a CS/HSPG copolymer

(Esko, 1991). The haematopoietic and epithelial cell adhesion molecule CD44, a putative metastasis molecule, is a chondroitin sulphate proteoglycan (CSPG). When overexpressed by non-metastasizing pancreatic adenocarcinoma cells, this molecule confers full metastatic behaviour (Gunthert *et al.*, 1991). CD44 has also been demonstrated to play a role in collagen I-mediated melanoma cell motility and invasion (Faassen *et al.*, 1992), and to bind the heparin-binding domain of fibronectin (Jalkanen & Jalkanen, 1992). Many of those proteoglycans whose functions have been identified and characterized are reviewed by Esko (1991) and Birchmeier *et al.*, 1991).

3.1.4. Proteoglycans and cell growth

Specific heparan sulphate species are capable of modulating cell growth, and certain growth factors, including EGF, bFGF and aFGF are known to be sequestered by heparin-like molecules (Harper & Reisfeld, 1983; Maciag *et al.*, 1984; Gordon *et al.*, 1987; Levine *et al.*, 1989). Other species influence cell growth more directly with growth control being linked to the synthesis of specific HSPG species. Endothelial cell-derived HSPG is a potent inhibitor of smooth muscle cell proliferation (Castellot *et al.*, 1982, Benitz *et al.*, 1990) and is antigenically similar to basement membrane HSPG (Benitz *et al.*, 1990). A cell surface HSPG synthesized and released by confluent hepatocytes synthesize inhibits cell proliferation when internalized (Ishihara *et al.*, 1987). Fedarko & Conrad (1986) demonstrated that the growth inhibitory activity of a nuclear fraction HS-GAG was related to its enrichment in a unique chemical structure. High concentrations of exogenous heparan sulphate are also growth inhibitory for fibroblasts (Winterbourne & Salisbury, 1981), and normal hepatocyte heparan sulphates restore hepatoma density-dependent growth inhibition

(Kawakami & Terayama, 1981). In contrast, exogenous heparan sulphate or heparin promotes endothelial cell proliferation and their long-term culture (Hook *et al.*, 1984).

Evidence strongly suggests a role for GAG and proteoglycans in growth regulation, but they do not appear to be essential for cell division, at least for all cell lines, since cells deficient in GAG do not exhibit abnormal growth patterns in vitro (Esko, 1991), although this observation may be an artefact of in vitro culture.

3.1.5. Proteoglycans and the extracellular matrix

The polyanionic nature of GAGs conceivably permits their interaction with molecules which are positively charged at physiological pH. Accumulated evidence therefore implicates a major role for cell surface proteoglycans in both cell adhesion processes and as receptors for circulating and immobilized molecules. These activities are not primarily the domain of the GAG side-chains. Cartilage CSPG binds to HA through a specific core protein domain (Ruoslahti, 1988a), but other matrix proteins possess positively charged heparin-binding domains. HA-binding proteins, including CD44, have been detected on both normal and transformed cells. These may play a role in endocytosis (Hook *et al.*, 1984), but more importantly, they may help create and maintain highly hydrated, HA-rich, microenvironments that facilitate cell movement and differentiation.

The interactions between GAG and matrix proteins are believed to have functional importance in the structural organization and biological activity of the ECM. HS-GAG may promote matrix stabilization and be indirectly involved in the maintenance of cell differentiation and growth

control through sequestrating and localizing growth factors (Spooncer *et al.*, 1983; Gospodarowicz *et al.*, 1987), or by maintaining gap junctional cell communication (Spray *et al.*, 1987). Cell motility is also influenced by GAG. Heparin and HS-GAG stimulate tumour cell invasion of artificial matrices (Robertson *et al.*, 1989), and CD44-like molecules are implicated in K1735 murine melanoma motility and invasion responses to collagen I (Faassen *et al.*, 1992).

In addition to integrin-like functions, proteoglycan-matrix protein interactions modulate the physiology and integrity of connective tissues. The orderly arrangement of proteoglycans along the length of collagen fibres modulates fibre diameter and subsequently strength (Vogel *et al.*, 1984; Rapraeger *et al.*, 1987), and additionally, GAG-collagen interactions contribute to the interactions between collagen and fibronectin (Ruoslahti & Engvall, 1980).

Fibronectin and laminin have an affinity for sulphated polysaccharides and, in vivo, HSPG is in close association with these proteins. HSPG augments the affinity of fibronectin for its cell surface receptor and co-distributes with actin and fibronectin during cell spreading (Woods *et al.*, 1984). HSPG is also critical for the reorganization of actin microfilaments into stress fibres and the subsequent formation of focal adhesions (LeBaron *et al.*, 1988). It is possible that proteoglycans constitute initial mediators of cell adhesion responses and modulate the strength of interaction between integrins and their ligand. By virtue of their ability to occupy a large volume of space relative to their size, and since their chemical interactions are primarily charge based, they may initiate cell adhesion by acting as an initial weak link between cell and

adhesive substrate, thereby permitting the appropriate integrin receptors time to fully interact.

3.1.6. Glycosaminoglycans and cell transformation

The relevance of the reported qualitative and quantitative changes in GAG biosynthesis upon malignant transformation of cells is somewhat unclear. Several investigators have reported that upon malignant transformation, GAG biosynthesis shifts from the production of sulphated GAG species to that of HA (Kimata *et al.*, 1983; Bouziges *et al.*, 1990), a modification that has important implications for cell locomotion and matrix properties. Cell differentiation however is often associated with increased proteoglycan biosynthesis (Baldwin *et al.*, 1989). Other investigators have reported the synthesis of undersulphated HS-GAG upon cell transformation (David & van den Berghe, 1983; Robinson *et al.*, 1984), an observation that has also been reported for cell lines selected for high tumourigenicity (Winterbourne & Mora, 1981). These structural alterations have the potential to modify the interactions between GAG and other pericellular molecules. Virally transformed cells that synthesize undersulphated HS-GAG fail to assemble a pericellular matrix even though they synthesize and secrete fibronectin, laminin and procollagen (Hook *et al.*, 1984), and cells chemically-induced to synthesize undersulphated CSPG (Walters *et al.*, 1988) or HSPG (Brauer *et al.*, 1988; Keller *et al.*, 1989) also fail to assemble a matrix. These reports suggest that GAG-matrix interactions may be mediated via discrete saccharide sequences. This is further supported by the observation that chemically modified heparins and undersulphated HS- and CS-GAG compete less effectively in cell adhesion assays.

Undersulphated HS-GAG has a lower affinity for fibronectin (Robinson *et al.*, 1984), and consequently, GAG alterations are often associated with changes in the cell adhesive apparatus and properties of cells (Couchman *et al.*, 1988; Keller *et al.*, 1988; LeBaron *et al.*, 1988). The adhesion and spreading of fibroblasts and endothelial cells appears to involve a HSPG-dependent mechanism, but neither HSPG or CSPG is necessary for maintaining the adhesion (Laterra *et al.*, 1983; Gill *et al.*, 1986). The degree of HS-GAG sulphation, however, does not appear to influence the processes of cell adhesion and spreading (Brauer *et al.*, 1988).

A role for proteoglycans in tumorigenesis was suggested by Esko (1991) who, with co-workers, observed that GAG-deficient CHO cells were non-tumorigenic; mutants expressing reduced amounts of the wild-type levels of proteoglycan (≥10%) were tumorigenic. Subsequently, HS-GAG was shown to be necessary and sufficient for tumour formation since only mutants that synthesized HS-GAG were tumorigenic whereas mutants that exclusively synthesized CS-GAG were not.

In this study the B16 melanoma cell lines were analysed for qualitative and/or quantitative differences in GAG synthesis which might be related to metastatic potential, and encompassed both the heterogeneous and highly clonal cell populations.

SECTION 4. MATRIX DISSOLUTION

Page
59: 3.2.1. Tumour-cell mediated ECM proteolysis
59: 3.2.2. Proteolysis and cell migration
60: 3.2.3. Basement membrane degradation
a) collagenase IV
b) heparanase
c) other proteinases
65: 3.2.4. Proteolysis and cell growth
65: 3.2.5. The influence of the ECM

3.2.1.Tumour cell-mediated ECM proteolysis

As previously discussed, many investigators have observed that the ECM of malignant cells is more sparse and disorganized. This often a consequence of the defective synthesis of matrix components (Barsky *et al.*, 1983; Meyer *et al.*, 1985; Charpin *et al.*, 1986; d'Ardenne *et al.*, 1986), the synthesis of components with altered properties (Hook *et al.*, 1984; Brauer *et al.*, 1988; Walters *et al.*, 1988), and/or enhanced cellular proteolytic activity (reviewed by Moscatelli & Rifkin, 1988; Tryggvason *et al.*, 1987). Several cell- and matrix-associated proteinases are capable of degrading ECM components with a resultant loss in its mechanical and structural integrity. This cell-mediated destruction or remodelling of the matrix and subsequent invasion into the zone of matrix modification is integral to the process of tumour cell invasion and metastasis (Liotta, 1986).

Matrix remodelling and matrix invasion are normal physiological processes. Within the ECM are latent proteinases are utilized by both normal and malignant cells during such processes. Malignant cells can directly initiate matrix destruction through the secretion of specific matrix-degrading enzymes (Chen & Chen, 1987), and the production of tumour cell-derived proteinases can correlate with metastatic potential: these include lysosomal hydrolases such as cathepsins (Sloane *et al.*, 1981; Rozhin *et al.*, 1990), collagenases (Liotta *et al.*, 1980, 1982; Reich *et al.*, 1988b), and plasminogen activator (PA) (reviewed by Saksela & Rifkin, 1988). Other neoplastic cells also initiate matrix proteolysis indirectly through stimulating adjacent normal cells to secrete the necessary proteinases (Biswas & Nugent, 1987; Prescott *et al.*, 1989).

3.2.2. Proteolysis and cell migration

Matrix proteolysis liberate factors which are are both chemokinetic and

chemotactic for cells. This includes not only proteolytic fragments derived from matrix proteins but also matrix-associated proteinases.

The release of degradation products during the process of invasion may direct tumour cell migration towards regions of matrix dissolution by both chemotactic and haptotactic mechanisms. Degradation products derived from the major ECM structural proteins, collagen I and collagen IV, have been demonstrated to be chemotactic and chemokinetic for hepatoma cells (Nabeshima *et al.*, 1986). Similarly, the degradation products from elastin, in particular the hexapeptide VGVAPG which is recognized by a class of high affinity cell surface receptors (Blood *et al.*, 1988), are also chemotactic, and the chemotactic and haptotactic properties of fibronectin and laminin and their degradation products are well documented (McCarthy & Furcht, 1984).

3.2.3. Basement membrane degradation

The BM is a principle regulatory barrier to tumour cell metastasis. The role of enzymes able to degrade this structure have received much attention in studies of cell invasion and metastasis.

a) Collagenase IV

The metalloproteinases (MMP) family includes the collagenases which degrade the major structural component of the ECM. The MMP family is enzymatically active at neutral pH, has tightly bound Zn^{2+}, and requires Ca^{2+} for activity (reviewed by Khokha & Denhardt, 1989; Emonard & Grimaud, 1990). Interstitial collagenase (MMP-1) degrades native collagens I, II, and III, cleaving the triple helix at a point located 1/4 of the distance from the C-terminus. In contrast, collagenase IV (MMP-2)

degrades native collagen IV at a specific point 1/4 of the distance from the N-terminus, and will degrade denatured fibrillar collagens (I, II, III). The resultant loss of collagen tertiary structure that arises from the actions of collagenases permits other proteinases to participate in further collagen degradation.

Collagenase IV appears to be highly important in the invasion of the BM. Tumour cell-derived enzymes appear to be responsible for the degradation of both BM collagen IV and HSPG. Tumour cell invasion of reconstituted BM gels correlates with a highly metastatic phenotype and can be used to select for such cell phenotypes (Tullberg *et al.*, 1989). Elevated serum levels of collagenase IV activity have been associated with metastatic disease in humans (Hashimoto *et al.*, 1988). Collagenase IV is associated with the cell surface (Nakajima *et al.*, 1989) and the level of enzyme activity correlates with the metastatic potential of several tumour types (Garbisa *et al.*, 1988; Reich *et al.*, 1988b), but is not always indicative (Garbisa *et al.*, 1988).

Natural and synthetic inhibitors of collagenase IV which depress tumour cell invasion through reconstituted BM can also reduce the incidence of lung tumours in mice injected with metastatic murine melanoma cells (Reich *et al.*, 1988a). Antibodies directed against collagenase IV (Hoyhtya *et al.*, 1990) and PA (Reich *et al.*, 1988a) also inhibit tumour cell invasion in vitro. Retinoic acid has been observed to inhibit tumour cell-mediated subendothelial matrix and collagen IV degradation and the invasion of reconstituted BM (Nakajima *et al.*, 1989; Emonard & Grimaud, 1990). The in vivo and in vitro anti-tumour activity of retinoic acid may therefore be a consequence, at least in part, of its inhibitory effect on the levels of

collagenase IV.

b) Heparanase

HSPG is believed to be important to the structural integrity of the BM through its potential to bind to and link several other BM proteins (reviewed by Gallagher *et al.*, 1986), and its loss or degradation may result in a loss of this integrity. Heparanase activity correlates with metastatic potential for both murine B16 melanomas and fibrosarcomas (Nakajima *et al.*, 1983; Ricoveri & Cappalletti, 1986), and the ability of both normal and malignant blood-borne cells to extravasate also correlates positively with their ability to degrade subendothelial BM HSPG via cell-derived heparanase (Savion *et al.*, 1987; Vlodavsky *et al.*, 1988).

HS-GAG can be degraded by lysosomal heparanases (Ricoveri & Cappalletti, 1986) and by plasma membrane-associated heparanase (Gallagher *et al.*, 1988). The cell surface heparanase is active at physiological pH (Gallagher *et al.*, 1988) and is ideally situated to directly interact with HSPG. This heparanase may be responsible for modulating cellular interactions mediated via HSPG or release biologically active HS-GAG fragments from the cell periphery, e.g. fragments with growth regulatory activity (Fedarko & Conrad, 1986) or with anticoagulant activity (Marcum *et al.*, 1986). The lysosomal heparanases have an acidic pH optimum and may become involved in matrix degradation when acidic conditions, often associated with metastatic progression (Young *et al.*, 1988; Hill, 1990; Young & Hill, 1990), subsequently arise within tumours.

Heparanase has multiple potential effects upon the nature of the ECM: not only has heparanase the potential to disrupt BM integrity, but it can

modulate the degradative activities of other matrix- and cell-associated proteinases and the availability and activity of growth factors for both normal and malignant cells thereby influencing invasive growth phenomena including angiogenesis.

c) Other proteinases

Plasmin and collagenases are both prominent in the processes of invasion and metastasis, and specific inhibitors of these proteinases are able to block these processes (Reich *et al.*, 1988a, 1988b; Nakajima *et al.*, 1989; Hoyhtya *et al.*, 1990). Plasmin and its latent proenzyme form plasminogen, are bound to both the cell surface and to the ECM where it is immobilized and protected from inactivation. Activated plasmin degrades most matrix components but also activates other latent proteinases including the procollagenases (Salo *et al.*, 1982; Emonard & Grimaud, 1990; Reich *et al.*, 1988b). The resultant amplification or cascade of proteolytic activity functions to increase the efficiency of ECM destruction.

Proteinases are associated with the cell surface of both malignant and normal cells. Urokinase-type plasminogen activator (u-PA) may be of particular importance in the localized destruction of the ECM. u-PA and its related proteinase, tissue-type PA (t-PA), generate activated plasmin from plasminogen, and high affinity u-PA receptors have been identified on the surface of normal and malignant cells (reviewed by Saksela & Rifkin, 1988; Laiho & Keski-Oja. 1989). The u-PA receptor-ligand complex is enzymatically active and able to degrade matrix components. u-PA is distributed beneath cells at cell-cell and cell-substratum contact sites (Pollanen *et al.*, 1988) and the receptor-ligand complexes are localized to the leading front of migrating monocytes (Estreicher *et al.*, 1990). The

direct catalytic action of u-PA on ECM components and the presence of ECM destruction at regions of cell-matrix contact (Fairbairn *et al.*, 1985) suggest that the u-PA receptor may be functionally important in both localizing, and concentrating, the proteolytic activity of u-PA to regions of cell contact and to the invasive front of migrating cells.

Matrix destruction is not singularly a function of tumour cell-derived proteinases. In situ, tumour cell-normal cell interactions can alter both the composition and structure of the matrix and the levels of matrix-degrading enzymes. Such indirect effects may be responsible for the ECM alterations associated with tumour cell invasion. The plasma membrane protein, tumour cell collagenase stimulatory factor (TCSF), is present on certain neoplastic epithelial cells including B16 melanomas. This protein is responsible for the stimulation of collagenase synthesis and secretion by some normal fibroblast cell lines in response to intimate contact with the tumour cells (Biswas & Nugent, 1987; Prescott *et al.*, 1989).

Since tissues and their matrices are heterogeneous in nature and composition, it unlikely that any one enzyme is capable of removing all connective tissue barriers. Subsequently several classes of proteinases are implicated in tumour invasion (Tryggvason *et al.*, 1987). The specific conditions under which each is active make it unlikely that they function simultaneously in the same microenviroment. The physiological conditions within tumours vary through time, requiring cellular adaptative responses. The existence of multiple degradative mechanisms may permit cells to remodel their enviroment or maintain normal proteinase-dependent functions irrespective of changes in physiological conditions.

3.2.4. Proteolysis and cell growth

Proteolytic activity is readily modulated by growth factors (reviewed by Gospodarowicz *et al.*, 1987; Laiho & Keski-Oja, 1989), possibly released from the ECM upon its degradation, and by the presence of pericellular and extracellular proteinase inhibitors (reviewed by Tryggvason *et al.*, 1987; Saksela & Rifkin, 1988; Khokha & Denhardt, 1989, Rozhin *et al.*, 1990). The matrix-degrading activity of cells is balanced by the presence and local concentration of cell-associated and matrix-associated inhibitors. These include tissue inhibitor of metalloproteinases (TIMP) and plasminogen activator inhibitors (PAI) and the levels of these inhibitors are often modulated in a similar fashion by the factors which modulate proteinase activity.

Several growth factors bind to highly sulphated GAG (Gospodarowicz *et al.*, 1987; Roberts *et al.*, 1988), and degradation of ECM heparan sulphate releases growth factors stored within the matrix. GAG-binding growth factors include the angiogens, acidic- (aFGF) and basic fibroblast growth factor (bFGF). aFGF and bFGF are potent endothelial cell mitogens (Gospodarowicz *et al.*, 1987; Vlodavsky *et al.*, 1988) and bFGF is also an important mitogen for B16 melanoma cells. Normal and malignant cells, heparin, HS-GAG and heparanase rapidly liberate FGFs from the ECM. The binding of HS-GAG and heparin to FGFs stabilizes the angiogen's tertiary structure and protects it from inactivation (reviewed by Gospodarowicz *et al.*, 1987; Gordon *et al.*, 1989; Mueller *et al.*, 1989; Sommer & Rifkin, 1989).

3.2.5. The influence of the ECM

The production and secretion of proteolytic enzymes by both normal and

malignant cells is modulated by the ECM (Emonard *et al.*, 1990). Contact with BM laminin apparently initiates tumour cell invasion by stimulating the production of collagenase IV (Turpeenniemi-Hujanen *et al.*, 1986; Emonard *et al.*, 1990). The synthesis of collagenase IV is also stimulated by purified laminin and BM matrigel; BM matrigel and collagen I also stimulate interstitial collagenase activity in normal and malignant trophoblasts (Emonard *et al.*, 1990). Degradative activity is influenced by the composition and assembly of the ECM; matrigel, but not soluble laminin, stimulates interstitial collagenase activity. Somatic cell hybrid studies suggest that collagenase IV activity and the effect of laminin on its secretion may be genetically linked to the metastatic phenotype (Turpeenniemi-Hujanen *et al.*, 1985). Plasmin activation is also influenced by the matrix; the secretion of u-PA and its rate of proteolytic conversion to a more active form is influenced by highly sulphated GAG, in particular heparin (Falcone, 1989; Watahiki *et al.*, 1989).

Heparanase activity (Nakajima *et al.*, 1983; Ricoveri & Cappelletti, 1986) and collagenase IV activity (Poste & Fidler, 1980) have been observed to correlate with the metastatic potential of various B16 melanoma cell lines. In order to determine whether this relationship existed for the B16 melanoma variants developed in this study, and to further characterize these cell lines, cells were assayed for their collagenase and heparanase activities through their ability to liberate ^{3}H-proline (collagenase) and $^{35}SO_4$ (heparanase) from metabolically-labelled CPAE subendothelial matrices.

SECTION 5. MATERIALS AND METHODS

Page
67: 4.1.1. Materials and methods

4.1.1. Materials and methods:

All reagents and chemicals used were of analytical grade.

Cell Culture; The B16 melanoma cells were maintained in complete medium, comprising Eagle's Minimal Essential Medium (MEM) containing 10% foetal calf serum (FCS) or newborn calf serum (NCS), 2mM L-glutamine, 100IU/ml penicillin and 100µg/ml streptomycin, (Life Technologies). Cell culture medium was routinely changed on day 3 and cells passaged on day 4. In order to minimise any potential phenotypic drift within the B16 melanoma cell populations experiments were conducted with cells which had not exceeded passage five. Calf pulmonary arterial endothelial cells (CPAE) were maintained in Dulbecco's Minimal Essential Medium (DMEM) (Life Technologies) containing 10% FCS and the aforementioned concentrations of antibiotics and L-glutamine. All cells were maintained at $37^{o}C$ in a humidified atmosphere of 5% CO_2 in air.

B16 melanoma cells were seeded in $80cm^2$ and $25cm^2$ culture flasks (Nunc) at cell densities which produced $5.0\text{-}6.0x10^6$ cells and $1.5\text{-}2.0x10^6$ cells/flask, respectively, after four days culture.

For cell growth curves B16 cells were plated at very low density in 35mm petri dishes and grown for up to 6 days. Each day cells from triplicate dishes were detached with 0.05% trypsin/0.02% EDTA and the cells counted electronically in a Coulter counter.

Cell treatments; B16 melanoma cell cultures were treated with a series of chemical agents which influenced GAG and PG biosynthesis.

1. Sodium chlorate; 1M sodium chlorate was prepared in PBS pH7.2, and sterilized by filtration. For experiments, cells were cultured in sulphate-depleted complete medium for 4 days, supplemented daily with 50mM sodium chlorate. Sodium chlorate is an inhibitor of phosphoadenosylphosphosulphate (PAPS) biosynthesis. PAPS is the sulphate donor in GAG biosynthesis and its inhibition causes a reduction in GAG charge density.

2. Heparin; A stock solution of 1mg/ml heparin in PBS was filter sterilized and 20μg/ml of heparin added daily to cell cultures.

3. N,N-Dimethylformamide (DMF); B16 melanoma cells were grown in complete medium containing 0.5% DMF (v/v) for 4 days. The medium was replaced routinely on day 3 and on day 2 for GAG studies.

4. 5-Hexyl-2-DeoxyUridine (HUdR/KL103); HUdR/KL103 was a kind gift from Dr. Josef Timar, (Semmelweis Medical University, Budapest). HUdR/KL103 was dissolved in Eagle's MEM or PBS at 50^{o}C for 10mins to a maximum of 5mg/ml, filter sterilized and stored at -20^{o}C. Cell cultures received 50μg HUdR/KL103 per ml of complete medium on a daily basis, and 75μg HUdR/KL103 per ml of medium on day 3 prior to the harvesting of cells on day 4.

5. p-nitrophenyl-ß-D-xylopyranoside (ß-D-xyloside); ß-D-xyloside was prepared as a stock solution of 80mg/ml in dimethyl sulphoxide (DMSO). Under experimental conditions, B16 cells were maintained in complete medium containing 0.5mM ß-D-xyloside for 4 days with fresh medium being added on day 3. For controls, cells were maintained in complete medium containing an equal volume of DMSO only.

B16 Murine Melanomas; All B16 melanoma cells were derived from B16F1 melanoma cells kindly provided by Dr.Ian R. Hart (ICRF, London). The B16F1 melanoma is a poorly metastatic cell line propagated from the parent B16 melanoma. B16F1 cells were used to generate cell populations with a greater lung colonization potential using the experimental metastasis method described by Fidler (1973a). B16F1 melanoma cells were injected into the tail vein of 6 week old syngeneic C57BL mice and after 18-21 days the mice were sacrificed, their lungs removed, and tumours excised for growth in culture. After 3 and 5 such passages through the lungs, the melanoma cells were designated B16F1M3 and B16F1M5 respectively.

Production of B16 melanoma cell clones; B16 clones were derived from the B16F1M3 melanoma by limiting dilution. 100µl of B16F1M3 melanoma cell suspension (5 cells/ml) was plated into each well of a 96-well culture plate. Each well was checked daily, and those containing only one cell per well marked as being monoclonal. Once grown to confluence, the clones were trypsinised and transferred to $25cm^2$ culture flasks for expansion. Stocks of cells were frozen down in liquid nitrogen. The metastatic potential of each clone was assayed by the artificial metastasis/lung colony formation model.

Tumour Spheroids; 2% Agar (Life Technologies) was melted and maintained at 43^oC. The agar was diluted to 0.5% with preheated complete medium and 2-3ml added to 35mm petri-dishes. The agar was allowed to set and equilibrate with complete medium at 37^oC for 2 hours. Dishes were seeded with $8x10^4$ cells and incubated without agitation for 24 or 96 hours for aggregation and growth studies respectively.

Engelbrecht-Holm-Swarm Sarcoma (EHS); The EHS sarcoma is a basement membrane-rich murine tumour and was propagated in DBA mice by the subcutaneous implantion of minced tumour tissue (Orkin *et al.*, 1977). The tissue was harvested when the tumour mass was 2-4cm in diameter.

Experimental Metastasis/Lung Colonization Model; B16 melanoma cells were grown for four days in culture flasks until confluent. Cells were detached with trypsin/EDTA and washed three times with sterile 0.9% NaCl. The cell suspension was adjusted to $5x10^5$ cells/ml and 200µl of suspension injected into C57BL/6 mice via the tail vein. After 18-21 days, the mice were sacrificed and their lungs excised and washed in PBS before being fixed in formyl saline. The number of visible lung tumours were then counted to provide a measure of metastatic potential.

Production of subendothelial matrix; (Vlodavsky *et al.*, 1982). $4x10^4$ CPAE cells were grown to confluence in 35mm petri dishes in 1.5ml of culture medium (DMEM, containing 10%FCS, 2mM L-glutamine, 100IU/ml penicillin, 100µg/ml streptomycin, and supplemented with 5% (w/v) dextran [average M.W. 40kD]). Filter sterilized ascorbic acid in PBS was added daily to a final concentration of 50µg/ml. Cells were cultured for 10-12 days with medium being replaced on days 3 and 7. To harvest the endothelial ECMs, cell layers were washed once with sterile PBS, and then treated for up to 10mins with sterile 0.5% Triton X-100 in PBS (v/v) at room temperature to dissolve the cell layer and leave the underlying matrix intact. The remaining nuclei and cytoskeletons were removed by a brief exposure (2-3mins) to sterile 25mM NH_4OH, followed by 4 washes with PBS. The matrix-coated dishes could be stored in PBS for several weeks at 4^oC.

Radiolabelling of subendothelial matrix; (Vlodavsky *et al.*, 1982). $4x10^4$ CPAE cells were grown to confluence in 35mm petri dishes under the aforementioned cell culture conditions. To radiolabel subendothelial matrix collagens, the culture medium was replaced on day 3 with 1ml proline-depleted medium containing 5% (w/v) dextran, 10% FCS, 100IU/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine and 5µCi/ml ^{3}H-proline. Ascorbic acid continued to be added on a daily basis until the matrices were harvested. On day 7 an additional 1ml proline-depleted medium containing 5µCi/ml ^{3}H-proline was added to the dishes. On day 12, the radiolabelled matrix was prepared as described previously. For radiolabelling matrices with $^{35}SO_4$, the radiolabelling procedure for ^{3}H-proline was slightly modified. The radiolabelling medium was sulphate-depleted DMEM (Life Technologies) containing 5% (w/v) dextran, 10% FCS, 100IU/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine, and up to 30µCi/ml $^{35}SO_4$, and the aforementioned radiolabelling and harvesting procedures were followed.

If dishes containing radiolabelled matrix were to be used immediately they were first preincubated for at least 3 hours to liberate any free radiolabel still present. If not, dishes were stored in PBS at 4^{o}C.

Subendothelial matrix degradation assay; Petri-dishes containing radiolabelled subendothelial matrices were seeded with $1.5\text{-}2.0x10^6$ cells in complete medium and incubated for 2 hours. Non-adherent cells and contaminating serum were removed by gentle washing with sterile PBS. The numbers of adherent cells in control and treated cultures were similar and exceeded 90%. Attached cells were incubated in 1ml serum-free MEM containing 100IU/ml penicillin, 100µg/ml streptomycin,

and 2mM L-glutamine for 48 hours at 37^{o}C after which the medium was removed, centrifuged, and two aliquots taken for scintillation counting. Results were expressed as disintegrations per minute (dpm)/number of cells seeded.

Uronic Acid Determination; In the generation of an ion-exchange chromatographic profile of non-radiolabelled GAGs, the uronic acid content of column fractions was determined using the method of Blumenkrantz & Asboe-Hansen (1973). 200µl samples of eluted GAG were set in crushed ice to which 1.2ml of 12.5mM sodium tetraborate in concentrated H_2SO_4 was added and thoroughly mixed. The samples were then heated to 100^{o}C for 5 minutes and the samples then cooled on ice. 20µl of 0.15% m-hydroxydiphenyl in 0.5% NaOH was added and mixed, and the absorbance measured at 520nm. A standard plot of absorbance at 520nm versus uronic acid concentration was generated by dissolving a range of glucuronic acid (GlcU) standards (0-20µg) in 0.2ml of buffer, and treating accordingly with tetraborate.

Protein Estimation; Protein concentrations were determined using the method of Bradford (1976). 100µl of test solution was mixed with 5ml of filtered Bradford reagent [0.1% (w/v) Coomassie Brilliant Blue G250, 4.5% EtOH (v/v), 10% phosphoric acid], and the absorbance at 595nm measured. A standard curve was generated using a bovine serum albumin (10-100µg).

Basement Membrane and Extracellular Matrix Components; Basement membrane proteins (laminin/nidogen and type IV collagen) were extracted from the EHS sarcoma, type I/III collagens from rat tail tendon, and fibronectin from human plasma.

Fibronectin; Plasma fibronectin was prepared using a modification of the method of Miekka *et al.* (1982). 3.8% tri-sodium citrate-treated human plasma containing 5.0mM benzamidinium chloride was centrifuged at room temperature (10,000g, 30min). The supernatant was applied to a Gelatin-Sepharose affinity column (70mmx25mm) equilibrated with 0.1M NaCl, 50mM 6-amino-hexanoic acid, 3.0mM sodium azide, 50mM tris/HCl, pH7.4, after an initial separation via a Sepharose 4B gel filtration pre-column (40-70mm). Non-specifically bound protein was eluted with 100ml of 1M NaCl, 5.0mM benzamidinium chloride, 50mM 6-amino-hexanoic acid, 3.0mM sodium azide, 50mM tris/HCl pH7.4, followed by 50ml 50mM tris/HCl, 1.0mM EDTA, pH7.4. The Sepharose 4B pre-column was then removed and the bound fibronectin eluted with 3M urea, 50mM tris/HCl, 1.0mM EDTA, pH7.4, and the eluted material monitored at 280nm. All steps were performed at room temperature. Fibronectin prepared in this manner contains only minor impurities. The maximum expected yield was 14-16mg fibronectin per 100ml plasma. The fibronectin could be stored at -20^{o}C in the 3M urea solution. Prior to use, aliquots of the fibronectin stock were dialysed against 0.15M NaCl, 50mM tris/HCl, pH7.4 and the concentration determined using the Bradford assay.

Laminin-Nidogen Complex; The complex was prepared according to the method of Paulsson *et al.* (1987a). Frozen EHS tissue was homogenized in 0.15M NaCl, 50mM tris/HCl, pH7.4, containing 0.5mM N-ethylmaleimide (N-EM), and 0.5mM phenylmethylsulphonylfluoride (PMSF) as protease inhibitors. The homogenate was centrifuged (8000rpm for 20mins in a Beckman centrifuge) and the pellet re-homogenized in the same buffer including 10mM EDTA. The homogenate was stirred for 1 hour at 4oC, centrifuged at 8000rpm for 20mins, and the supernatant collected and stored at -20^{o}C. Aliquots of the supernatant were separated on a Biogel

A5M (BioRad) gel filtration column (26mm x 900mm) equilibrated in 0.15M NaCl, 50mM tris/HCl, pH7.4, 0.5mM N-EM, and 0.5mM PMSF. The elution of proteins was monitored at 280nm. The laminin-nidogen complex which eluted at the void volume (V_0) was pooled and concentrated by ultra-filtration through an Amicon PM10 membrane to approximately 1mg/ml and stored at -20^{o}C.

Type I/III Collagen; Collagen was prepared according to the method of Schor (1980). 1g of rat tail tendon was sterilized in EtOH and dissolved in 250ml of pre-cooled 0.5M acetic acid by stirring for 48 hours at 4^{o}C. The solution was centrifuged (10,000g, for 30mins at 4^{o}C) and the pellet discarded. An equal volume of pre-cooled 20% NaCl (w/v) was added to the supernatant and stirred overnight at 4^{o}C. The collagen-rich, salt-insoluble material was recovered by centrifugation (10,000g, for 30mins at 4^{o}C) and resuspended in 200ml of pre-cooled 0.25M acetic acid before being dialysed against 3-4 changes of pre-cooled 0.1% acetic acid, and was sterilized by centrifugation (20,000g, for 6 hours at 4^{o}C). The collagen concentration was determined by weighing a freeze-dried aliquot of the solution.

Type IV Collagen; DBA mice subcutaneously implanted with EHS sarcoma tissue were fed with 0.3% ß-aminoproprionitrile fumarate (ß-APN) in their drinking water. ß-APN is a lathyritic agent that inhibits the cross-linkage of collagen IV monomers. The extraction of collagen IV was performed using the method of Kleinman *et al.* (1983, 1986). After excision, 200-400g of EHS sarcoma tissue was washed in ice cold 3.4M NaCl, 50mM tris/HCl, pH7.4, 2mM N-EM, 8mM EDTA. The tissue was homogenized in ice cold 0.5M NaCl, 50mM tris/HCl, pH7.4, 2mM N-EM, 8mM

EDTA, and extracted twice by centrifugation (10,000g) with the same buffer at 4°C. This removed the cellular proteins and laminin-nidogen complex. The residue was extracted at 4°C with pre-cooled 2M guanidine HCl, 50mM tris/HCl, pH7.4, 2mM N-EM, 8mM EDTA, 0.1mM PMSF, followed by the same buffer containing 2mM dithiothreitol (DTT). This second extract, containing most of the solubilized collagen IV, was dialyzed against 10 litres of pre-cooled 1.7M NaCl, 50mM tris/HCl, pH7.4, 2mM DTT, 1mM EDTA, 0.1mM PMSF until a precipitate was formed. The precipitate was collected by centrifugation (12,000g, for 1 hour at 4°C), dissolved in pre-cooled 2M guanidine/HCl, 50mM tris/HCl, pH7.4, 2mM DTT, 1mM EDTA, 0.1mM PMSF, and dialysed against pre-cooled 4M urea, 0.25M NaCl, 50mM tris/HCl, pH8.6, 2mM DTT, 1mM EDTA, 0.1mM PMSF. The dialysed collagen IV was mixed with DEAE-cellulose equilibrated with the same buffer and the unbound material collected and dialyzed against the 2M guanidine/HCl buffer. This was then centrifuged at 60,000g for 90mins at 4°C to remove aggregates. The supernatant, reported to contain approximately 80% dimeric collagen IV, was dialysed against 2M urea, 50mM tris/HCl, pH7.4, 2mM DTT, 5mM glycine, 1mM EDTA, 0.1mM PMSF. After a second round of ultracentrifugation, the collagen IV can be further purified on Sephacryl S-1000 (5mmx95mm) equilibrated in the same buffer. Collagen IV was stored at -20°C in 2M guanidine HCl buffer.

Adhesion Assay; 35mm petri dishes were coated with ECM components at 40μg/dish, (an optimum standard concentration used in cell adhesion assays in this laboratory) and treated with 10mg/ml bovine serum albumin (BSA) in PBS to block any unoccupied adhesive sites. Fibronectin and laminin were prepared in 0.15M NaCl, 50mM Tris/HCl pH 7.4, and collagens I and IV in 0.1% acetic acid for coating petri dishes. CPAE and B16 melanoma cells were grown to confluence in 35mm dishes for

cell-cell adhesion assays. B16 melanoma cells cultures which had been grown for four days to near confluency were detached with trypsin/EDTA, washed, and their cell densities adjusted to $2.0x10^5$-$2.5x10^5$ cells/1.5ml in serum-free MEM. Cells were then plated at the above density onto the substrates and incubated at 37°C for set time periods. Non-adherent cells were washed off and the remaining cells detached with trypsin/EDTA and two aliquots taken for electronic cell counting in a Coulter counter. The percentage cell attachment was expressed as the [number of cells adherent at time **x** / number of cells added per dish at time 0 (or alternatively, the number of cells adhered to culture plastic after 2 hours)] x100.

For assays of adhesion to cell monolayers and subendothelial matrices, B16 melanoma cells were labelled with 100μCi of ^{51}Cr /80cm^2 flask overnight. The same assay procedure was followed for the ECM and BM substrates, except that after the washing off of non-adherent cells, the remaining cells were solubilized with 0.1M NaOH. Two aliquots were taken for counting in a Wilj gamma-counter. The percentage attachment was expressed as [dpm at time **x** / dpm cells seeded] x100. For all experiments, triplicate dishes were used per cell line per time point and all experiments were performed in triplicate.

Measurement of cell spreading in vitro; B16 melanoma cells grown on tissue culture plastic were photographed at a magnification of X125. The outlines of cells were carefully cut from photographs and weighed, and cell spread area was then expressed in milligrammes.

Melanin and Tyrosinase Assays; A modified procedure was used from those reported by Lotan and Lotan (1980) and Halaban *et al.* (1983). Duplicate

cultures of melanoma cells were grown in 60mm petri dishes for 4 days until confluent. The cell layers were washed with ice cold PBS, pH 7.2 and scraped in 1ml of the PBS. From this both the melanin and tyrosinase assays were performed. A 100µl aliquot was used for the tyrosinase assay, 2x100µl aliquots for cell counts and the remaining 700µl for the melanin determination.

For the tyrosinase assay, the cells were gently pelleted and then resuspended and lysed in 0.2ml of PBS pH7.2 containing 1.0% Nonidet P-40. The solution was then centrifuged at 16,000g for 5 mins and the supernatant transferred to clean eppendorf tubes containing 0.2ml of PBS containing $2x10^{-4}$M L-DOPA, $4x10^{-4}$M L-tyrosine and 2.0µCi L-[3,5-^{3}H] tyrosine, and incubated at 37^{o}C for one hour. The reaction was stopped by the addition of 0.5ml of cold trichloroacetic acid (TCA) and centrifuged.

The supernatant was transferred to 1.5ml eppendorf tubes containing 100mg of activated charcoal, mixed and centrifuged. 0.5ml samples were taken from each tube and mixed with 5ml of Ecoscint scintillation cocktail prior to being counted in a Packard Tri-Carb 300C scintillation counter. Tyrosinase activity was measured as the release of ^{3}H-radiolabel (as $^{3}H_2O$) per $1.0x10^{6}$ cells.

For the melanin assay the cell suspension was initially subjected to two cycles of freeze/thawing. Perchloric acid was added to a final concentration of 0.5M and the suspension kept on ice for 10 mins before being centrifuged at 16,000g for 5 mins. The pellets were extracted twice with 0.5M perchloric acid, twice with a cold 3:1 v/v mixture of EtOH:ether, followed by a final extraction with ether and then air dried. Pellets were

then dissolved by boiling in 1.0ml 0.85M KOH for 10 mins. The insoluble material was pelleted and the absorbance of the supernatant read at 400nm. Absorbances were compared with those obtained for preprepared melanin standards and results expressed as μg of melanin/10^5 cells. All experiments were performed in triplicate.

Radiolabelling of Glycosaminoglycans (GAG): For qualitative GAG analyses different B16 melanoma cells, grown in 80cm^2 flasks for 4 days until confluent, were radiolabelled for the final 48 hours with either 5μCi ^{3}H-GlcN or 2μCi ^{14}C-GlcN (ICN-Flow Radiochemicals) per ml of medium. On day 4 the medium was removed and the cell layer washed twice with Ca^{2+}- and Mg^{2+}-free PBS and pooled with the medium. This was designated the medium GAG fraction. The cell layer was detached with trypsin/EDTA and the cells resuspended in complete medium. The cells were pelleted by centrifugation and the medium removed. The cell pellet was washed twice with Ca^{2+}- and Mg^{2+}-free PBS, centrifuged and the washings pooled with the medium. This was designated the trypsin-releasable GAG fraction. The cell pellet was then resuspended in Ca^{2+}- and Mg^{2+}-free PBS and designated the cell-associated GAG fraction.

0.5mg of both chondroitin sulphate and heparin (Sigma) was added as carrier GAG to all fractions. The fractions were boiled for 5 mins and prepared for Pronase digestion. Identical GAG fractions from different cell lines which were differentially labelled with either ^{3}H- or ^{14}C-GlcN could be pooled for direct comparison of their GAG properties.

For quantitative GAG analyses, the previous procedure was followed except that triplicate 80cm^2 flasks were dual labelled with 20μCi ^{35}SO4 and 5μCi ^{3}H-GlcN per ml of medium and all fractions kept separate. After

48 hours incubation the cells were switched to sulphate-depleted complete medium for radiolabelling.

Pronase Digestion; GAG fractions dialysed against 50mM tris/HCl pH7.6, were digested for 24 hours with 0.5mg Pronase (Sigma) in 50mM tris/HCl pH7.6, per ml of sample at 50^{o}C, with a further addition after 6 hours incubation. Samples were then boiled and dialysed against column buffer in preparation for DEAE-ion exchange chromatography.

Ion Exchange Chromatography (IEC); GAGs were initially separated on DEAE-cellulose (Whatman Biosystems Ltd) ion exchange columns (80mmx10mm) using a linear 0.05M-0.7M NaCl gradient in 10mM tris/HCl, pH7.6. DEAE is cationic, with molecular structure -CH2-$N^{+}(CH_3)_3$. Fractions were collected as 2.5ml volumes and an aliquot taken from every second fraction for scintillation counting on a Packard Tri-Carb 300C. Radioactive peaks corresponding to the sulphated GAGs were pooled, split into equal sized fractions, and prepared for either nitrous acid deamination at low pH or chondroitinase ABC (Sigma) digestion to generate, respectively, intact chondroitin sulphates (CS) or heparan sulphates (HS) only. These were then dialysed against column buffer for further qualitative determination on DEAE-cellulose. In quantitative GAG analyses, the relative proportions of individual GAG species were estimated by gel filtration chromatography on Sephadex G-50 columns (Pharmacia/LKB Biotechnology) following sequential degradation of void volume material with chondroitinase ABC and nitrous acid.

For qualitative analyses, Mono-Q-Sepharose (Pharmacia/LKB) was used to provide a measure of the degree of GAG sulphation. Mono-Q-Sepharose is a

highly polycationic ion exchange resin and within the range of pH2.0-4.0 the binding of GAGs to the resin is due to charged sulphate groups (Couchman *et al.*, 1988). GAG was eluted from the Mono-Q-Sepharose column (90mm x 15mm) with a linear gradient of 0.2M-1.8M NaCl in 50mM tris/HCl, pH2.0, or alternatively, in 50mM sodium acetate, pH4.0, containing 0.1%(v/v) Triton X-100 (Sigma). Fractions were collected as 2.5ml samples and an aliquot taken from every second fraction for scintillation counting.

Gel Filtration Chromatography; Freeze-dried GAG was rehydrated in 0.2ml 0.5M NaCl for gel filtration chromatography on Sephadex G-50 columns (600mmx5mm) equilibrated in the same buffer. 700µl samples were collected and 100µl samples analysed for their radioactive content. For quantatative GAG analyses, samples treated with either chondroitinase ABC or nitrous acid were separated on Sephadex G-50 columns into two peaks; the void volume (V_0) represented intact GAG, and the included volume (V_i), which elutes near to the total volume (V_t), degraded GAG. These peaks permit the levels of heparan sulphate and chondroitin sulphates to be calculated.

Proteoglycan analysis; B16 melanoma cells were radiolabelled for qualitative analysis as previously described and their proteoglycans isolated and analysed by the method described by Couchman *et al.* (1988). The medium fraction consisted of the culture medium plus three washes with PBS at 37^{o}C. The cell layer was solubilized overnight at 4^{o}C in 10ml of 4M urea, 1.0% (v/v) Triton X-100, 25mM tris/HCl pH7.5, plus the following protease inhibitors; 0.2mM PMSF, 10mM N-EM, 50mM aminocaproic acid (ACA), 20mM EDTA, and 5mM benzamidinium chloride.

The two proteoglycan pools, medium and cell-lysate, were diluted 1:5 with 4M urea, 0.2M NaCl, 50mM tris/HCl, pH8.0, plus protease inhibitors, and applied to a 30mmx10mm column of DEAE-cellulose equilibrated in the same buffer with 0.1% Triton X-100. The ion-exchange column was washed with 10 column volumes of equilibration buffer followed by 5 column volumes of 4M urea, 50mM sodium acetate, pH4.0, 0.2mM NaCl, 0.1% Triton X-100, plus protease inhibitors. Radiolabelled GAGs and PGs were eluted in 10x1ml fractions of 4M urea, 50mM sodium acetate, pH4.0, 1.5M NaCl, 0.1% Triton X-100, plus protease inhibitors. Under these conditions no unincorporated radiolabel co-purified with the macromolecules.

The molecular size of the PGs and GAGs was estimated by Sephadex CL-4B gel filtration using 900mmx15mm columns equilibrated with 0.1% (w/v) SDS, 50mm tris/HCl pH8.0, 0.35M NaCl, 0.2g/L sodium azide and protease inhibitors. Radiolabelled macromolecules were eluted at 6ml/hour and collected as 1.5ml fractions. An aliquot was taken from each fraction for scintillation counting.

Heparan sulphate PG (HSPG) and GAG (HS-GAG) was isolated by digesting contaminating chondroitin sulphates (CS) with 0.1U/ml chondroitinase ABC in 50mM tris/acetate, pH8.0, 30mM sodium acetate, 0.1% Triton X-100, 0.1mg/ml BSA, 0.2mM PMSF, 5mM N-EM, and 10mM EDTA overnight at 37^{o}C. Chondroitin sulphates were isolated by deamination of heparan sulphates with nitrous acid at low pH.

Preparation of Heparan and Chondroitin Sulphates; GAG fractions separated by DEAE-cellulose IEC were dialysed against distilled water, split into two equal samples and freeze dried. Chondroitin sulphates were digested with chondroitinase ABC (0.25units in 0.05M Tris-acetate buffer,

pH8.0, containing 0.15M NaCl and 0.1% (w/v) BSA overnight at 37^{o}C (Oldberg *et al.*, 1977). Heparan sulphates were degraded by deaminative cleavage at low pH (Shively and Conrad, 1976): 50µl of 3.6M acetic acid and 0.48M sodium nitrite were added to the sample to generate nitrous acid, and incubated for 90mins at room temperature. The reaction was terminated by neutralization with 2M sodium carbonate.

SECTION 6. RESULTS

Page
83: 4.2.1. Production of metastatic cell lines
84: 4.2.2. Lung colonization potential
86: 4.2.3. Cell growth
87: 4.2.4. Cell morphology
88: 4.2.5. Tumour spheroids
89: 4.3.1. Induction of differentiation: Melanin and tyrosinase
91: 4.3.2. Cell spreading and adhesion
95: 4.3.3. Degradation of the subendothelial matrix
99: 4.4.1. Proteoglycan analyses
100: 4.4.2. Qualitative differences in GAG biosynthesis
100: a) B16F1 versus B16F1M3
101: b) B16F1M3±0.5% DMF
102: c) B16F1 versus B16F1M5
102: d) B16F1H5 versus B16F1D2
103: e) B16F1M3±50mM sodium chlorate
103: f) B16F1M3±20µg/ml heparin
104: g) B16F1D2±0.5mM ß-D-xyloside
105: 4.4.3. Qualitative differences in proteoglycan synthesis
105: a) B16F1H5 versus B16F1D2
108: b) B16F1D2±0.5mM ß-D-xyloside
110: 4.4.4. Quantitative differences in GAG biosynthesis
110: a) B16F1 versus B16F1M3±DMF
117: b) B16F1H5 versus B16F1D2
123: c) B16F1D2±o.5mM ß-D-xyloside

FIGURES & ILLUSTRATIONS:

Page number or adjacent page number in parentheses:

(83): *Artificial Tumour Cell Metastasis.*
(i): Table.1. B16 Murine Melanoma Lung colonizing potentials

(86): *B16 Murine Melanoma Growth characteristics in vitro.*
(i): Fig.6a: B16 melanoma growth rate in vitro: B16F1 and B16F1M3±0.5% DMF
(ii): Fig.6b: B16 melanoma growth rate in vitro: B16F1 and B16F1M5±0.5% DMF
(iii): Fig.6c: B16F1M3 melanoma cell growth response to varying concentrations of DMF
(iv): Fig.6d: B16 melanoma growth rate in vitro: B16F1H5 and B16F1D2

(87): *B16 Murine Melanoma Cell Morphology.*
(i): Figs.7A-7H: B16 melanoma cell morphology in vitro:
A; B16F1 cells
B; B16F1M3 cells
C; B16F1+0.5% DMF
D; B16F1M3+0.5% DMF
E; B16F1H5 cells
F; B16F1D2 cells
G; B16F1D2 control cells (+DMSO)
H; B16F1D2+0.5mM ß-D-xyloside

(88): *B16 Murine Melanoma Tumour Spheroid Formation.*
(i): Figs.7I-7P: B16 melanoma cells as 24 hour spheroid cultures:
I; B16F1 cells
J; B16F1M3 cells
K; B16F1+0.5% DMF
L; B16F1M3+0.5% DMF
M; B16F1H5 cells
N; B16F1D2 cells

O; B16F1D2 control cells (+DMSO)
P; B16F1D2+0.5mM ß-D-xyloside

(89): *Melanin and Tyrosinase Levels.*
(i): Fig.8a: The effect of DMF on tyrosinase activity
(ii): Fig.8b: The effect of DMF on intracellular melanin content

(91): *Cell Spreading in vitro.*
(i): Fig.9: Cell spread area in vitro

(92): *Cell Adhesion Assays*:

Cell attachment to BSA
(i): Fig.10: B16F1 versus B16F1M3±0.5% DMF
(ii): Fig.11: B16F1D2 versus B16F1H5
(iii): Fig.12: B16F1 versus B16F1M5

Cell attachment to collagen type I
(iv): Fig.13: B16F1 versus B16F1M3±0.5% DMF
(v): Fig.14: B16F1D2 versus B16F1H5
(vi): Fig.15: B16F1D2±0.5mM ß-D-xyloside
(vii): Fig.16: B16F1 versus B16F1M5

Cell attachment to fibronectin
(viii): Fig.17: B16F1 versus B16F1M3±0.5% DMF
(ix): Fig.18: B16F1D2 versus B16F1H5
(x): Fig.19: B16F1D2±0.5mM ß-D-xyloside
(xi): Fig.20: B16F1 versus B16F1M5

(93): Cell attachment to laminin/nidogen
(i): Fig.21: B16F1 versus B16F1M3±0.5% DMF
(ii): Fig.22: B16F1 versus B16F1M5
(iii): Fig.23: B16F1D2 versus B16F1H5
(iv): Fig.24: B16F1D2±0.5mM ß-D-xyloside

Cell attachment to collagen type IV
(v): Fig.25: B16F1 versus B16F1M3±0.5% DMF
(vi): Fig.26: B16F1 versus B16F1M5

(vii): Fig.27: B16F1D2 versus B16F1H5
(viii): Fig.28: B16F1D2±0.5mM ß-D-xyloside

(94): Cell attachment to CPAE monolayers
(i): Fig.29: B16F1 versus B16F1M3±0.5% DMF
(ii): Fig.30: B16F1D2 versus B16F1H5

Cell attachment to CPAE subendothelial matrix
(iii): Fig.31: B16F1 versus B16F1M3±0.5% DMF
(iv): Fig.32: B16F1D2 versus B16F1H5

Homotypic ell attachment to B16 melanoma monolayers
(v): Fig.33: B16F1 versus B16F1M3±0.5% DMF

Degradation of Metabolically-Labelled Subendothelial Extracellular Matrix:

Release of ^{3}H-Proline from CPAE subendothelial matrices
(96): Fig.34a: B16F1 versus B16F1M3±0.5% DMF
(96): Fig.34b: B16F1D2 versus B16F1H5

Release of ^{35}Sulphate from CPAE subendothelial matrices
(98): Fig.35a: B16F1 versus B16F1M3±0.5% DMF
(98): Fig.35b: B16F1D2 versus B16F1H5

Qualitative Glycosaminoglycan Analyses:

(99): Chromatography elution standards
(i): Fig.36a: Initial DEAE-Cellulose IEC separation
(ii): Fig.36b: Sephadex G50 GPC/Chondroitinase ABC digestion eltion profile
(iii): Fig.36c: Sephadex G50 GPC/Nitrous acid deamination elution profile
(iv): Fig.36d: Sephadex GPC/Nitrous acid deamination of HS-GAG elution profile

(100): B16F1 versus B16F1M3: DEAE-Cellulose IEC
(i): Fig.37: Medium fraction CS-GAG
(ii): Fig.38: Medium fraction HS-GAG
(iii): Fig.39: Trypsin fraction CS-GAG
(iv): Fig.40: Trypsin fraction HS-GAG
(v): Fig.41: Cell fraction HS-GAG
(vi): Fig.42: Cell fraction HS-GAG

(101): B16F1 versus B16F1M3: Mono-Q-Sepharose IEC
(i): Fig.43: Medium fraction CS-GAG
(ii): Fig.44: Trypsin fraction CS-GAG
(iii): Fig.45: Trypsin fraction HS-GAG
(iv): Fig.46: Medium fraction HS-GAG

B16F1M3±0.5% DMF: DEAE-Cellulose IEC
(v): Fig.47: Medium fraction CS-GAG
(vi): Fig.48: Trypsin fraction CS-GAG
(vii): Fig.49: Cell fraction CS-GAG
(viii): Fig.50: Medium fraction HS-GAG
(ix): Fig.51: Trypsin fraction HS-GAG
(x): Fig.52: Cell fraction HS-GAG

(102): B16F1M3±0.5% DMF: Mono-Q-Sepharose IEC
(i): Fig.53: Medium fraction CS-GAG
(ii): Fig.54: Trypsin fraction CS-GAG
(iii): Fig.55: Medium fraction HS-GAG
(iv): Fig.56: Trypsin fraction HS-GAG

B16F1 versus B16F1M5: DEAE-Cellulose IEC
(v): Fig.57: Medium fraction HS-GAG
(vi): Fig.58: Trypsin fraction HS-GAG

B16F1D2 versus B16F1H5: DEAE-Cellulose IEC
(vii): Fig.59: Medium fraction CS-GAG
(viii): Fig.60: Trypsin fraction CS-GAG
(ix): Fig.61: Trypsin fraction HS-GAG
(x): Fig.62: Medium fraction HS-GAG

(103): B16F1D2 versus B16F1H5: Mono-Q-Sepharose IEC
(i): Fig.63: Medium fraction CS-GAG
(ii): Fig.64: Trypsin fraction HS-GAG
(iii): Fig.65: Trypsin fraction CS-GAG
(iv): Fig.66: Medium fraction HS-GAG

B16F1M3±50mM sodium chlorate: DEAE-Cellulose IEC
(v): Fig.67: Medium fraction HS-GAG
(vi): Fig.68: Cell-Lysate fraction HS-GAG
(vii): Fig.69: Medium fraction CS-GAG

B16F1M3±20µg/ml heparin: Mono-Q-Sepharose IEC
(viii): Fig.70: Medium fraction HS-GAG

(104): B16F1D2±ß-D-xyloside: DEAE-cellulose IEC
(i): Fig.71: Cell-lysate fraction CS-GAG
(ii): Fig.72: Medium fraction HS-GAG
(iii): Fig.73: Medium fraction CS-GAG
(iv): Fig.74: Cell-lysate fraction HS-GAG

TABLES & ILLUSTRATIONS:

Quantitative Glycosaminoglycan Analyses:

B16F1 versus B16F1M3±0.5% DMF
(112): Table.2: % Glycosaminoglycan composition
(114): Table.3a: Quantity of glycosaminoglycan as measured by ^{3}H-GlcN incorporation
(115): Table.3b: Total glycosaminoglycan as measured by ^{3}H-GlcN incorporation
(116): Table.3c: $^{35}SO_4$-incorporation into glycosaminoglycan

B16F1D2 versus B16F1H5
(117): Table.4: % Glycosaminoglycan composition
(118): Table.5a: Quantity of glycosaminoglycan as measured by ^{3}H-GlcN incorporation
(119): Table.5b: Total glycosaminoglycan as measured by ^{3}H-GlcN incorporation
(121): Table.5c: $^{35}SO_4$-incorporation into glycosaminoglycan
(122): Table.5d: $^{3}H/^{35}S$ ratio
(123): Table.5e: HS-GAG N-sulphate: O-sulphate ratio

B16F1D2±0.5mM ß-D-xyloside
(124): Table.6: % Glycosaminoglycan composition
(126): Table.7a: Quantity of glycosaminoglycan as measured by ^{3}H-GlcN incorporation
(127): Table.7b: Total glycosaminoglycan as measured by ^{3}H-GlcN incorporation
(128): Table.7c: $^{35}SO_4$-incorporation into glycosaminoglycan
(129): Table.7d: $^{3}H/^{35}S$ ratio
(129): Table.7e: HS-GAG N-sulphate: O-sulphate ratio

4.2.1. Production of metastatic cell lines

From a poorly metastatic B16 murine melanoma cell line, B16F1, a series of cell lines was derived that differed in their metastatic potential, growth rates, and morphology. Metastatic potential (capacity) was assessed as a measure of a cell line's ability to colonize the lungs of syngeneic C57BL mice following bolus administration via tail vein injection into experimental animals. The moderately metastatic cell lines B16F1M3 and B16F1M5 (Table.1) were generated from B16F1 cells via this artificial metastasis system described by Fidler (1973a). B16F1M3 and B16F1M5 cells were developed from the lung colonies formed by B16F1 cells that were sequentially passaged through the lungs of syngeneic C57BL mice three and five times respectively. B16F1M5 melanoma cells proved to be phenotypically unstable in vitro, and therefore most of the experimental work concentrated on the more stable B16F1M3 melanoma cell line.

A panel of B16 melanoma cell clones were generated from the B16F1M3 melanoma cell line by the process of limited dilution. From this panel of B16F1M3-derived clones, two clones were identified which possessed widely contrasting metastatic capacities and were stable in vitro for the duration of the experiments: B16F1H5 was poorly metastatic whereas B16F1D2 was highly metastatic (Table 1).

The major aim of the study was to determine to what extent tumour cell-derived proteoglycans were involved in the latter phases of tumour cell metastasis as measured by an artificial metastasis model. A series of chemical agents reported to influence the biosynthesis and chemical

Table.1 Lung Colonization Efficiencies

Lung colony formation after intravenous injection of B16 melanoma cells ($1x10^5$ cells/0.2ml saline)

Cell Line	mean (s.d)	Range	n
B16F1 (a)(b)(g)	3.0 (3.3)	0-14	31
B16F1+0.5%DMF (a)	7.7 (7.6)	0-23	14
B16F1M3(b)	38.6 (46.8)	0-158	47
B16F1M3+0.5% DMF(b)	118.6 (67.6)	14->200	28
B16F1M3+50mM sodium chlorate	37.7 (15.4)	10-62	6
B16F1M3+50g/ml HUdR/KL103	38.5 (49.9)	0-163	13
B16F1M3+20µg/ml heparin	25.4 (14.9)	2-60	12
B16F1M5(g)	34.7 (21.7)	6-78	6
B16F1D2(c)	100.7 (86.3)	1-234	17
B16F1H5(c)	1.1 (1.0)	0-3	10
B16F1D2+DMSO carrier(d)	104.1 (102.4)	17-286	15
B16F1D2+0.5mM ß-D-xyloside(d)	42.2 (32.2)	0-94	17
B16F1M3(LT)(e)	47.8 (54.7)	10->200	12
B16F1M3+0.5% DMF(LT)(e)	124.0 (65.9)	13->200	11
B16F1M3(LT:C4D1)(f)	152.0 (59.3)	16->200	9
B16F1M3(LT:D4C1)(f)	47.8 (38.2)	4-116	10

Abbreviations:

LT: long term culture (five passages total)
C4D1: four passages in complete medium followed by a final passage in complete medium+0.5%DMF.
D4C1: four passages in complete medium+0.5%DMF followed by a final passage in complete medium only.

Significance values (Student's t-test):
(b)(c)(e)(f): 0.001>P
(a)(d): 0.05>P
(g): 0.01>P

characteristics of proteoglycans was assayed for their ability to interfere with the metastatic ability of B16 melanoma cells. The ability of these agents to interfere with metastatic potential may therefore be related to, or be a consequence of, proteoglycan modifications.

4.2.2. Lung colonization potential

B16 melanoma cells were assayed for their metastatic ability using the method described by Fidler (1973a) which measures the cells' ability to colonize a target organ, in this case the lungs of syngeneic C57BL mice (Table 1).

DMF, a putative reversible inducer of differentiation (Spremulli & Dexter, 1984) was analysed for its biological effects on B16 melanoma cells. The induction of a more differentiated cell phenotype is usually associated with an inhibition of metastatic ability. Other chemical agents widely reported to modify proteoglycan biosynthesis were also assayed for their effects on lung colonization; these agents were sodium chlorate, heparin, ß-D-xyloside, and 5-hexyl-2-deoxyuridine (HUdR/KL103). Briefly, sodium chlorate is an inhibitor of proteoglycan sulphation (Humphries & Silbert, 1988) whereas heparin promotes GAG sulphation (Nader *et al.*, 1989). ß-D-xylosides interfere with proteoglycan biosynthesis by competing with proteoglycan core proteins for GAG chain synthesis (Schwartz *et al.*, 1974), and HUdR/KL103 is described as a specific inhibitor of GAG synthesis (Lapis *et al.*, 1987). The effects of these agents on lung colonization is summarized in Table 1.

Heparin, sodium chlorate and HUdR/KL103 had no significant effect on the lung colonization potential of moderately metastatic B16F1M3 melanoma

cells. However, the effect of 0.5% DMF and 0.5mM ß-D-xyloside were both significant but contrasting. Pretreatment with 0.5% DMF resulted in a significant increase in the lung colonizing potential of both the poorly metastatic B16F1 cells and the moderately metastatic B16F1M3 cells ($P<0.001$). This was in contrast to the significant reduction in lung colonization when highly metastatic B16F1D2 melanoma cells were treated with 0.5mM ß-D-xyloside ($P<0.05$).

In order to minimise the effect of phenotypic drift that can arise through prolonged culture of tumour cells, long-term cultures of B16F1M3 melanoma cells were prepared and their lung colonization potential determined (Table 1). B16 melanoma cells which underwent 24 days (5 passages) of continuous culture possessed a similar lung colonization potential as early passage cells. Prolonged cell culture in the presence of 0.5%DMF provided similar values for lung colonization as early passage cells treated with DMF. Switching cells from DMF-medium to normal medium after 20 days (passage 4) resulted in the reacquisition of the moderately metastatic phenotype (D4C1). In contrast, the addition of DMF to long-term cultures after 20 days induced the highly metastatic phenotype.

These results would indicate that at passage 5, any phenotypic drift that had occurred in culture was minimal, and that the cell populations were stable up to that point. Pretreatment with DMF did not apparently select for subpopulations of tumour cells with increased lung colonization potential since its removal resulted in the reacquisition of the original potential. DMF-induced changes in cell phenotype were rapid and reversible. In consequence, all experimental work was performed with

cells between passages 2 and 5.

4.2.3. Cell growth

The B16 melanoma cell lines used displayed differences in their initial in vitro growth rates. The kinetics of cell growth in vitro were measured as cell doubling times. Cell viability in culture (as measured by trypan blue exclusion) was in excess of 92%, and pretreatment of cells with different GAG-altering chemical agents assayed did not affect the viability.

Poorly metastatic B16F1 cells had a cell doubling time ranging from approximately 23 hours on days 2-3 and decreasing to 12 hours on days 3-4. The moderately metastatic B16F1M3 cell line was relatively faster growing with cell doubling times of approximately 13-14 hours over days 2-4 (fig.6a). B16F1M5 melanoma cells were slower growing with a relatively stable cell doubling time of 17 hours over days 2-4 (fig.6b). Variability in cell growth rates was observed in different vials of cryopreserved cells that were thawed for expansion in vitro. These variations may probably reflect differences in cell sensitivity and recovery from cryopreservation.

Treatment of the B16F1M3 melanoma cells with 0.5% DMF initially reduced cell growth rates over the first 48 hours. On days 2-3 the cell doubling time was approximately 16 hours, recovering to 13 hours on days 3-4 (fig.6a). B16F1M3 cells initially treated with 0.5% DMF and then allowed to recover after removal of the DMF did not significantly alter their growth rate during the recovery period and maintained a growth rate similar to that measured for their DMF-treated counterpart. DMF had very slight suppressive effect upon the cell doubling times of B16F1M5 cells

Fig.6a: B16 melanoma cell growth rate in vitro (mean ± SEM; n=12)

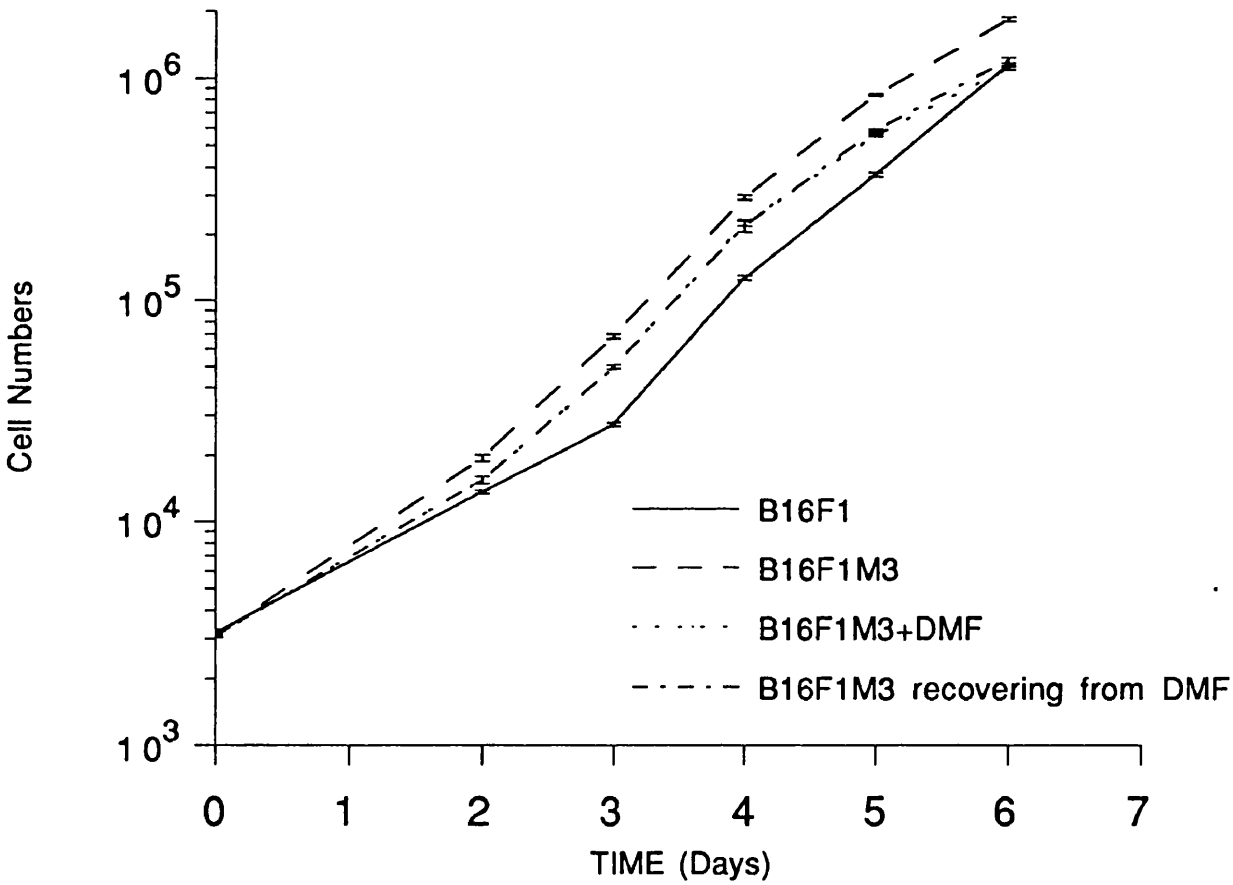


Fig.6b: B16 melanoma cell growth rate in vitro (mean ± SEM; n=12)

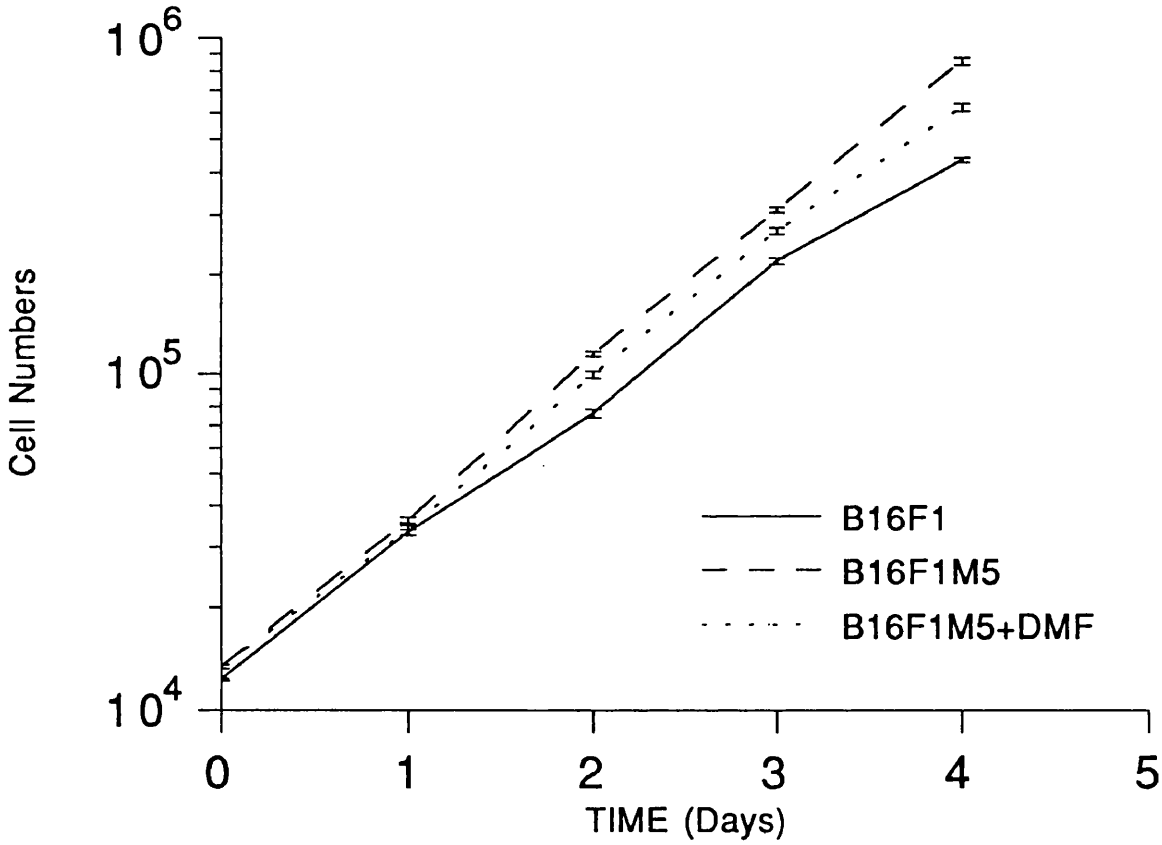


Fig.6c: B16F1M3 cell growth response to varying concentrations of DMF (mean ± SEM; n=12)

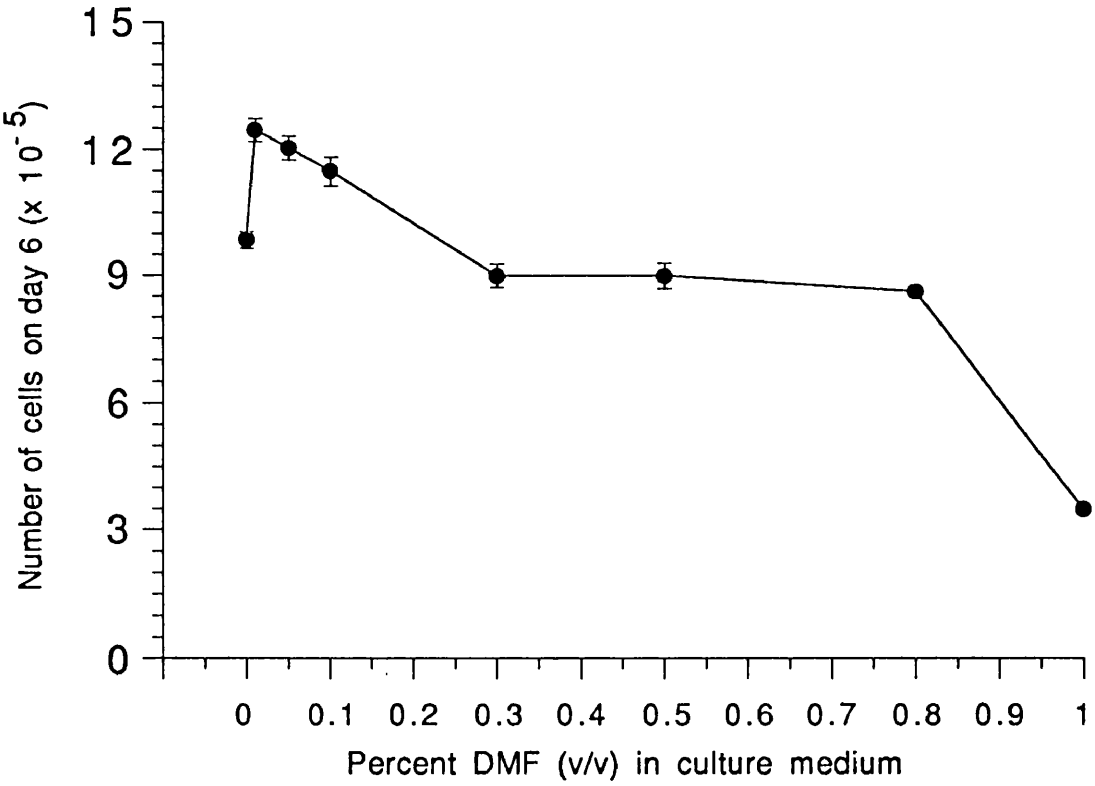
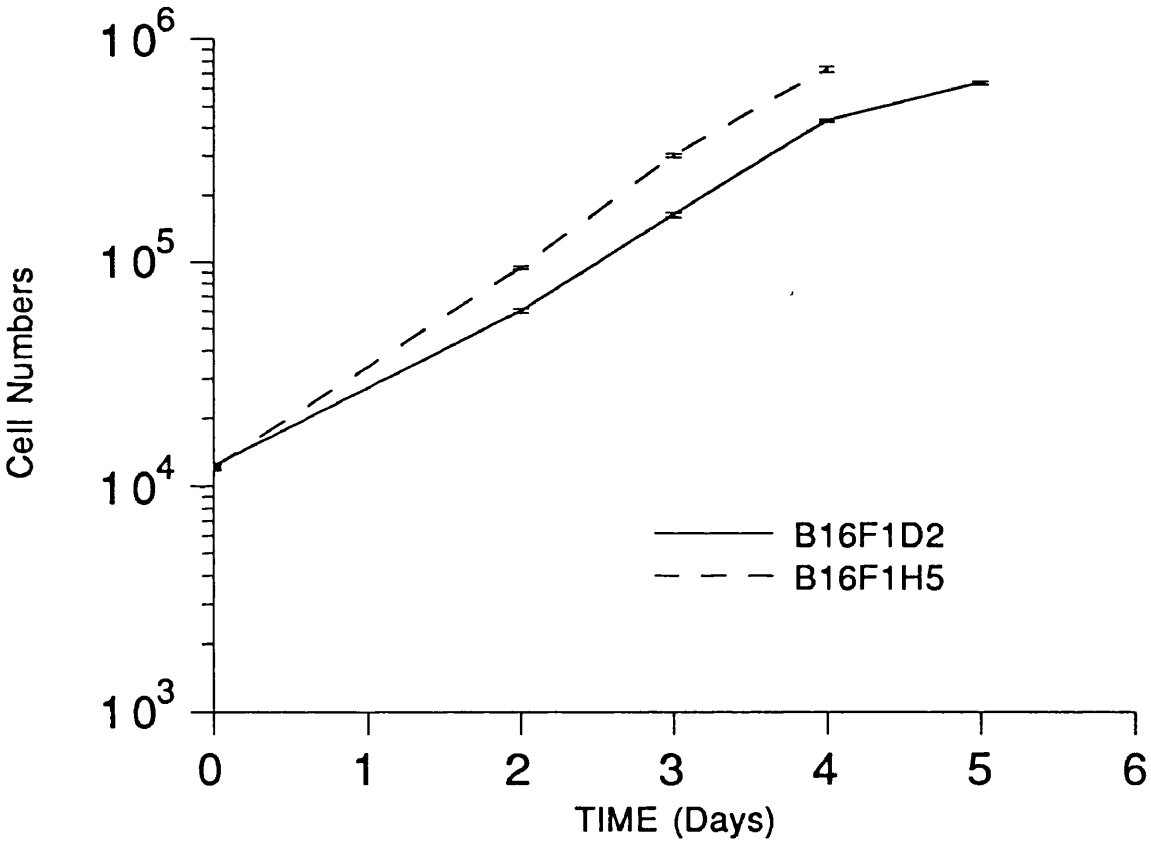


Fig.6d: B16 melanoma cell growth rate in vitro (mean ± SEM; n=6)



(fig.6b). The presence of HUdR/KL103, sodium chlorate, and heparin had no significant influence upon cell growth (not shown).

Low concentrations of DMF (≤0.1%) stimulated cell growth by up to 20%. At higher concentrations (0.3%-1.0%) a proportional but gradual reduction in cell numbers resulted with cell numbers being reduced to approximately

40% following 5 days incubation in 1.0% DMF (fig. 6c). DMF had no observable effect upon the initial rates of cell attachment and spreading on culture plastic since the great majority of cells were attached and spread within a few hours. One effect of DMF appears to be an initial inhibition of cell growth occuring within the first 48 hours of exposure (fig. 6a). After the first 48 hours the B16 melanoma cells appear to adapt to the presence of DMF and recover their usual growth rate.

The B16 melanoma cell clones derived from B16F1M3 cells also exhibited differences in cell doubling times (fig.6d). B16F1H5 cells had a doubling time of approximately 14 hours on days 2-3 and 18 hours on days 3-4. B16F1D2 melanoma cells had a more consistent cell doubling time over days 2-4 of 17 hours. It was observed that the in vitro culture of cells in the presence of 0.5mM ß-D-xyloside reduced the cell numbers by approximately 20% after 4 days, and is suggestive of the possible involvement of normal proteoglycan synthesis in the regulation of B16 melanoma cell growth.

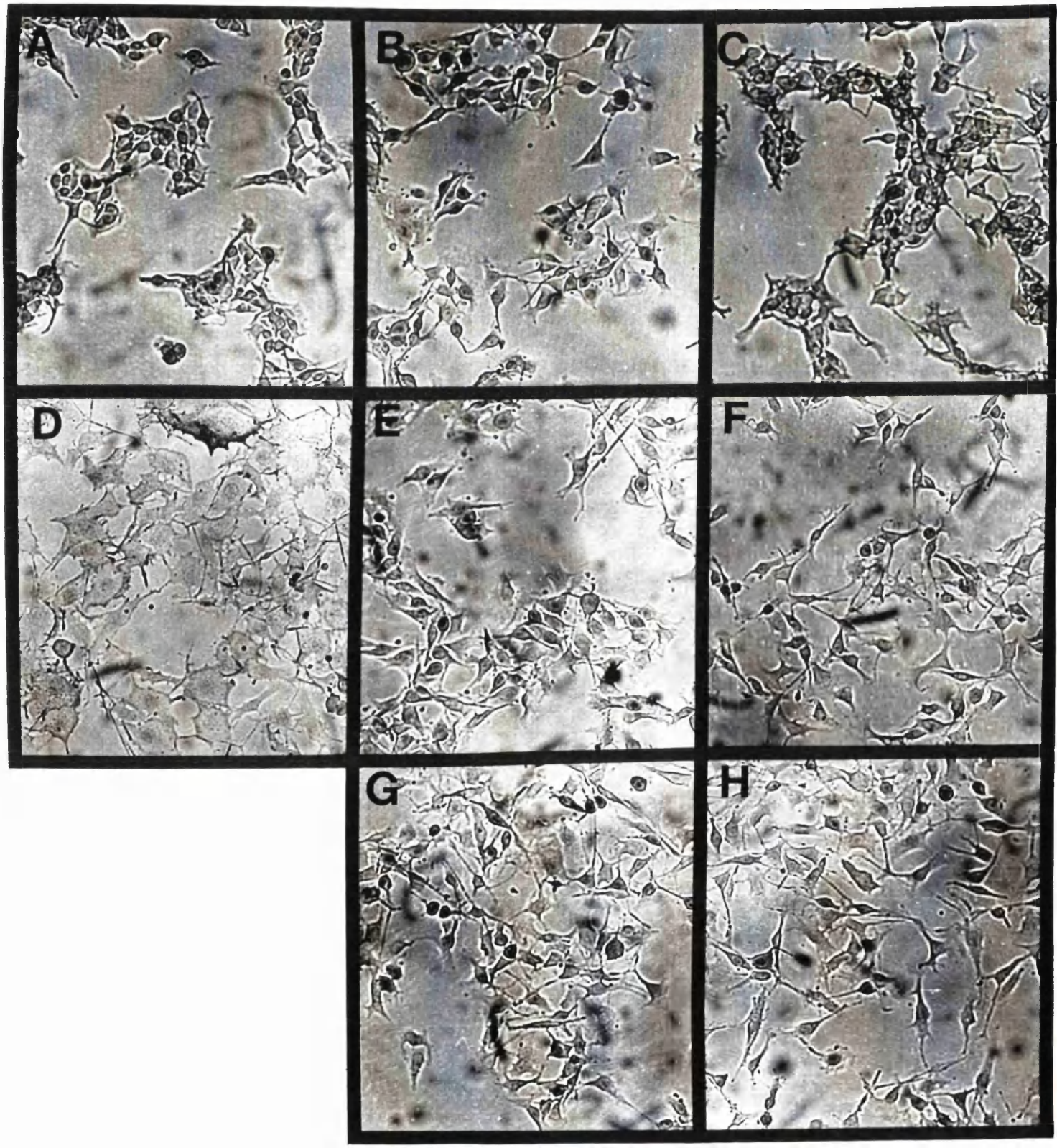
4.2.4. Cell morphology

The production of metastatic variants from B16F1 melanoma cells generated cells which differed in cell morphology and growth rates. In

Figures 7A-7H: B16 melanoma cell morphology in vitro

(Cells photographed x125 magnification using a Leitz Diavert microscope, Wild PhotoAutomat MPS55 and Ilford FP4 Plus f125 photographic film)

B16F1 (7A); B16F1M3 (7B); B16F1+0.5%DMF (7C); B16F1M3+0.5%DMF (7D); B16F1H5 (7E); B16F1D2 (7F); DMSO-treated B16F1D2 control cells (7G); and B16F1D2+0.5mM ß-D-xyloside (7H)



vitro, B16F1 cells (fig.7A) grew as poorly spread cells which formed tight colonies. B16F1M3 cells (fig.7B) grew as looser cell colonies and appeared flatter than the B16F1 cells. It was also more common to see individual B16F1M3 cells. Treatment of B16F1 cells with 0.5% DMF did not induce gross morphological alterations although the cells were more pigmented (fig.7C), but treatment of B16F1M3 cells with 0.5% DMF caused gross morphological alterations (fig.7D). Cells became epithelioid and flattened with irregular cell margins. Pretreatment of B16 melanoma cells with HUdR/KL103, heparin or sodium chlorate had no visible effects upon cell morphology (not shown).

B16F1M3-derived cell clones did not display morphological differences in vitro. Both the poorly metastatic B16F1H5 (fig.7E) and highly metastatic B16F1D2 (fig.7F) melanoma clones expressed similar morphology and cell spreading. The B16F1D2 cells though formed more diffuse colonies than B16F1H5 cells. Pretreatment of B16F1D2 cells with ß-D-xyloside had no visible effects upon cell morphology or cell spreading in vitro (figs.7G, 7H).

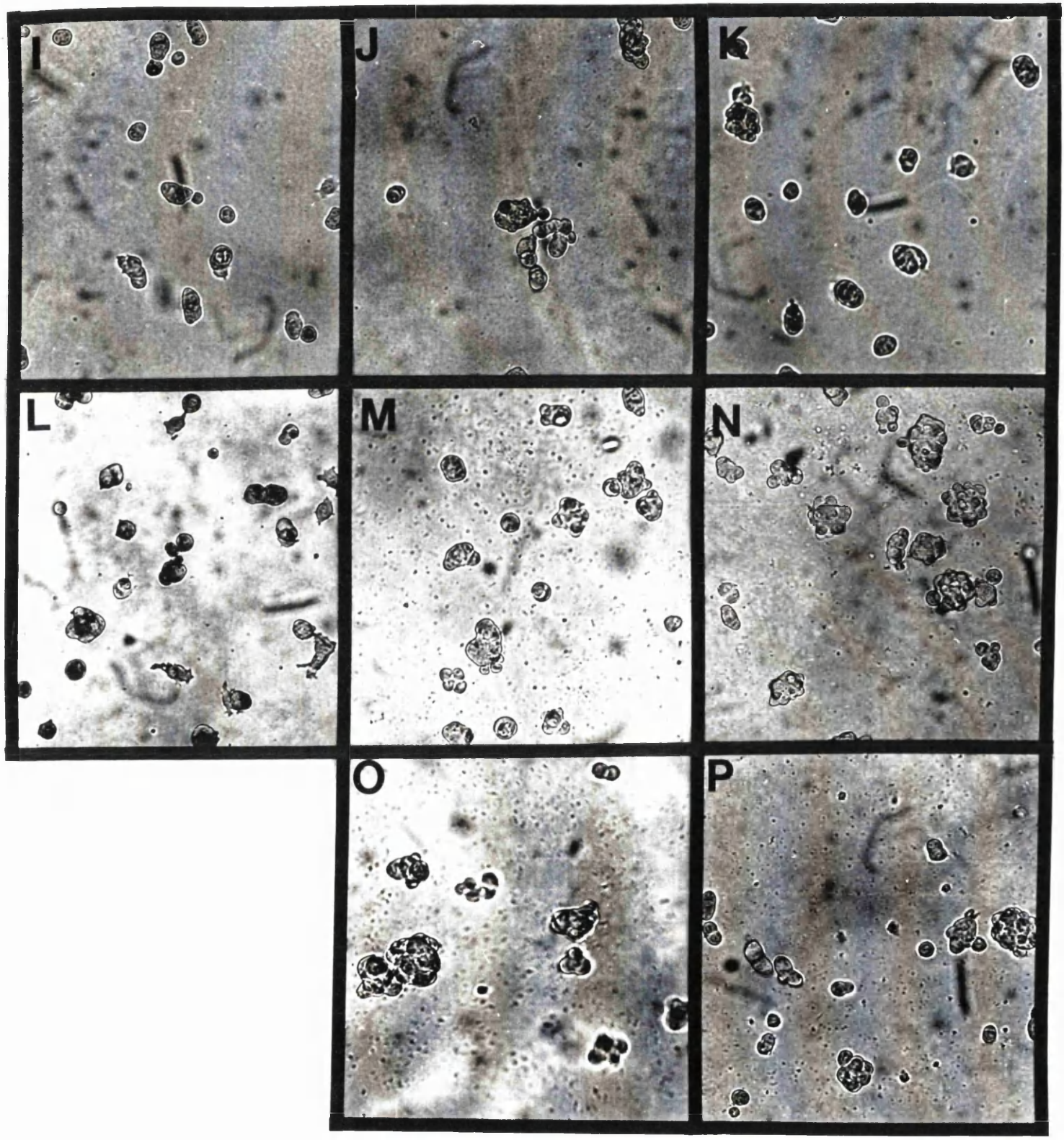
4.2.5. Tumour spheroids

It was observed that DMF-treated cells were more resistant to detachment with trypsin/EDTA from culture dishes than untreated cells, and that they rapidly formed cell aggregates when in suspension. Since cell aggregation and embolus formation are believed to afford physical protection to circulating tumour cells and facilitate tumour cell arrest, tumour cells were grown in suspension for 24 hours to measure aggregation (spheroid formation) and for 72 hours to permit measurement of cell growth.

Figures 7I-7P: B16 melanoma cell lines as 24 hour spheroid cultures

(Cells photographed x125 magnification using a Leitz Diavert microscope, Wild PhotoAutomat MPS55 and Ilford FP4 Plus f125 photographic film)

B16F1 (7I); B16F1M3 (7J); B16F1+0.5%DMF (7K); B16F1M3+0.5%DMF (7L); B16F1H5 (7M); B16F1D2 (7N); DMSO-treated B16F1D2 control cells (7O); and B16F1D2+0.5mM ß-D-xyloside (7P)



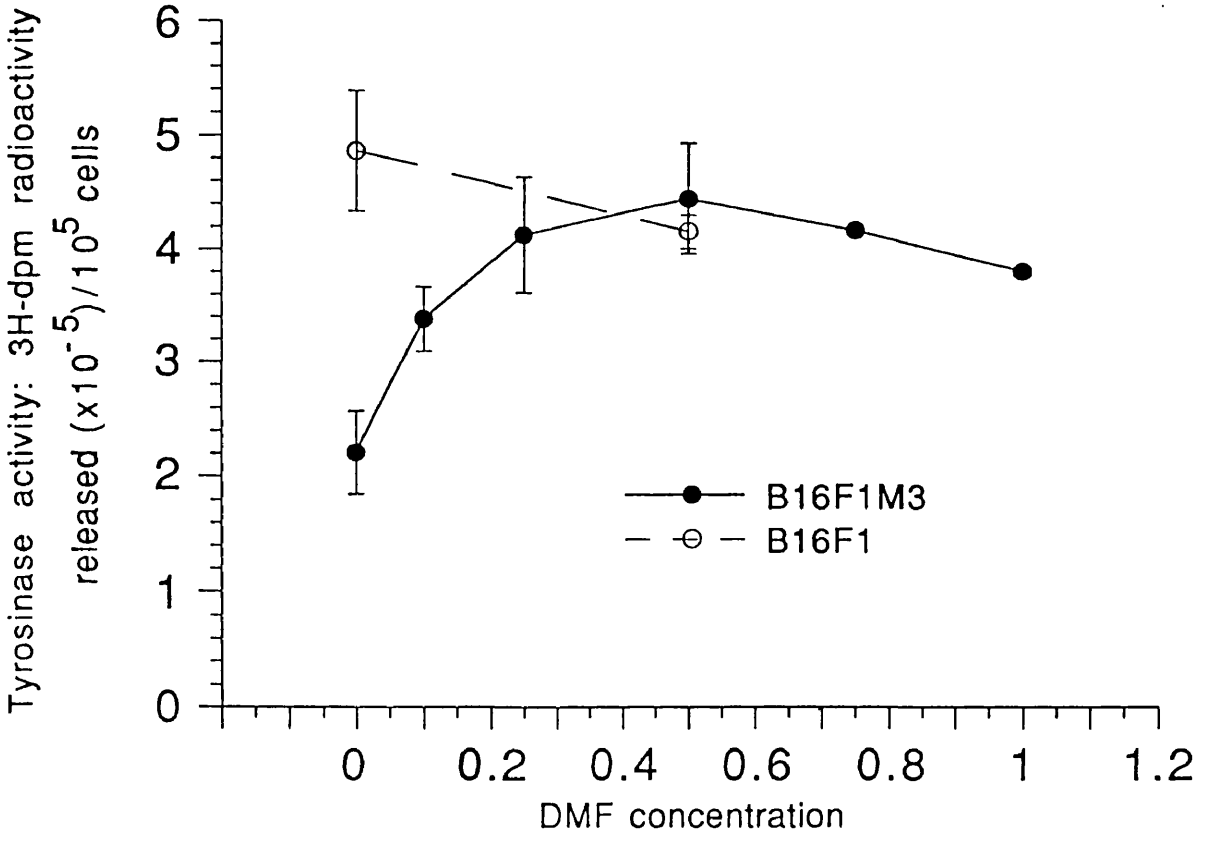
After 24 hours B16F1 cells and DMF-treated B16F1 cells both formed small tumour cell aggregates of similar size, the only observable difference being that DMF-treated cells were more pigmented (figs.7I, 7J). Tumour cell spheroids formed by DMF-treated B16F1M3 cells were no larger than those formed by control cells (figs.7J, 7L) except for being more pigmented. DMF had no obvious effect upon the size of tumour spheroids formed by B16F1 and B16F1M3 cells. At 72 hours DMF had no effect upon the growth rates of tumour spheroids. This was observed for all cell lines tested.

At 24 hours, B16F1D2 tumour cell spheroids appeared to be marginally larger than those of B16F1H5 cells (figs.7M, 7N), and ß-D-xyloside had no apparent effect upon spheroid formation (Figs.7O, 7P). It is therefore difficult to assess the contribution to metastatic potential that differences in spheroid formation in vitro made. The cell adhesion studies to cell monolayers may be more representative especially if since the 24 hour time interval used in this assay is too long for an accurate assessment of cell-cell interactions.

4.3.1. Induction of differentiation: Melanin and tyrosinase

The process of melanogenesis is the most commonly used marker for melanoma cell differentiation. Melanin synthesis is normally associated with differentiated cell function, and the enzyme tyrosinase is involved in several critical steps of the process. The B16 melanoma cell lines and cell clones were relatively amelanotic at low cell densities and melanotic at high densities. Due to the putative effects of DMF upon cellular differentiation, B16F1 and B16F1M3 cells were incubated in differing concentrations of DMF and their intracellular melanin and tyrosinase

Fig.8a: Effect of DMF on tyrosinase activity (as measured by the release of $^{3}H_2O$) in B16 melanoma cells (mean ± SEM; n=4)



levels measured for comparison with controls.

B16F1 melanoma cells were greater than 2-fold more melanotic than their B16F1M3 melanoma counterparts. DMF had a concentration-dependent effect upon melanin and tyrosinase levels with B16 melanoma cells exhibiting a concentration-dependent increase in melanin synthesis and tyrosinase activity (fig.8a). DMF stimulated tyrosinase activity with maximal stimulation occurring around 0.5% DMF. There was a 2-fold maximal stimulation over concentrations of 0.25%-1.0% DMF. The lowest concentration of DMF used (0.1%) produced a 50% stimulation of tyrosinase activity. At higher DMF concentrations there appeared to be a slight inhibition of tyrosinase activity from the maximal level recorded. The level of intracellular melanin followed a near-linear relationship with DMF concentration. Intracellular melanin levels were stimulated up to 6-fold by 1.0% DMF, and 0.5%DMF a greater than 4-fold increase (fig. 8b).

Incubation of B16F1 cells with 0.5% DMF produced a 2-3 fold stimulation of intracellular melanin levels without significantly altering the level of tyrosinase activity (fig.8b). Initially, B16F1 melanoma cells possessed approximately double the tyrosinase activity as B16F1M3 cells. DMF did not alter the tyrosinase activity measured in B16F1 cells and induced a doubling of this activity in B16F1M3 cells. These results might then indicate that the level of tyrosinase activity in B16F1 cells is maximal for these B16 melanomas.

Melanogenesis is influenced by pH but DMF had no affect on the pH of the culture medium. It would therefore appear that DMF does not mediate its effects upon melanogenesis through extracellular pH.

The levels of intracellular melanin synthesized by B16F1H5 and B16F1D2 melanoma cell clones were also measured and no difference recorded (not shown) although both were slightly more melanotic than B16F1 cells.

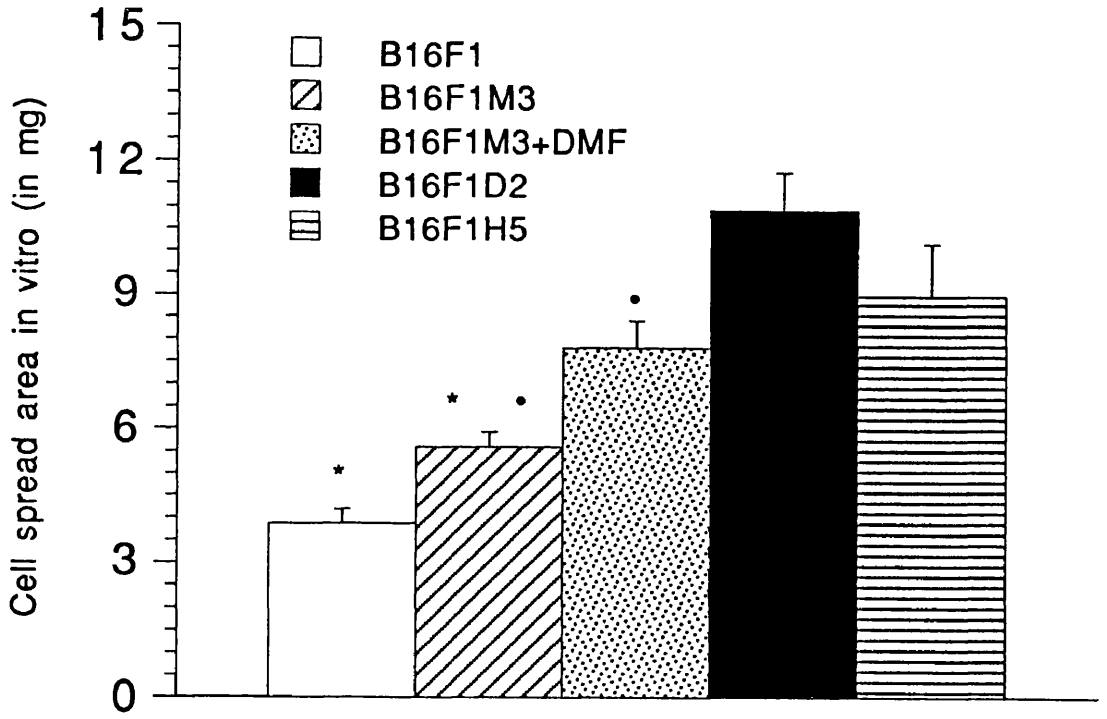
4.3.2. Cell spreading and adhesion

A positive correlation between lung colonization and cell spreading was observed in vitro. Increased lung colonization potential coincided with increased cell spreading for B16F1M3 and DMF-treated B16F1M3 cells (fig.9). However, this correlation did not hold with the B16 melanoma cell clones. This correlation was not evident when B16 melanoma cells were allowed to attach and spread upon ECM glycoproteins in vitro.

Heterogeneity in the in vitro cell adhesion responses to immobilized ECM proteins was observed with the different B16 melanoma cell lines. Cells were also tested for their ability to attach to pulmonary endothelial cell monolayers, BM-like matrices derived from pulmonary endothelial cells, and to melanoma cell monolayers to measure homotypic cell adhesion, A process comparable with spheroid formation.

The level of melanoma cell adhesion to bovine serum albumin (BSA) was also determined since BSA was used as the blocking agent in the in vitro adhesion assays to immobilized matrix proteins. All 35mm petri dishes were treated with 40μg of matrix protein in 1.5ml buffer. The actual amount of matrix protein which bound to the culture dish was not determined, but this concentration had proved to be sufficient for maximal cell adhesion in previous studies performed in this laboratory. Initially each of the B16 melanoma cell lines tested exhibited very poor attachment to BSA-coated dishes (figs.10, 11, 12). At later time points

Fig.9: Cell spread area in vitro (mean ± SEM; n=12)



Significance values (Student's t-test): * P<0.01; • P<0.01

increased levels of adhesion were often observed (figs.10,12) that might indicate the initiation or employment of other cellular process to facilitate cell adhesion.

The melanoma cell lines were tested for their abilities to attach to rat tail tendon collagen. The collagen derived from this source is a complex of interstitial collagens type I and III. Initially this substrate proved to be a relatively poor substrate for B16 melanoma cell adhesion (figs.13, 14, 15, 16). Within the first 30 minutes incubation the levels of cell attachment were $\leq$10% for all cell lines tested. After 60 mins incubation maximal attachment was usually in the range of 10%-30%. Some cell lines exhibited a significant increase in cell attachment by 90 minutes, and this might possibly reflect either degradation of collagen or the synthesis of factors which facilitate attachment to collagen (figs.13, 15).

Fibronectin provided a highly adhesive substrate for the attachment of all B16 melanoma cell lines tested (figs.17, 18, 19, 20). Cell attachment to fibronectin substrates was rapid with high levels of attachment being attained (60%-90% after 90mins) for all cell lines irrespective of metastatic potential.

Differences in cell attachment to BM-derived proteins was more evident. Moderately metastatic B16F1M3 cells displayed a higher maximal adhesion to petri dishes coated with laminin-nidogen complex when compared with poorly metastatic B16F1 cells (fig.21). Pretreatment of B16F1M3 cells with 0.5%DMF reduced this maximal adhesion to a level that was similar to that attained for B16F1 cells. B16F1M5 melanoma cells, which later proved to be unstable, displayed similar levels of cell attachment to

Fig.10: Cell attachment to BSA (mean ± SEM; n=6)

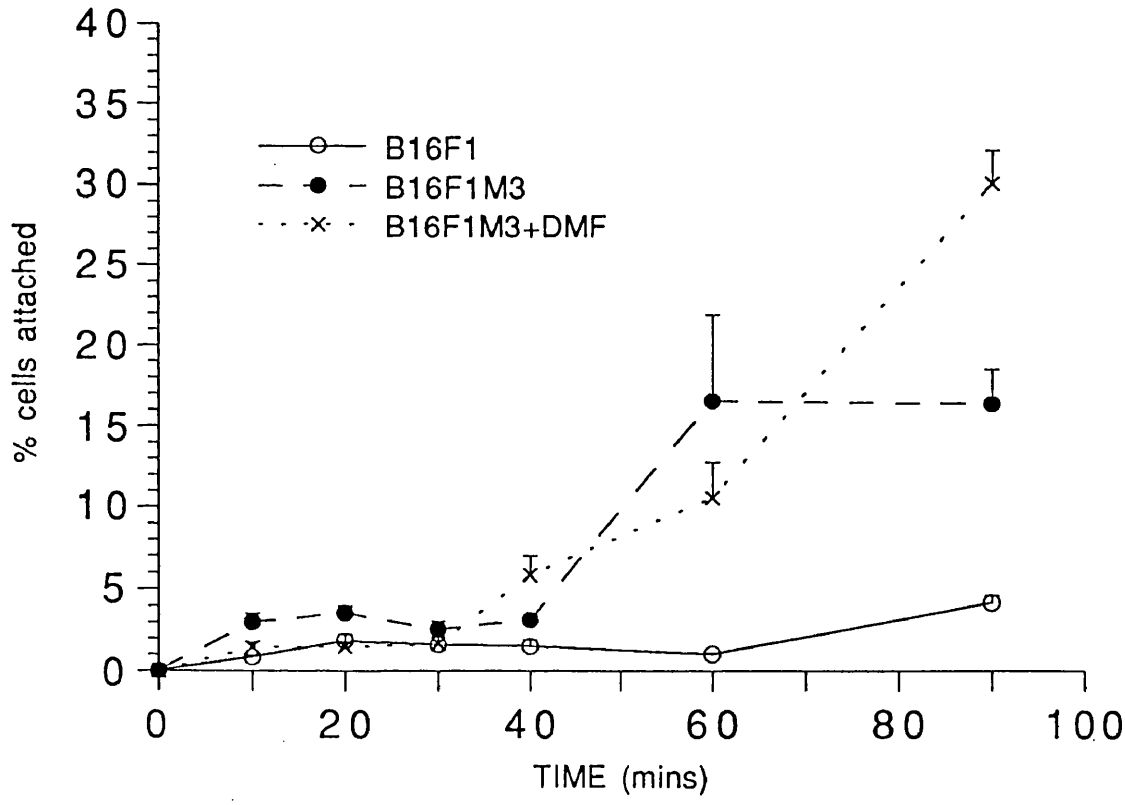


Fig.11: Cell attachment to BSA (mean ± SEM; n=6)

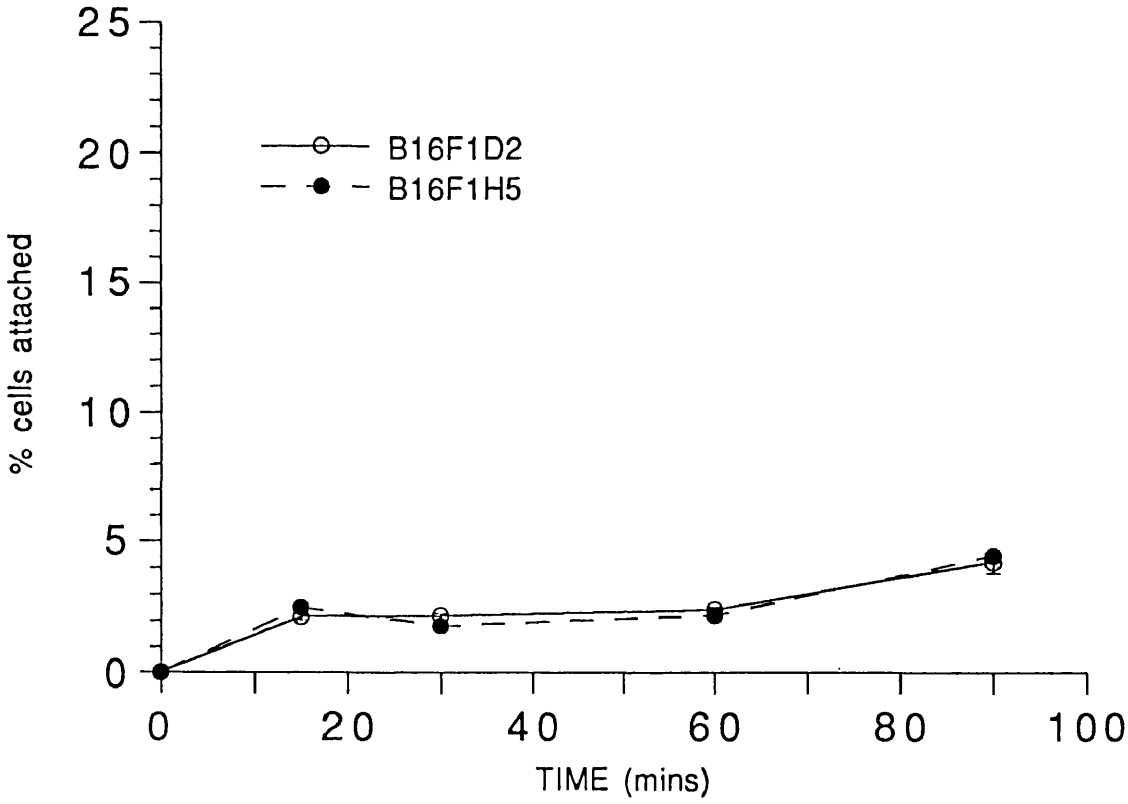


Fig.12: Cell attachment to BSA (mean ± SEM; n=6)

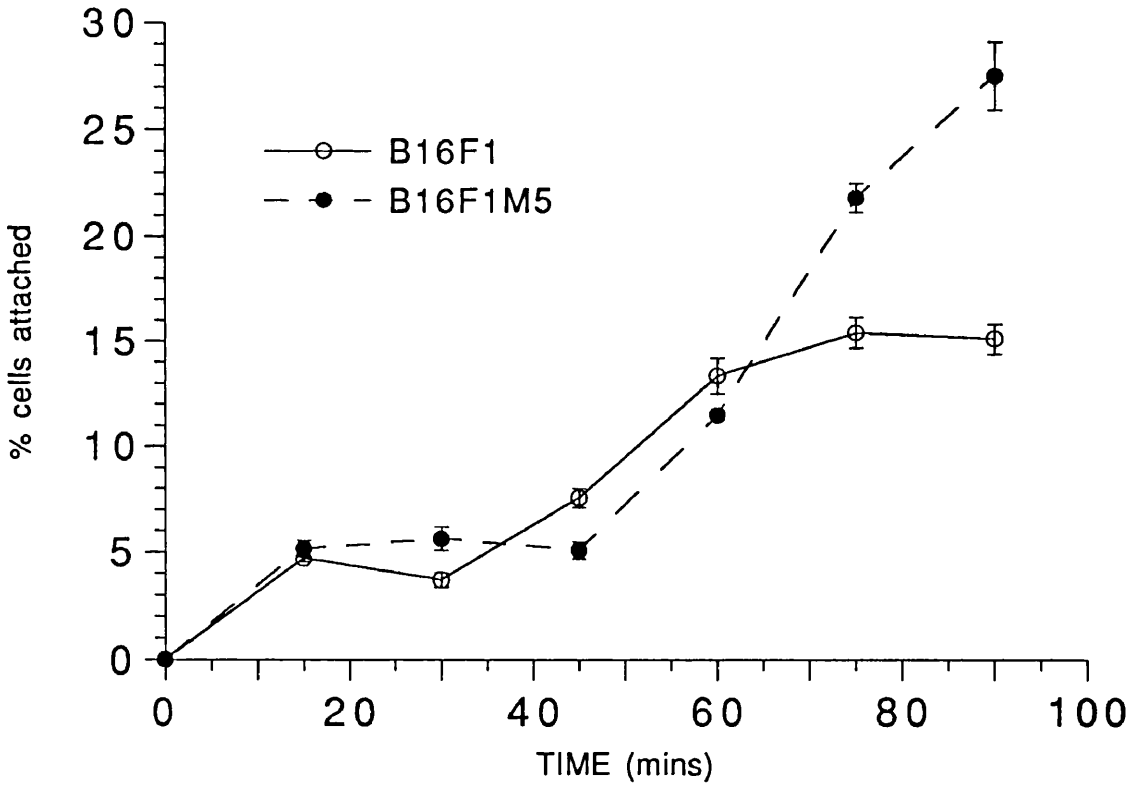


Fig.13: cell attachment to collagen type I (mean ± SEM; n=12)

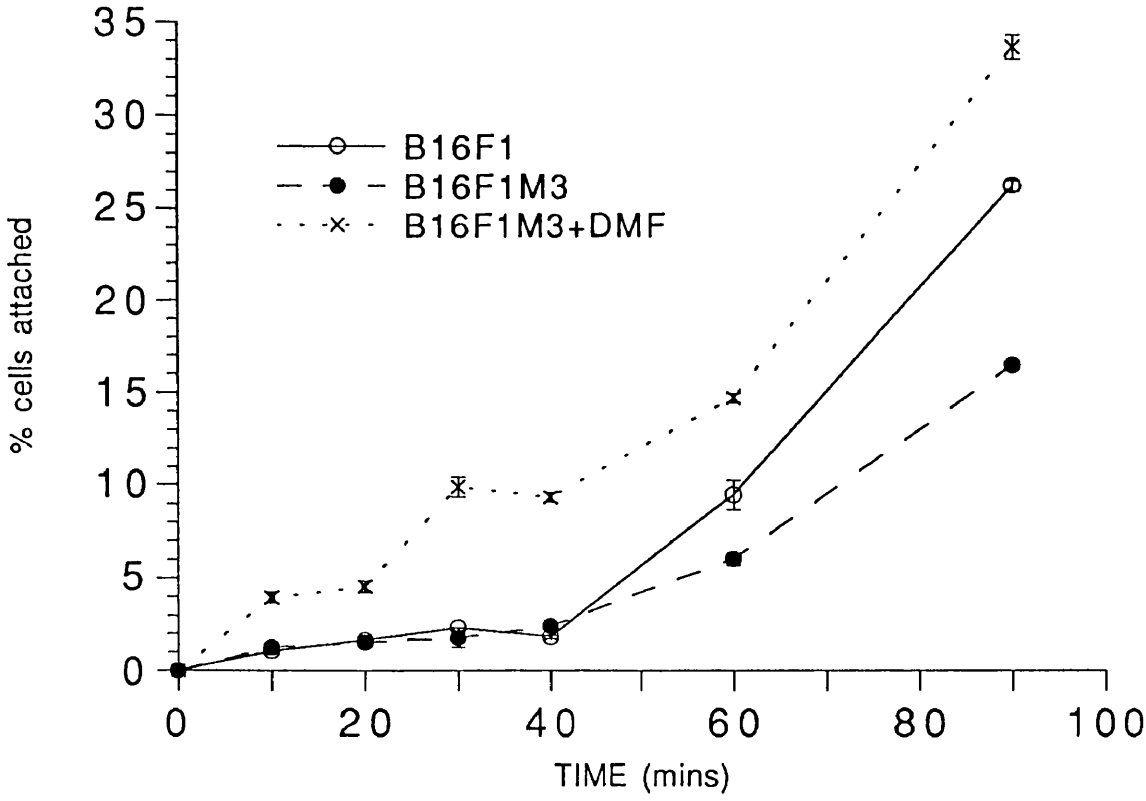


Fig.14; cell attachment to collagen type I (mean ± SEM; n=6)

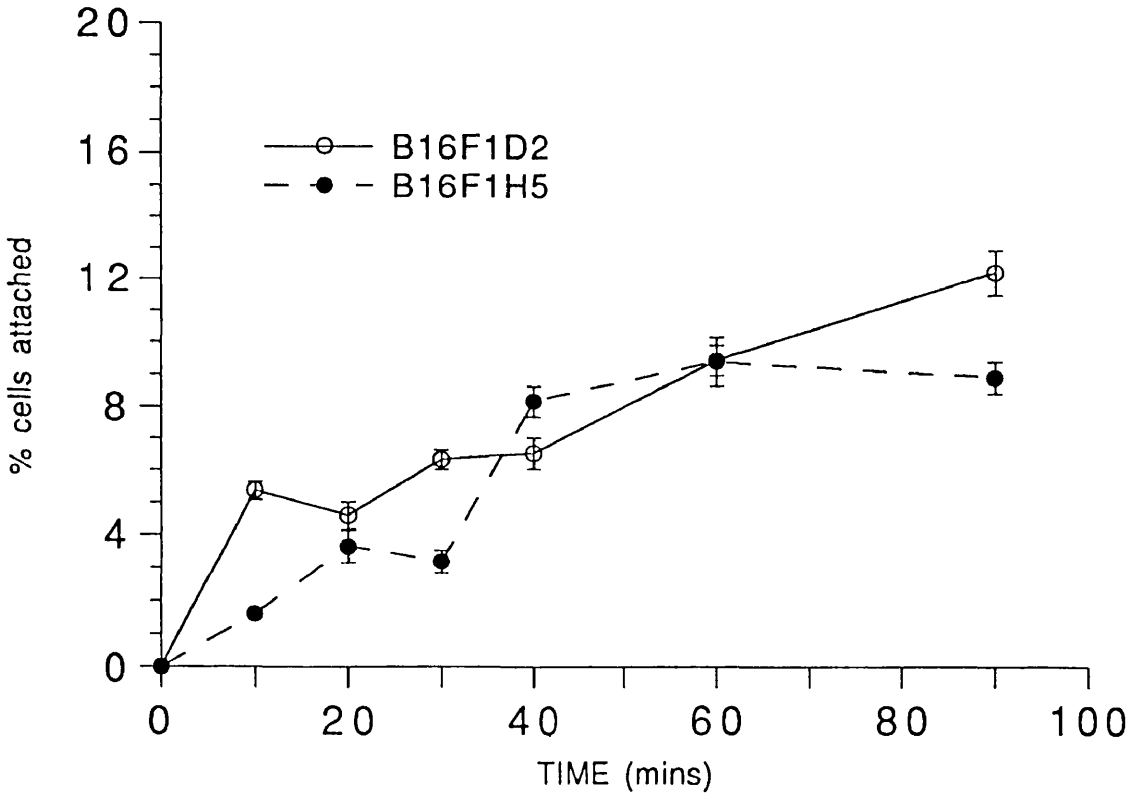


Fig.15: Cell attachment to collagen type I (mean ± SEM; n=6)

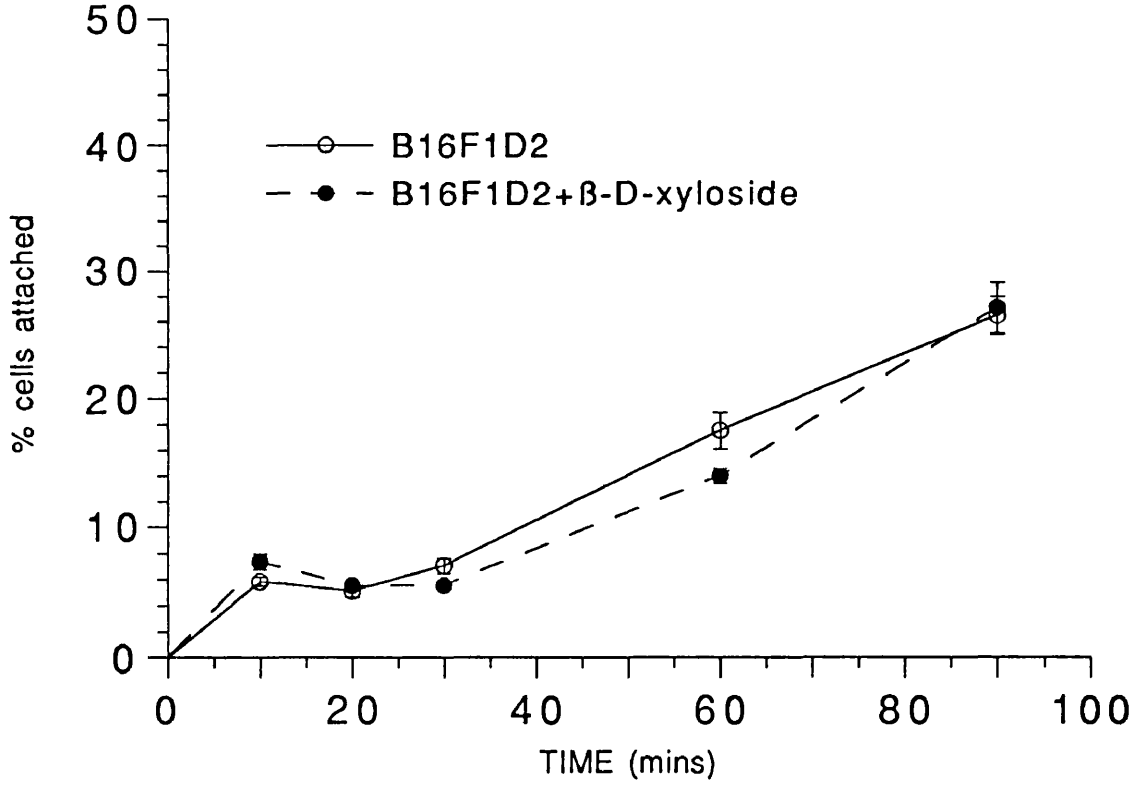


Fig.16: Cell attachment to collagen type I (mean ± SEM; n=12)

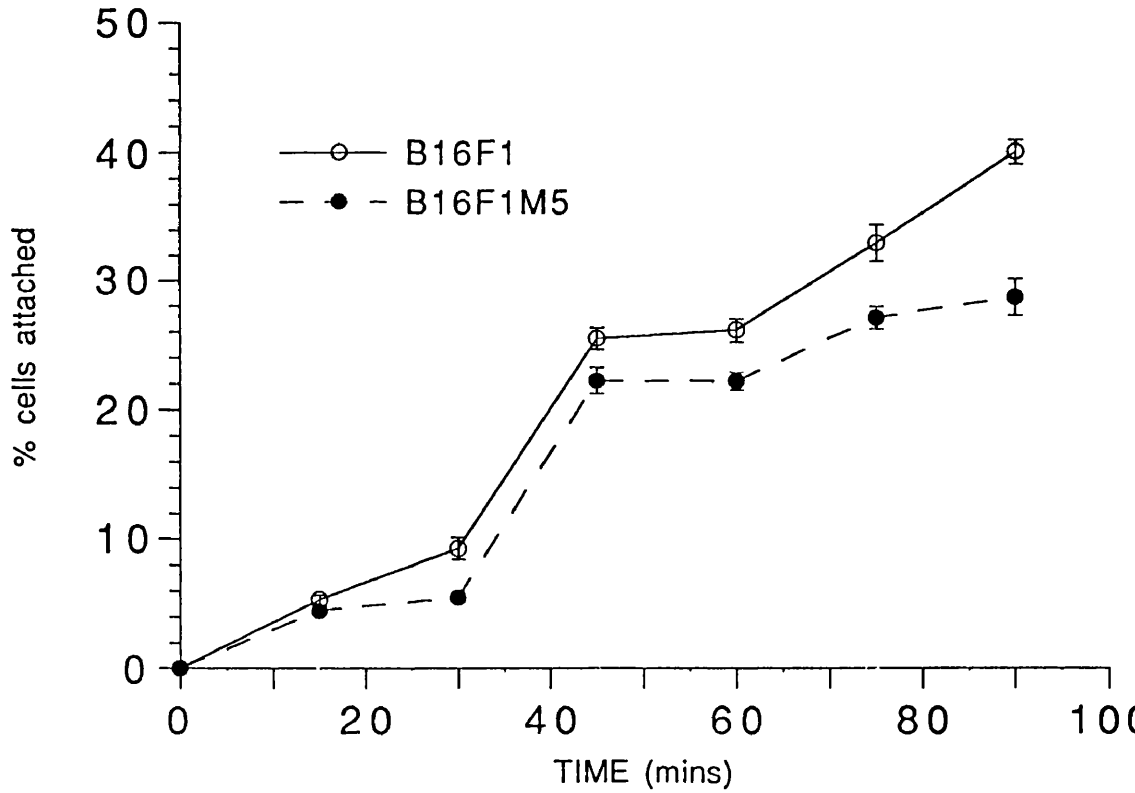


Fig.16: Cell attachment to collagen type I (mean ± SEM; n=12)

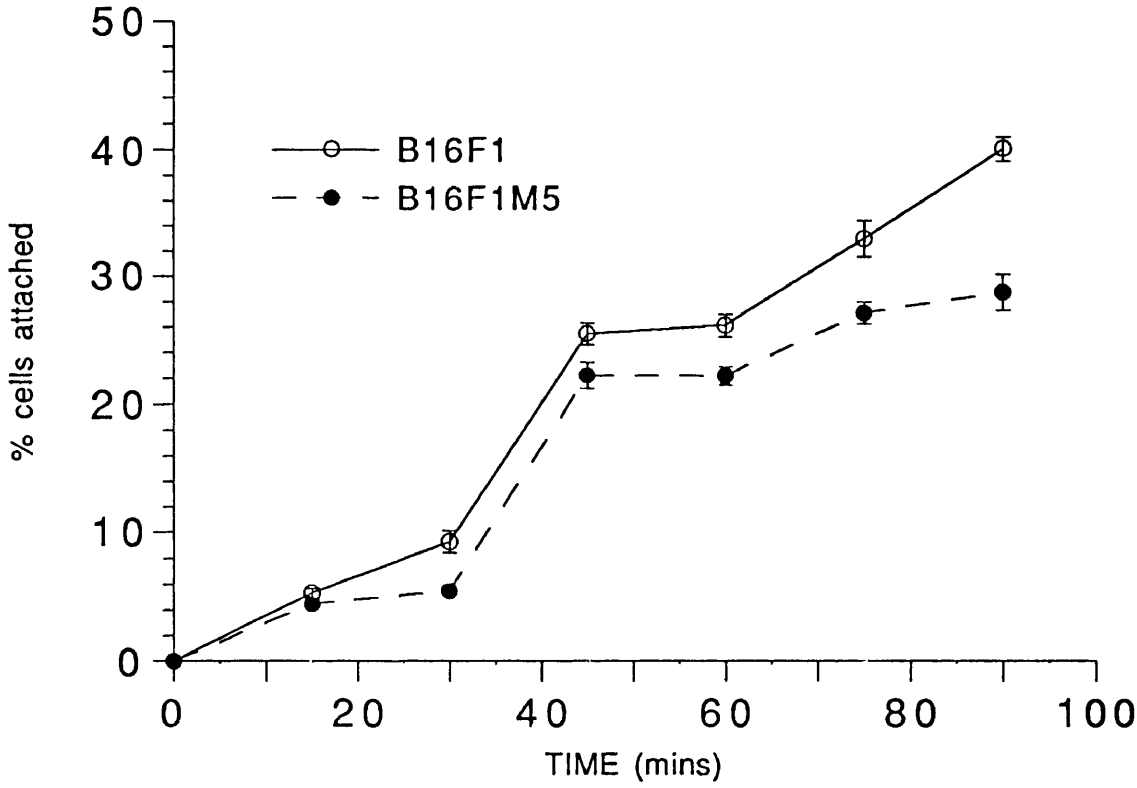


Fig.17: Cell attachment to fibronectin (mean ± SEM; n=12)

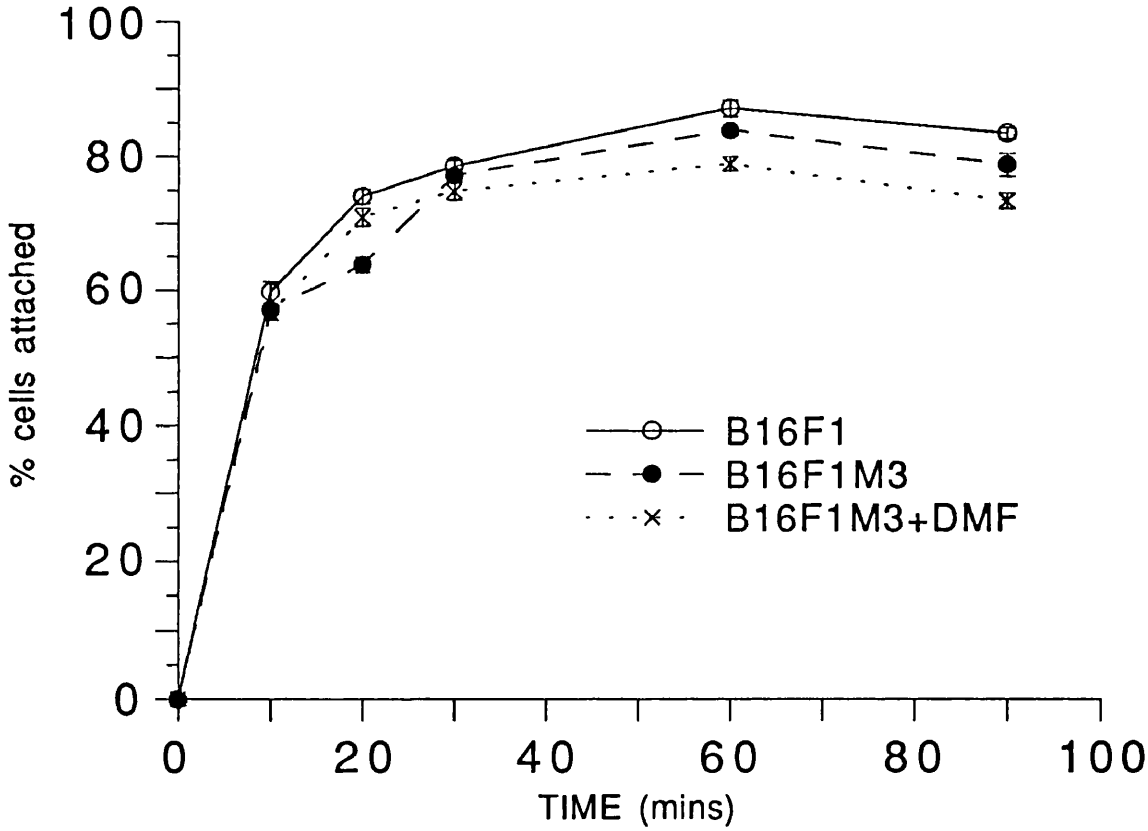


Fig.18: Cell attachment to fibronectin (mean ± SEM; n=6)

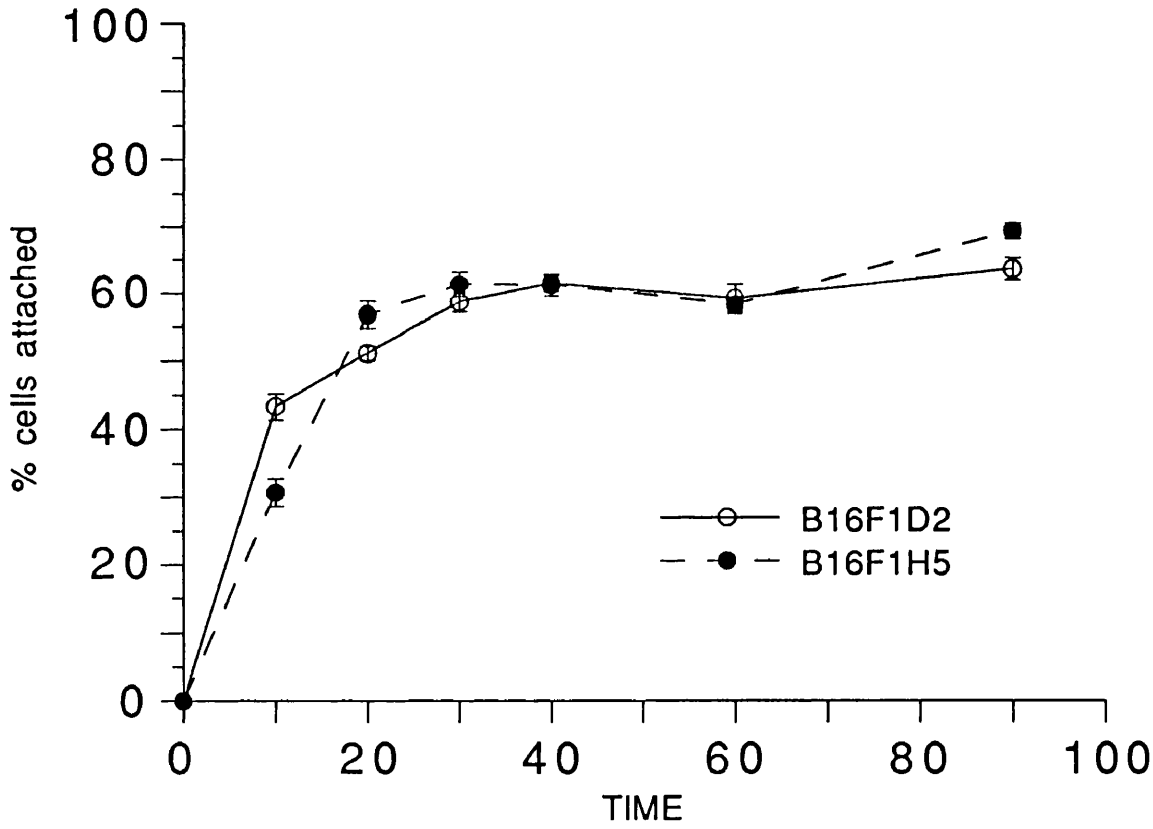


Fig.19: Cell attachment to fibronectin (mean ± SEM; n=6)

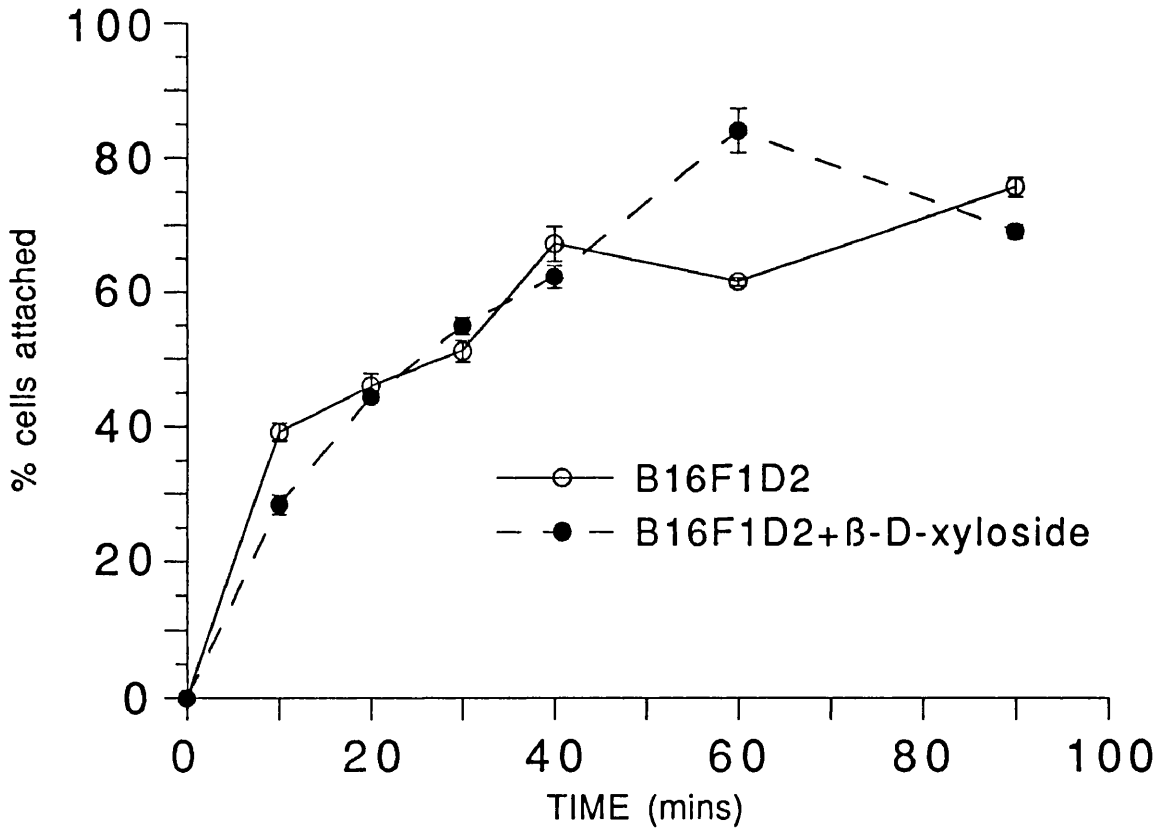
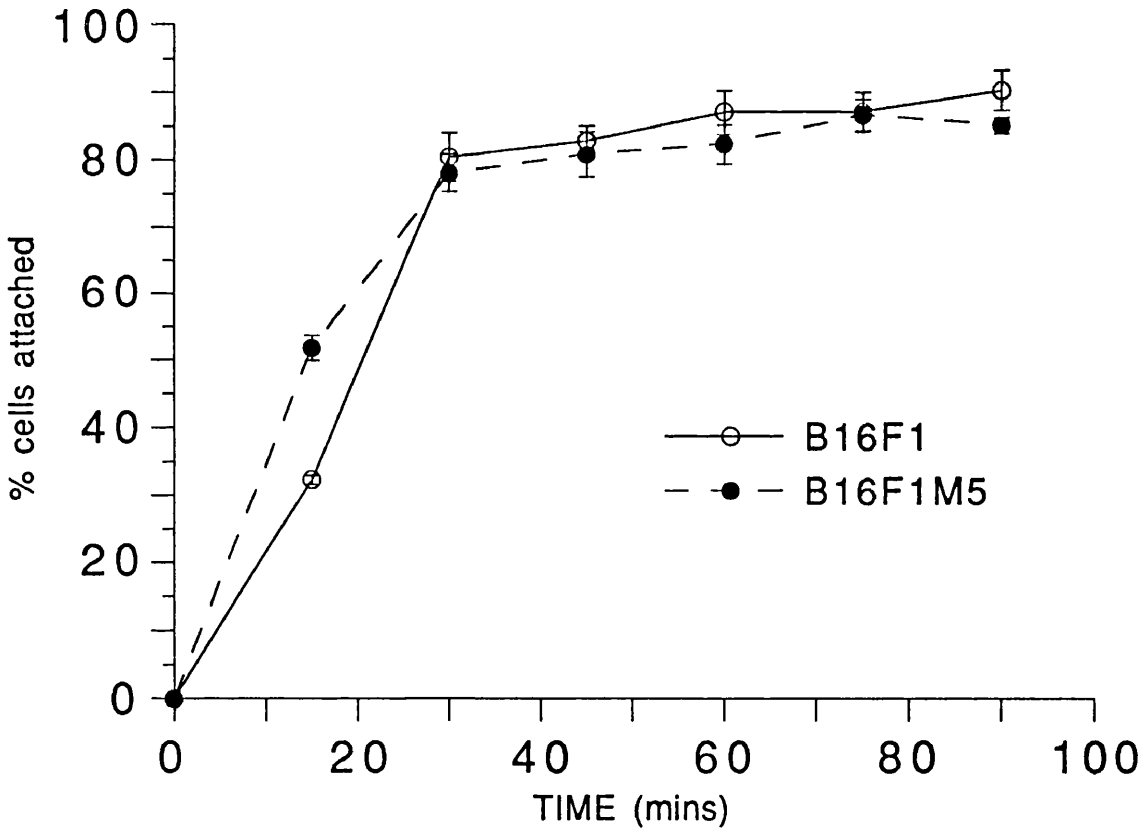


Fig.20: Cell attachment to fibronectin (mean ± SEM; n=6)



laminin-nidogen (fig.22) as B16F1 cells. Conversely, the B16F1H5 clone displayed a higher maximal adhesion to laminin-nidogen than the B16F1D2 clone (fig.23). Pretreatment of B16F1D2 melanoma cells with 0.5mM ß-D-xyloside did not influence cell attachment to laminin-nidogen-coated dishes (fig.24). The heterogeneity in the levels of cell adhesion to laminin-nidogen substrates in vitro by metastatic and poorly metastatic B16 melanoma cells suggests that no specific adhesion response to laminin-nidogen correlates with the metastatic potential of these cell lines. The ability to adhere to laminin, irrespective of efficiency, may only be required to initiate subsequent events which may be critical for the continuence or completion of the metastatic process.

In the adhesion assays, both B16F1M3 and DMF-treated cells exhibited a faster initial rate of attachment to BM collagen IV than poorly metastatic B16F1 cells (fig.25). The maximal levels of attachment were similar for B16F1, B16F1M3 and DMF-treated cells, although B16F1 cells had a slightly higher proportion of cells attached by 90 mins. B16F1M5 cells displayed a higher rate and maximal level of cell attachment than B16F1 cells (fig.26). Elevated levels of initial cell attachment may be crucial since the process of extravasation is complete within minutes in vivo. Once again the converse applied to B16 melanoma clones; poorly metastatic B16F1H5 cells had a faster initial rate of attachment to collagen IV than the highly metastatic B16F1D2 cells (fig.27) although the maximal level of attachment at 90 mins was similar for both. ß-D-xyloside treatment inhibited the attachment of B16F1D2 melanoma cells to BM collagen IV substrates (fig.28) suggesting that proteoglycans are potentially involved in this adhesion process.

Fig.21: Cell attachment to laminin/nidogen (mean ± SEM; n=12)

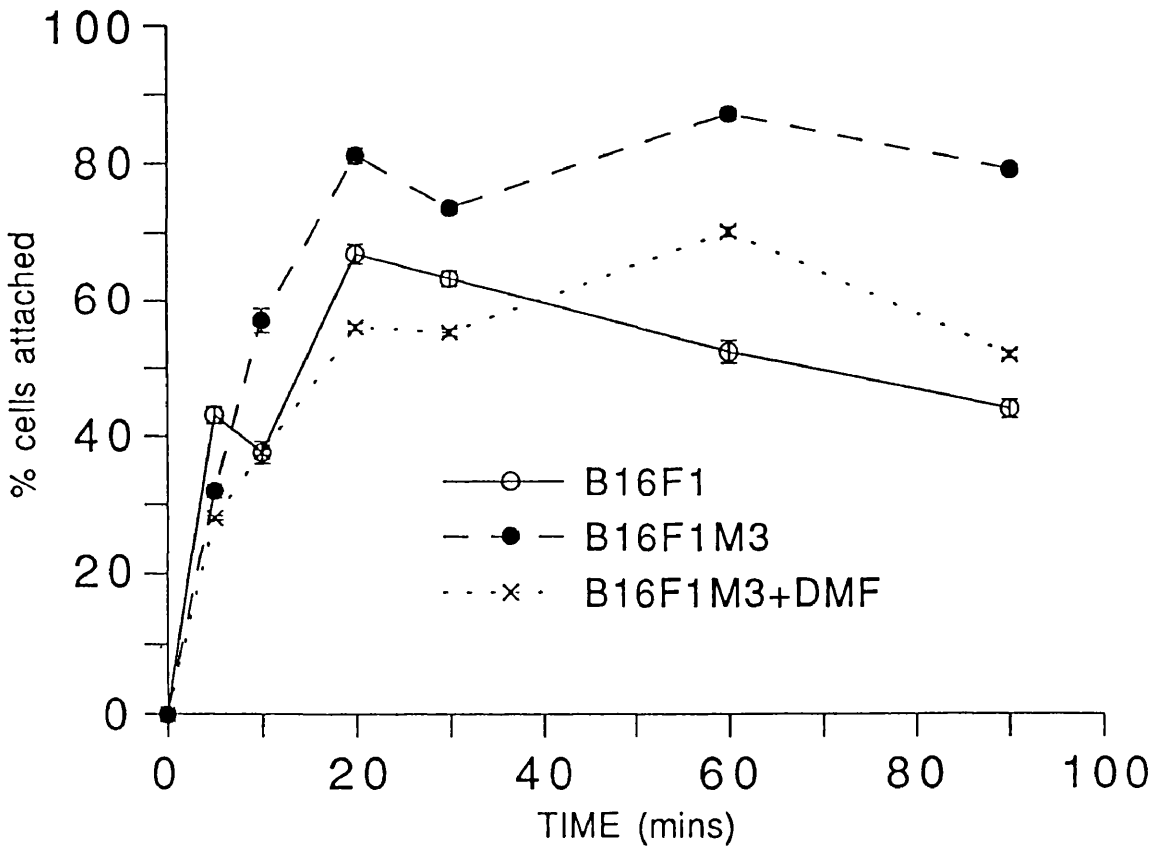


Fig.21; The adhesion values for obtained B16F1M3 cells over time points 20-90 mins were statistically significant (0.01>P; Student's t-test) when compared with those obtained for B16F1 and DMF-treated cells.

Fig.22: Cell attachment to laminin/nidogen (mean ± SEM; n=12)

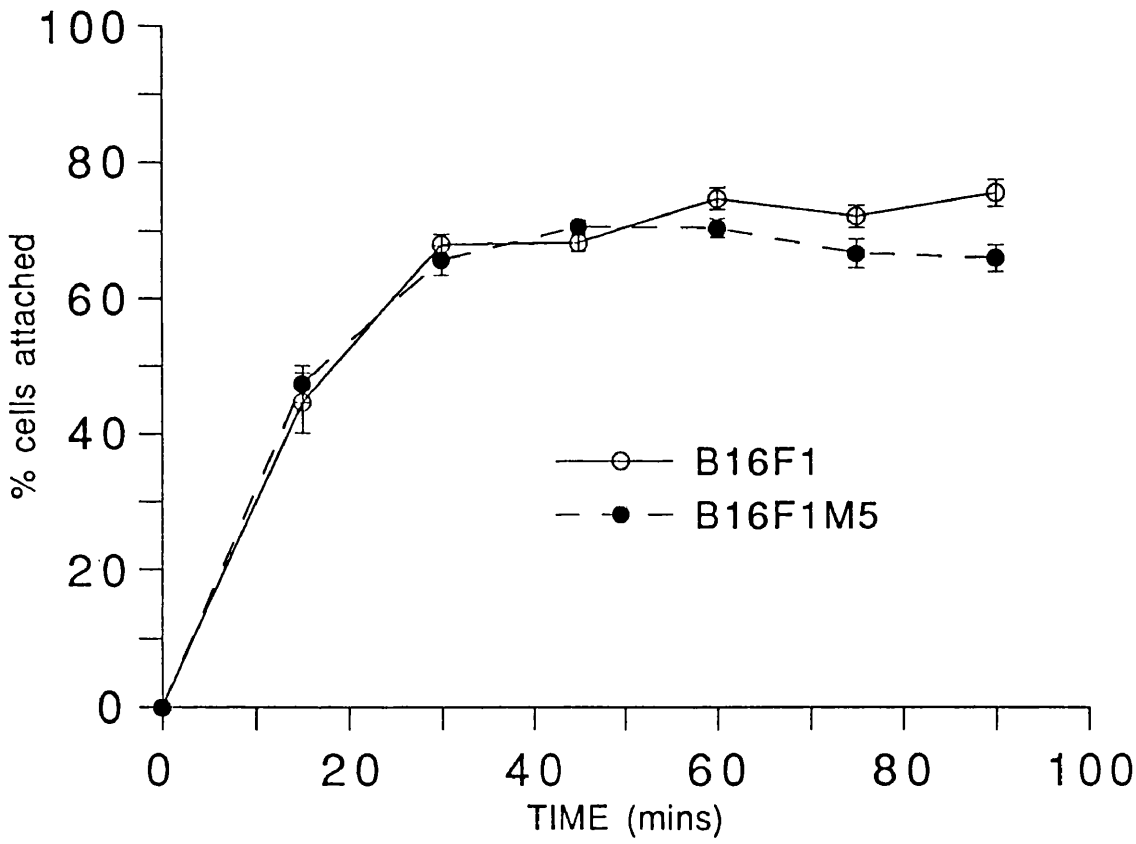


Fig.23; Cell attachment to laminin/nidogen (mean ± SEM; n=6)

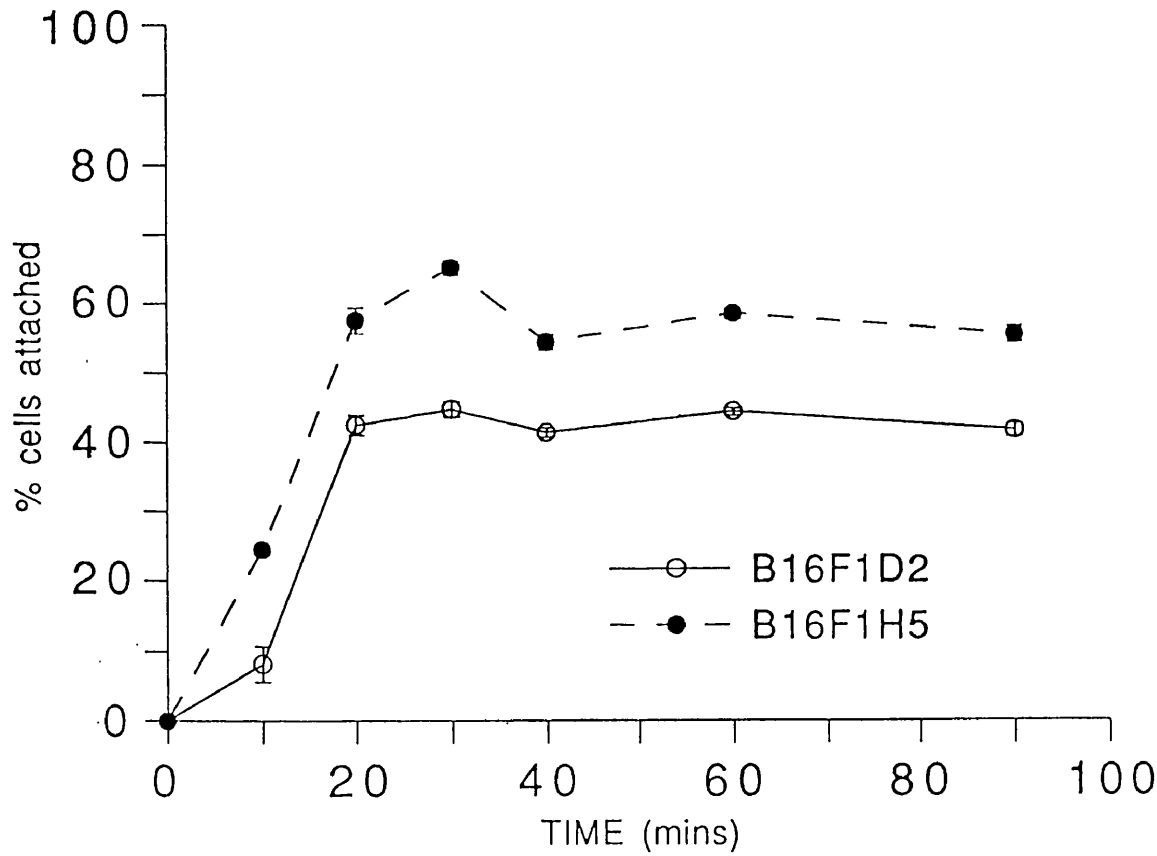


Fig.23; The adhesion values obtained for B16F1H5 cells over the time points 10-90 mins were statistically significant (0.01>P; Student's t-test) when compared with those obtained for B16F1D2 cells.

Fig.24: Cell attachment to laminin/nidogen (mean ± SEM; n=6)

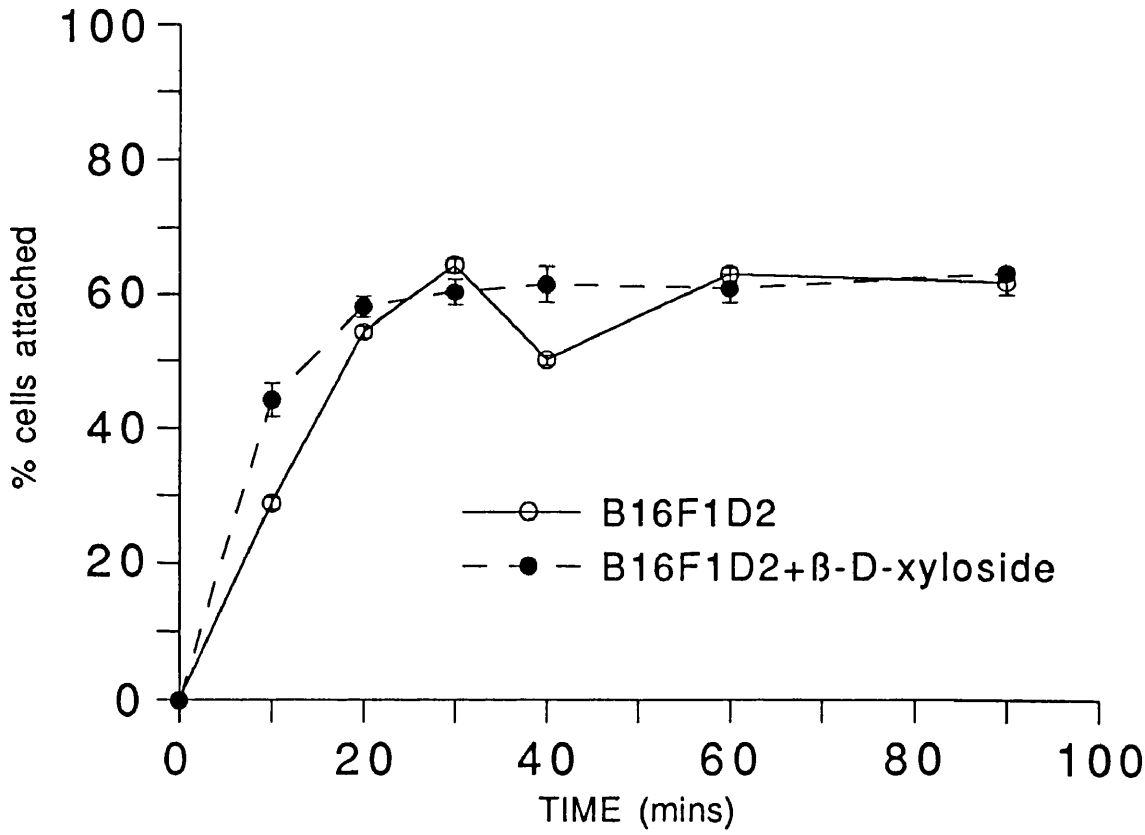


Fig.25; Cell attachment to collagen type IV (mean ± SEM; n=6)

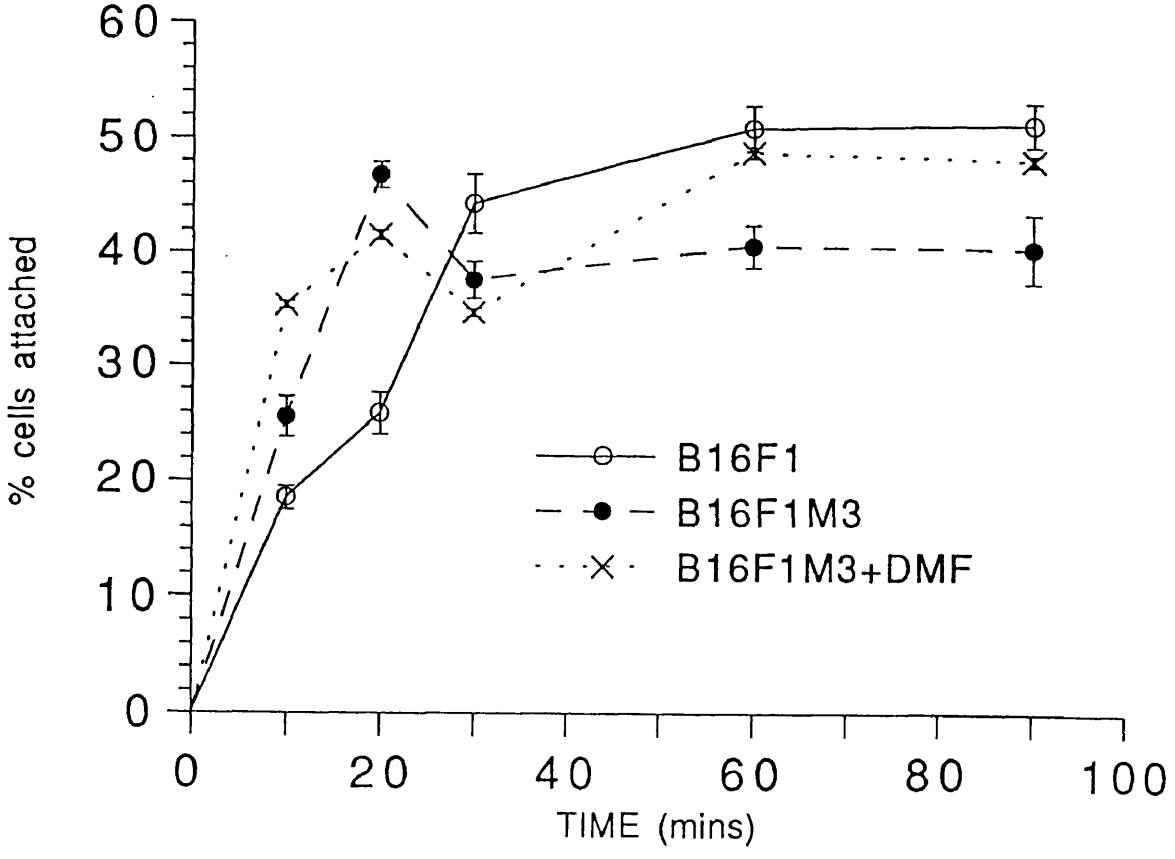


Fig.25; The adhesion values for B16F1M3 and DMF-treated B16F1M3 cells over the first 20 mins were statistically significant (0.05>P; Student's t-test) when compared with those obtained for B16F1 cells.

Fig.26; Cell attachment to collagen type IV (mean ± SEM; n=6)

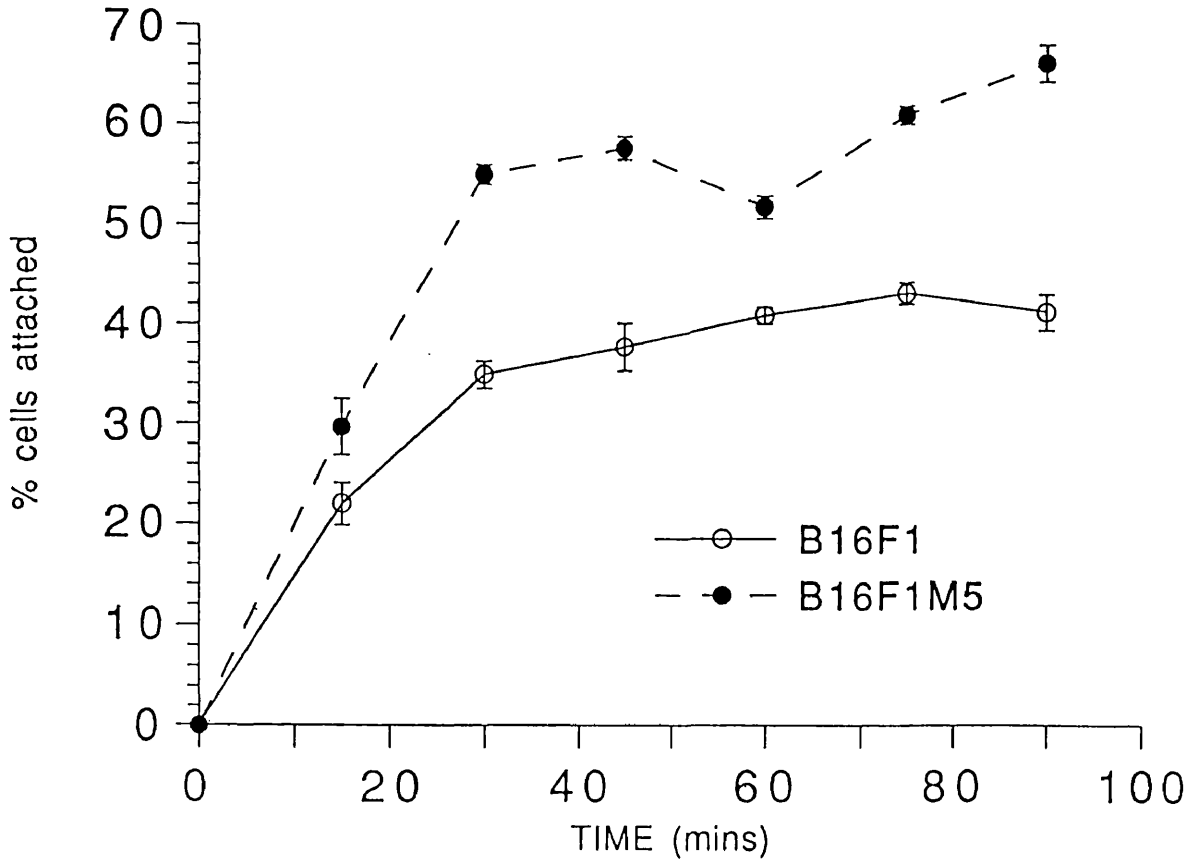


Fig.26; The adhesion values obtained for B16F1M5 cells over the time points 30-90 mins were statistically significant (0.01>P; Student's t-test) when compared with those obtained for B16F1 cells.

Fig.27; Cell attachment to collagen type IV (mean ± SEM; n=6)

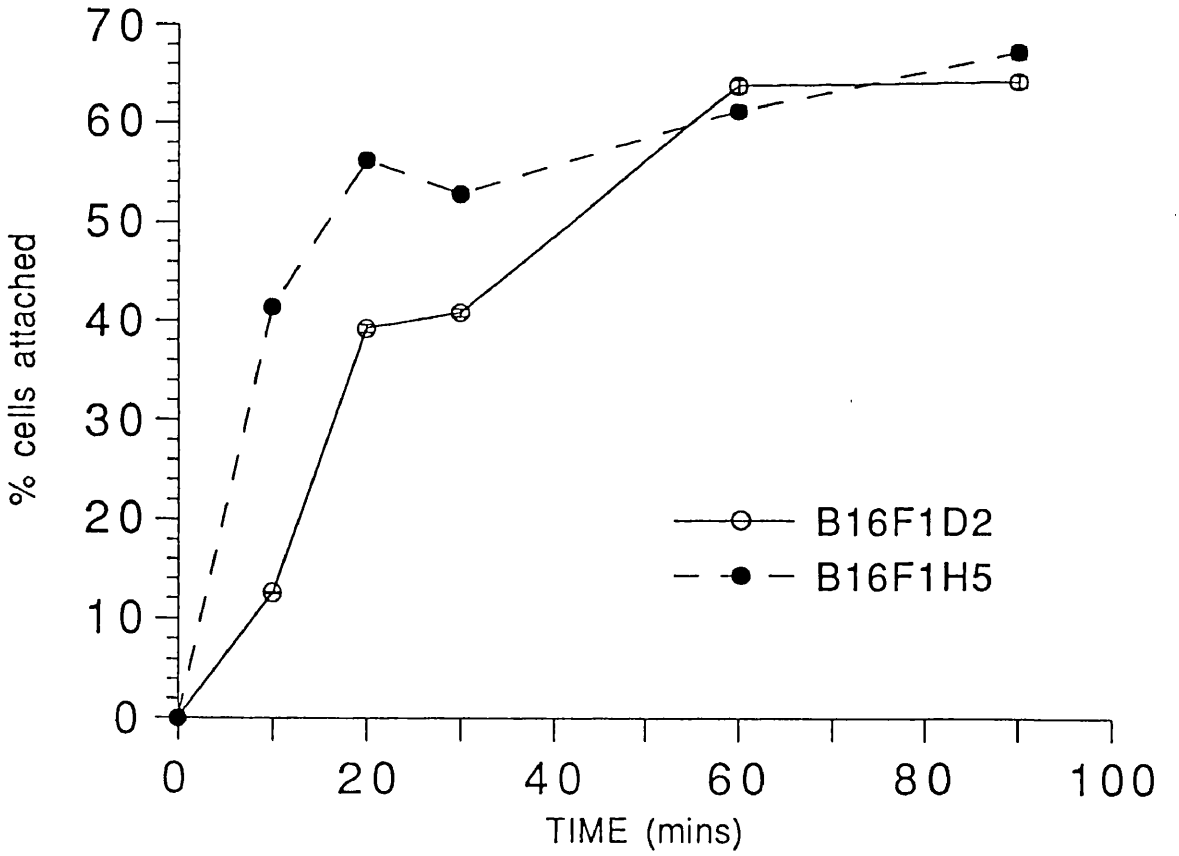


Fig.27; The adhesion values obtained for B16F1H5 cells over the time points 10-30 mins were statistically significant (0.01>P; Student's t-test) when compared with those obtained for B16F1D2 cells.

Fig.28; Cell attachment to collagen type IV (mean ± SEM; n=6)

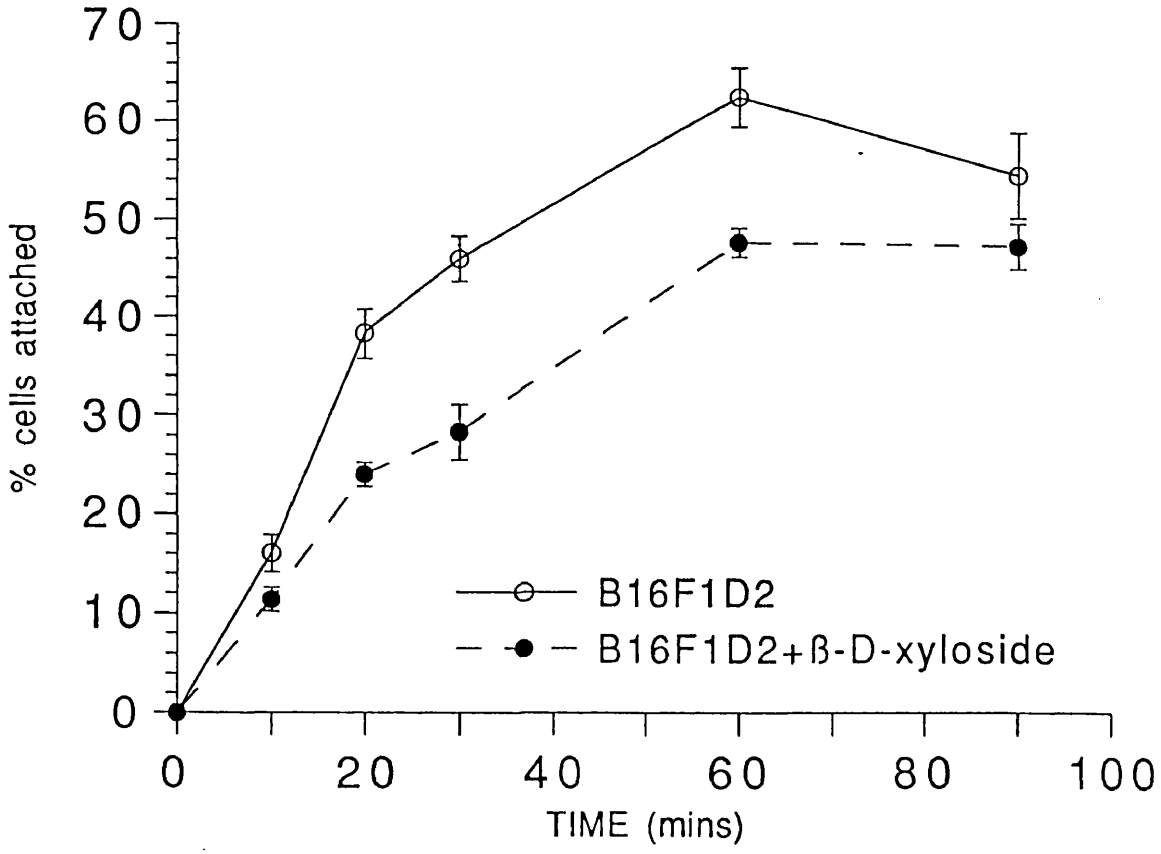


Fig.28; The adhesion values obtained for B16F1D2 cells over the time points 20-60 mins were statistically significant (0.01>P; Student's t-test) when compared with those obtained for ß-D-xyloside treated cells.

Pulmonary endothelial cells and their underlying BM-like matrix was assessed for their ability to support B16 melanoma cell attachment since these structures are more representative of naturally occuring substrates encountered by tumour cells in vivo. For those B16 melanoma cell lines tested, there was both a rapid rate of cell attachment to CPAE monolayers (figs.29, 30) and to isolated subendothelial matrices (figs.31, 32) and high levels of maximal cell attachment. No differences in the rate and level of cell attachment to these substrates could be identified between metastatic and and poorly metastatic B16 melanoma cell lines and clones. The fact that there are no discernible differences in the rate and level of attachment to the subendothelial matrix (figs.31, 32) questions the significance and relevance of cell adhesion assays to individual basement membrane proteins. The most important aspect may be the secondary events that are elicited by the interaction of cells with matrix proteins which can influence the metastatic process.

Once again heterogeneity in the adhesion responses of cell lines to the same substrate was observed (e.g. Figs.23 & 24). These differences may reflect changes in the cell populations through a measure of phenotypic drift or changes in the cell subpopulations that constitute the cell line. Differences in cell culture conditions may also have proved influential

A short series of homotypic cell adhesions was also performed to complement the spheroid studies in which B16 melanoma cell lines were assayed for their ability to attach to B16 melanoma monolayers. Poorly metastatic B16F1 melanoma cells and moderately metastatic B16F1M3 melanoma cells had similar rates and maximal levels of cell attachment to their monolayers (fig.33). However, DMF treatment resulted in the

Fig.29: Cell attachment to CPAE monolayers (mean ± SEM; n=6)

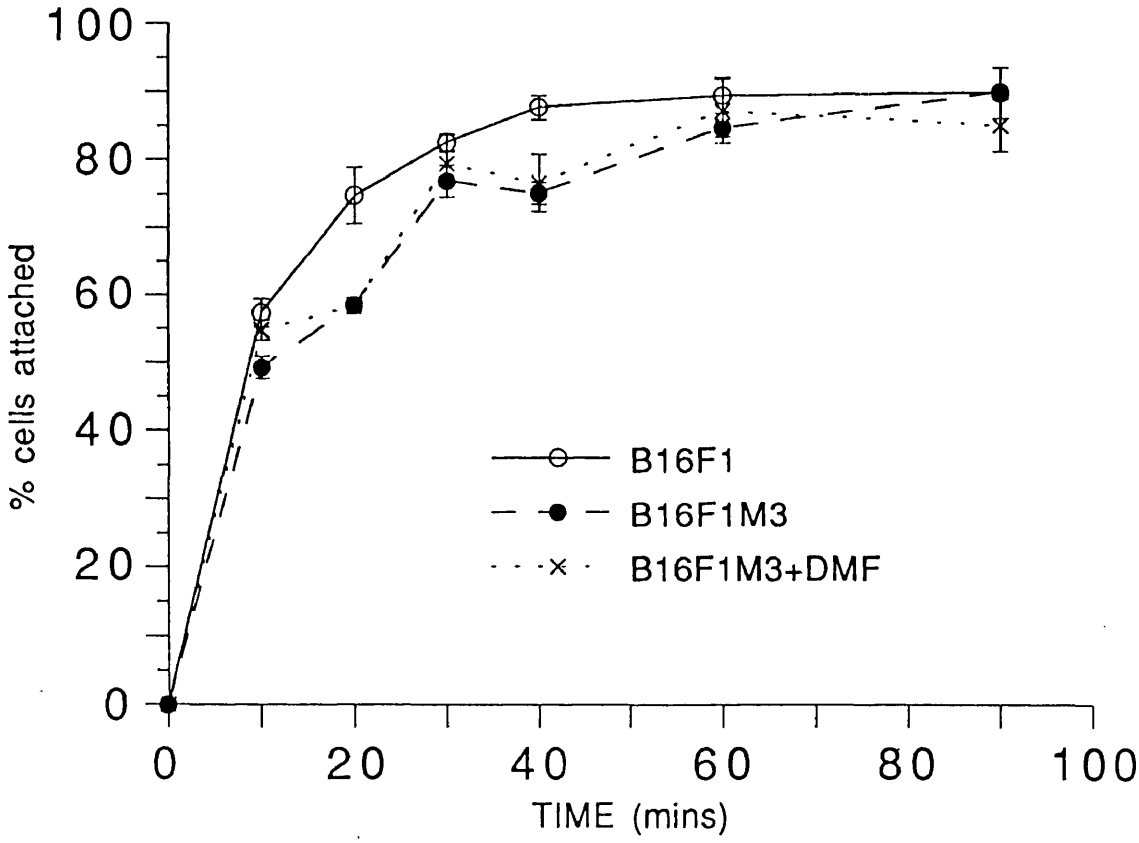


Fig.30; Cell attachment to CPAE monolayers (mean ± SEM; n=6)

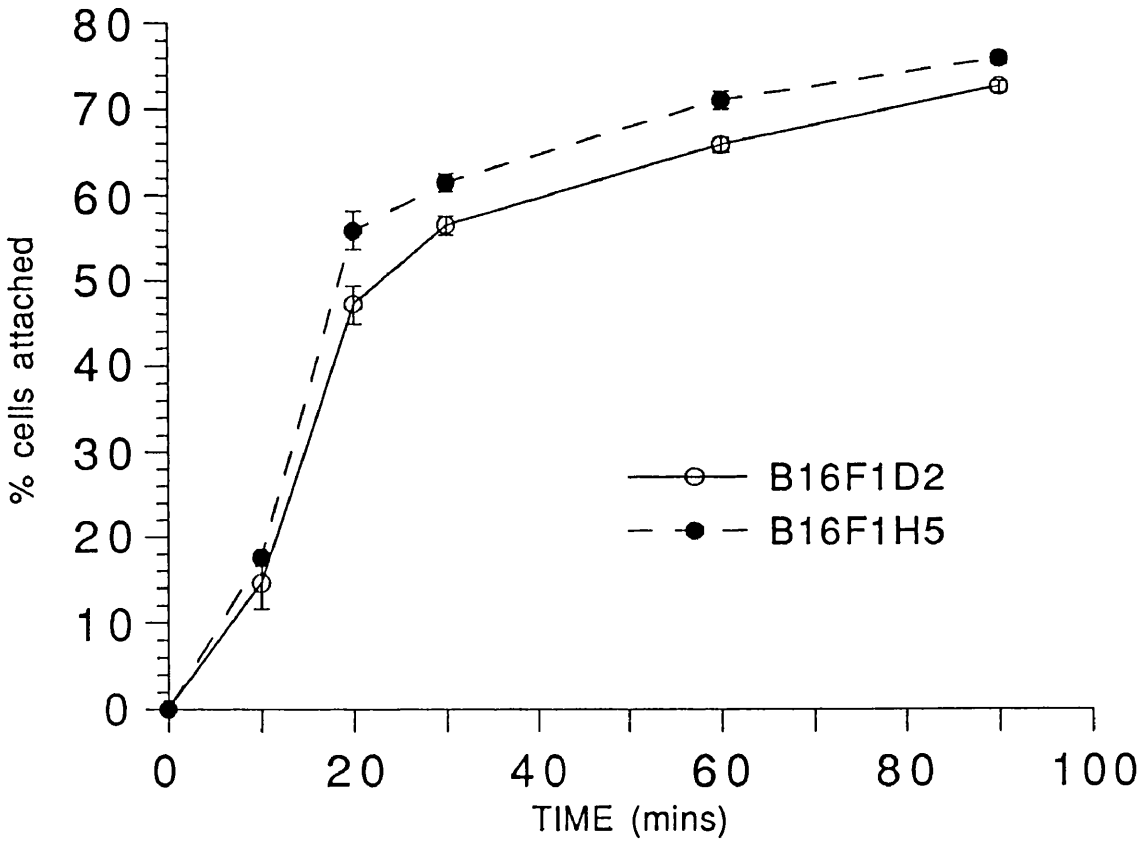


Fig.31: Cell attachment to CPAE subendothelial matrix
(mean ± SEM; n=6)

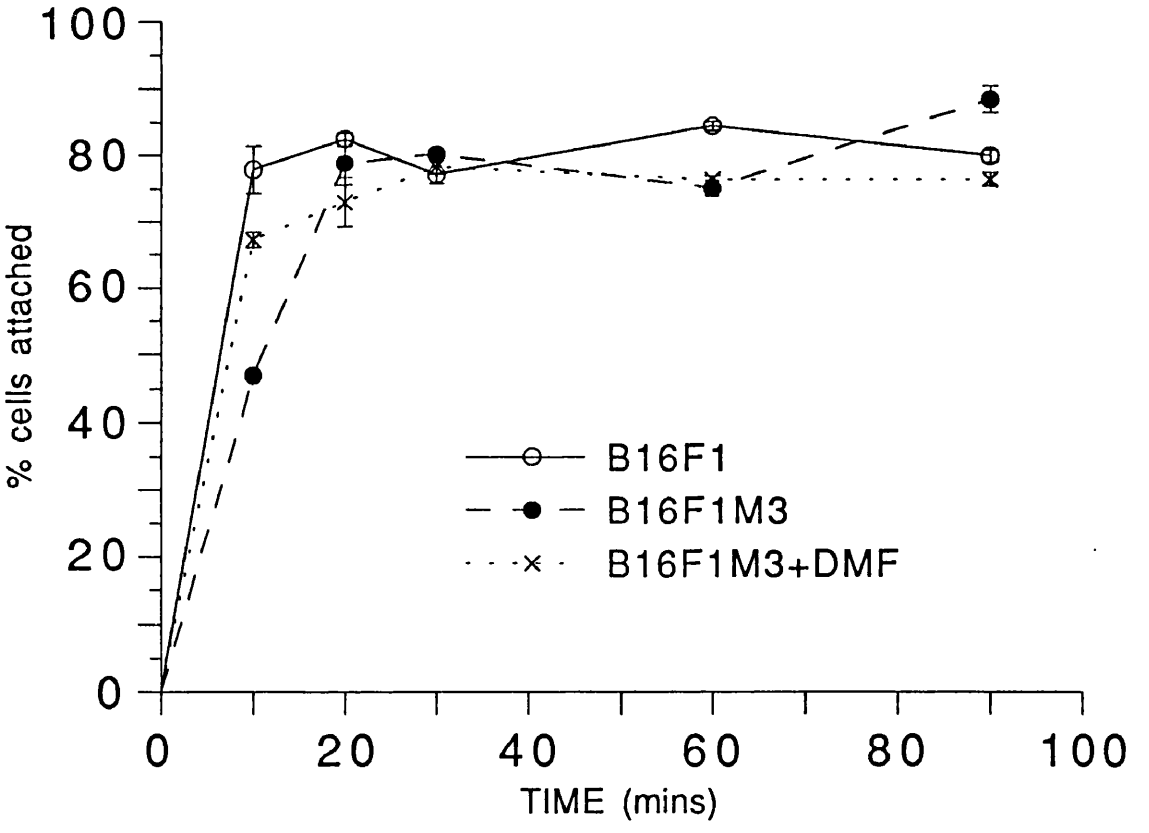


Fig.32: Cell attachment to CPAE subendothelial matrix
(mean ± SEM; n=6)

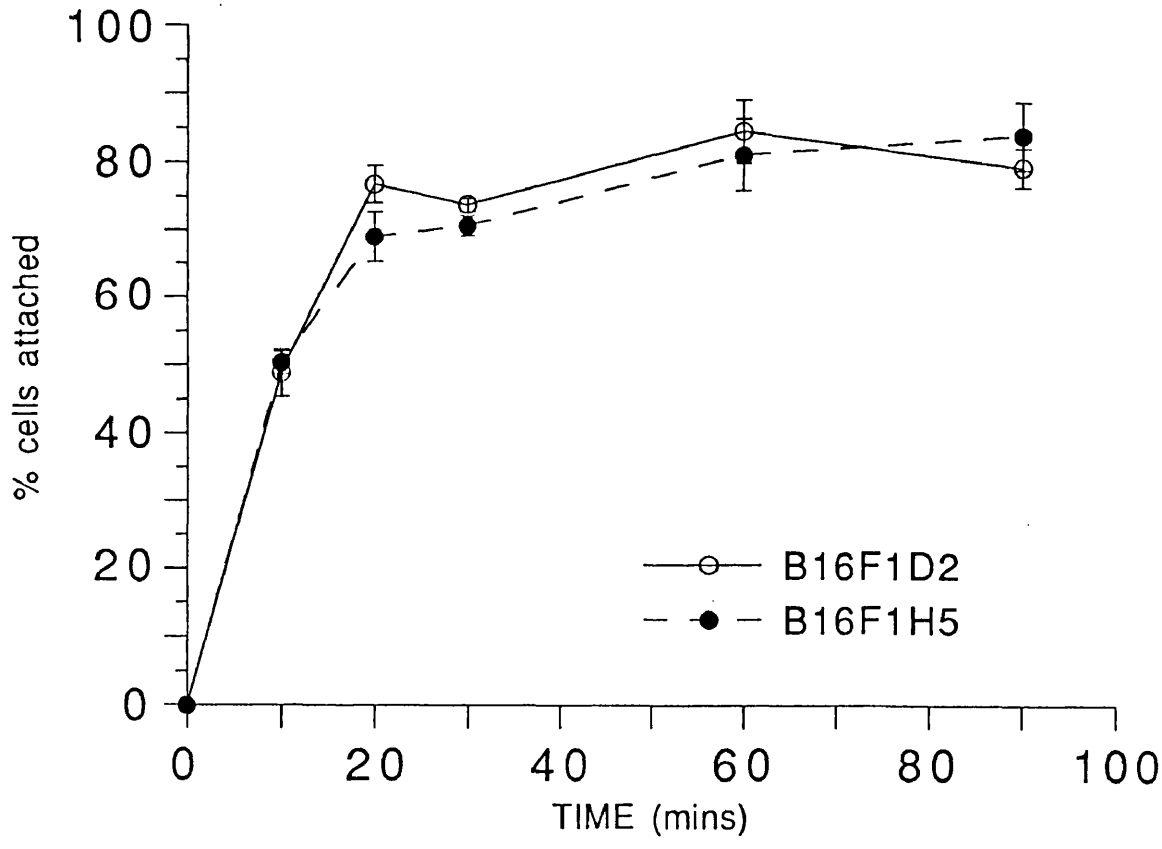


Fig.33; Cell attachment to (homotypic) B16 melanoma monolayers (mean ± SEM; n=6)

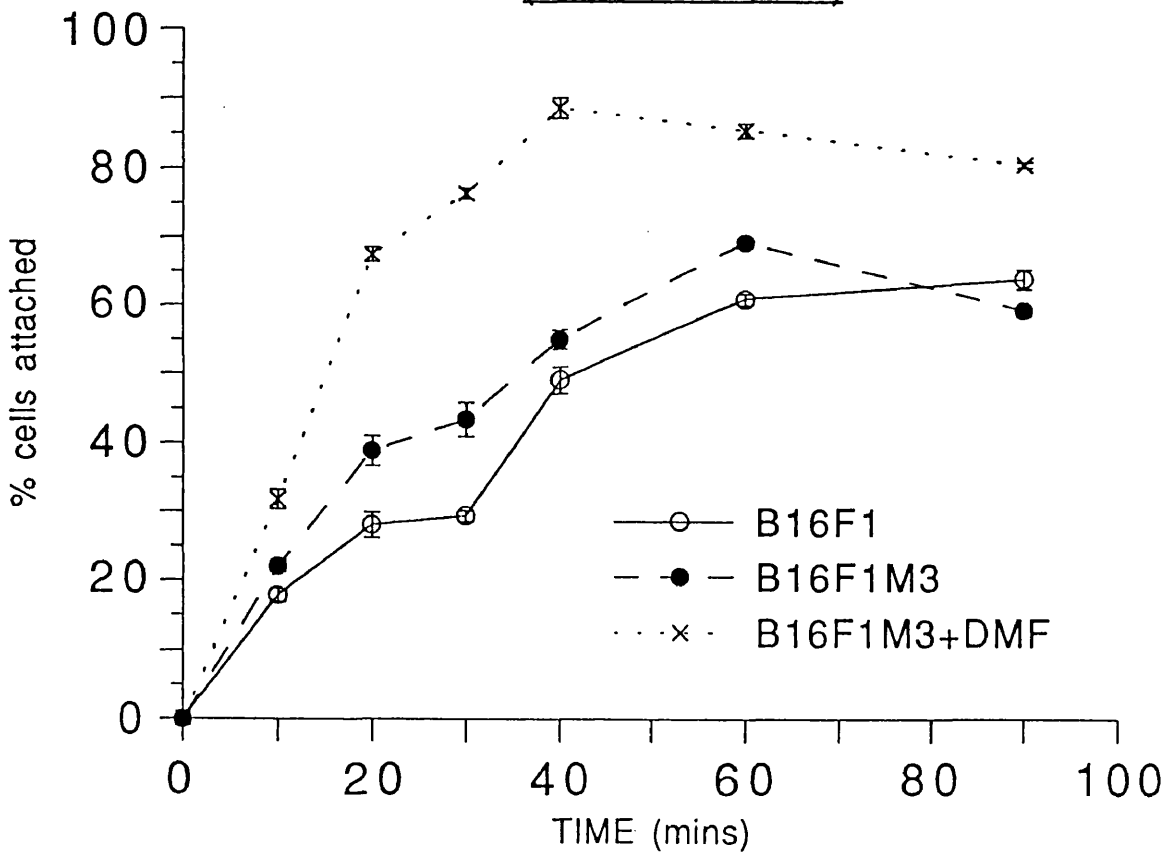


Fig.33; The adhesion values obtained for DMF-treated B16f1M3 cells over time points 20-90 mins were statistically significant (0.05>P; Student's t-test) when compared with those obtained for B16F1 and B16F1M3 cells.

stimulation of both the rate and maximal level of attachment to DMF-treated monolayers. DMF-treatment may therefore partly mediate its augmentation of organ colonization by stimulating cell aggregation and embolus formation.

4.3.3. Degradation of the subendothelial matrix

The subendothelial matrix synthesized by CPAE cells was metabolically labelled with ^{3}H-proline (^{3}H-Pro) or ^{35}sulphate (^{35}S) to predominantly radiolabel (putatively) collagen and proteoglycans respectively.

In vitro, no significant difference in the collagenolytic abilities of metastatic and poorly metastatic B16 melanomas could be determined. All B16 melanoma cell lines and clones were capable of stimulating the release of ^{3}H-Pro from subendothelial matrices. Similar levels of ^{3}H-Pro-releasing activity were detected in B16F1, B16F1M3, and DMF-treated melanoma cells (fig.34a). Similarly, the B16 melanoma cell clones, B16F1H5 and B16F1D2, exhibited near equivalent levels of ^{3}H-Pro-releasing activity (fig.34b).

Fig.34 Release of ^{3}H-Pro from metabolically labelled CPAE subendothelial matrices

a) Release of ^{3}H-Pro from radiolabelled matrices by B16F1, B16F1M3 and B16F1M3+0.5%DMF cells (mean ± SEM; n=3)

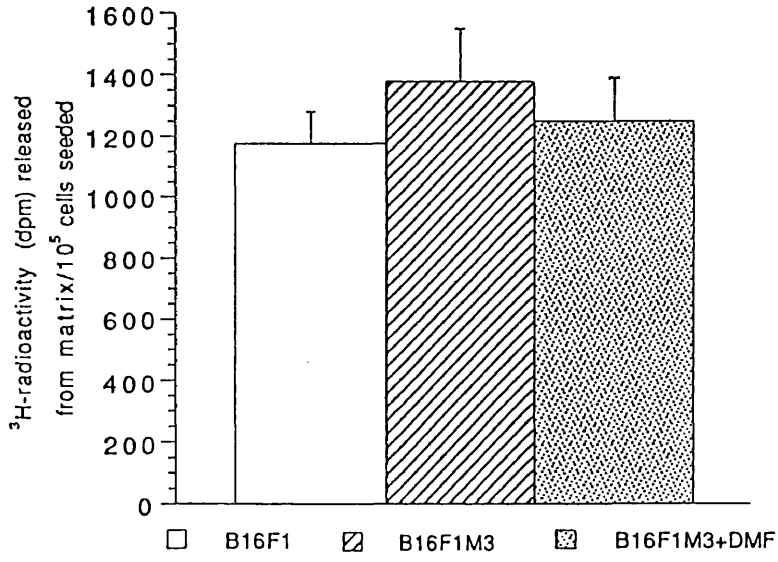
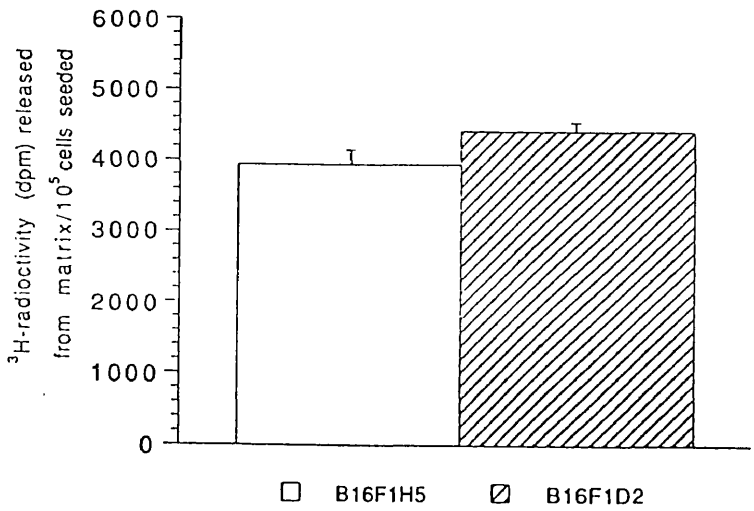


Fig.34a-b; The P-values indicated were calculated using Student's t-test

b) Release of ^{3}H-Pro from radiolabelled matrices by B16F1D2 and B16F1H5 cells (mean ± SEM; n=3)



The release of ^{35}S from radiolabelled subendothelial matrices was demonstrated to show a positive correlation with metastatic potential. B16F1M3 cells possessed a significantly higher level of ^{35}S-releasing activity than B16F1 cells ($P<0.01$); pretreatment with 0.5% DMF did not influence the ^{35}S-releasing activity of B16F1M3 cells (fig.35a). Similarly, this positive correlation was also detected in the B16 melanoma clones: a significantly higher level of ^{35}S-releasing activity ($P<0.01$) was associated with B16F1D2 cells when compared with B16F1H5 cells (fig.35b).

Fig.35 Release of ^{35}S from metabolically labelled CPAE subendothelial matrices

a) Release of ^{35}S from radiolabelled matrices by B16F1, B16F1M3 and B16F1M3+0.5%DMF cells (mean ± SEM; n=3)

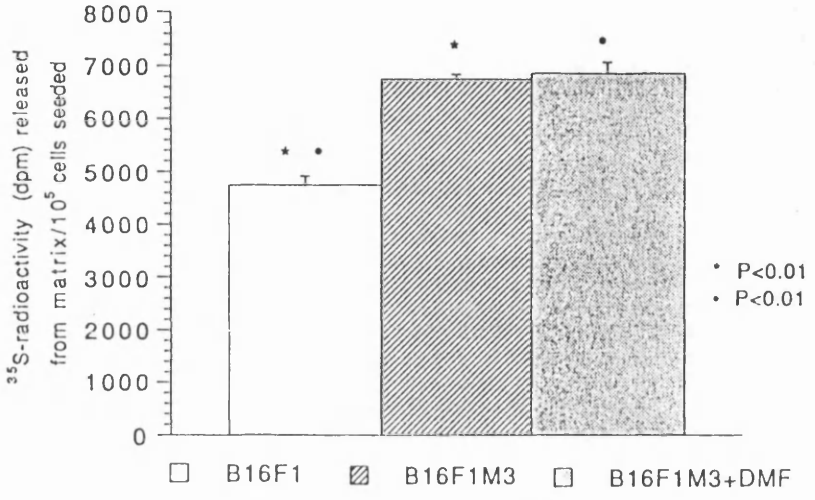
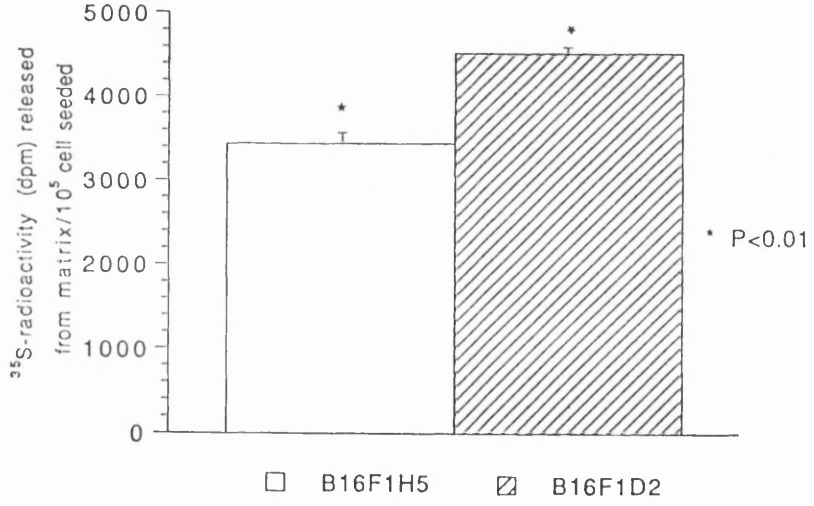


Fig.35a-b; The P-values indicated were calculated using Student's t-test

b) Release of ^{35}S from radiolabelled matrices by B16F1D2 and B16F1H5 cells (mean ± SEM; n=3)



4.4.1. Proteoglycan Analyses

Ion-exchange chromatography (IEC) and gel permeation chromatography (GPC) were employed to study the qualitative and quantitative differences In GAG and proteoglycan biosynthesis that existed between the B16 melanoma cell lines used in this study. In certain experiments, three GAG fractions were isolated and analysed: the medium (MEDIUM) fraction, the trypsin-releasable (TRYPSIN) fraction and the cell-associated (CELL) fraction. The medium fraction contained GAG released into the culture medium; the trypsin fraction the GAG present on the cell surface as proteoglycan or released by trypsin/EDTA; the cell fraction comprised internalized GAG and proteoglycan, trypsin-insensitive proteoglycans, and newly synthesized proteoglycan not yet expressed on the cell surface. The initial IEC separation generated a characteristic elution profile containing two distinct regions (fig.36a). The first major peaks contained small GAG fragments, glycopeptides and poorly charged molecules and the minor second peak contained the sulphated GAG of interest.

Constituent GAG species were separated by digestion with chondroitinase ABC or nitrous acid at low pH to generate HS-GAG or CS-GAG species alone respectively. These intact GAG species were separated from degraded GAG via Sephadex G50 GPC (fig.36b, fig.36c). This procedure does not however distinguish between HS/CSPG copolymers.

Two fractions, medium-associated and cell-lysate, were generated for proteoglycan analysis. The medium-associated fraction again comprised culture medium proteoglycan and GAG, and the cell-lysate fraction the cell surface and intracellular proteoglycans and GAG.

Fig.36a: Ion Exchange Chromatography
Initial DEAE-Cellulose Separation

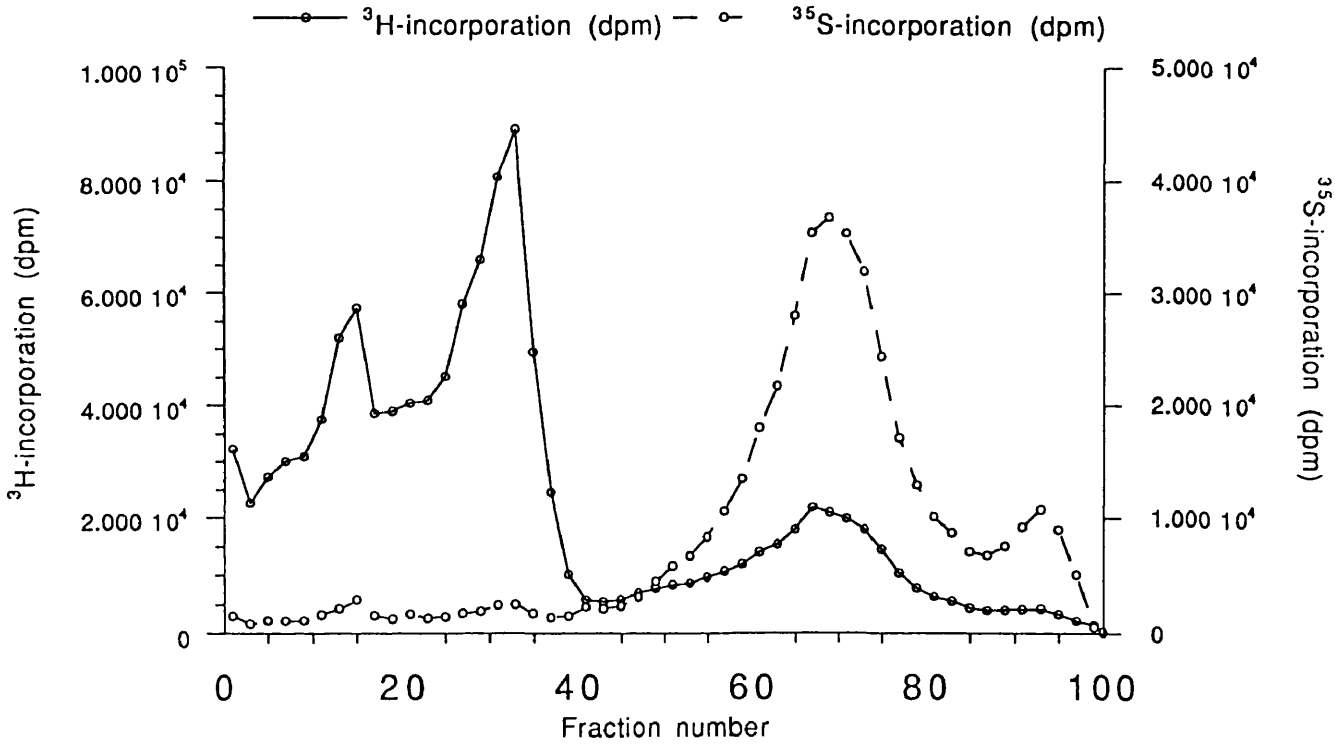


Fig.36b: Sephadex G50 GPC
Chondroitinase ABC digestion elution profile

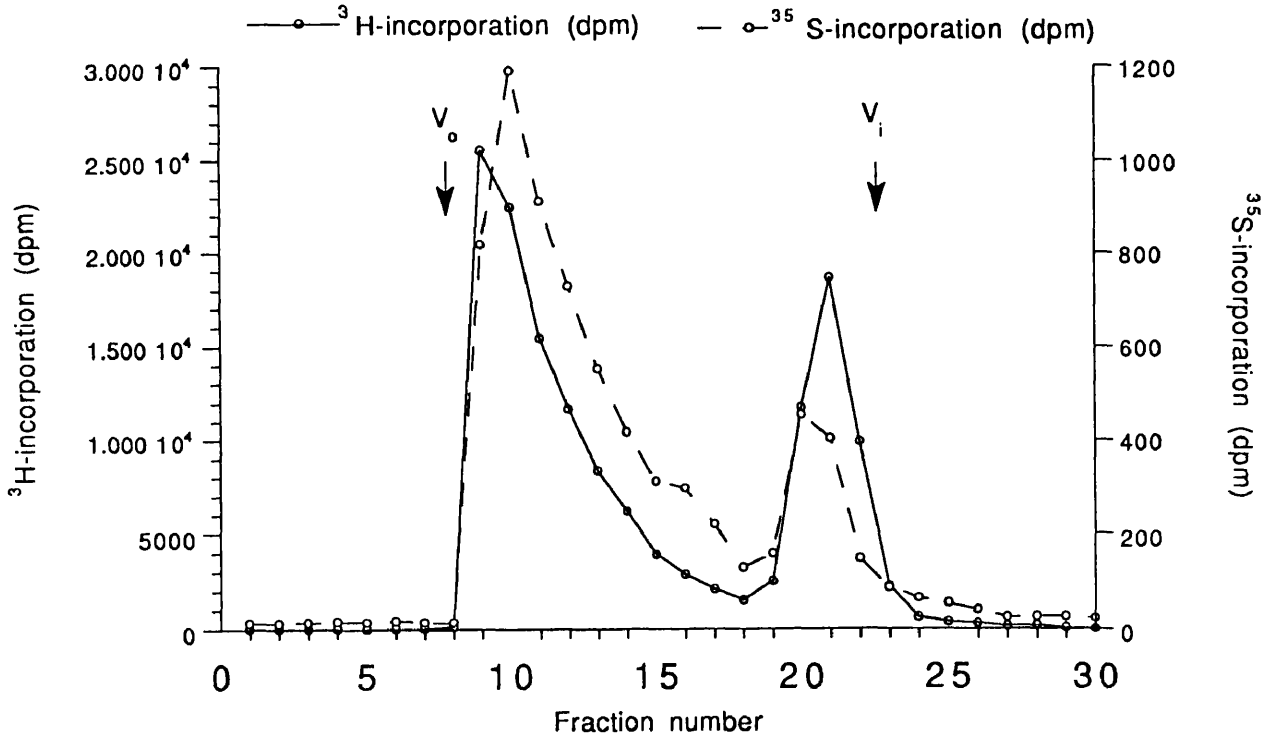


Fig.36c: Sephadex G50 GPC
Nitrous acid deamination elution profile

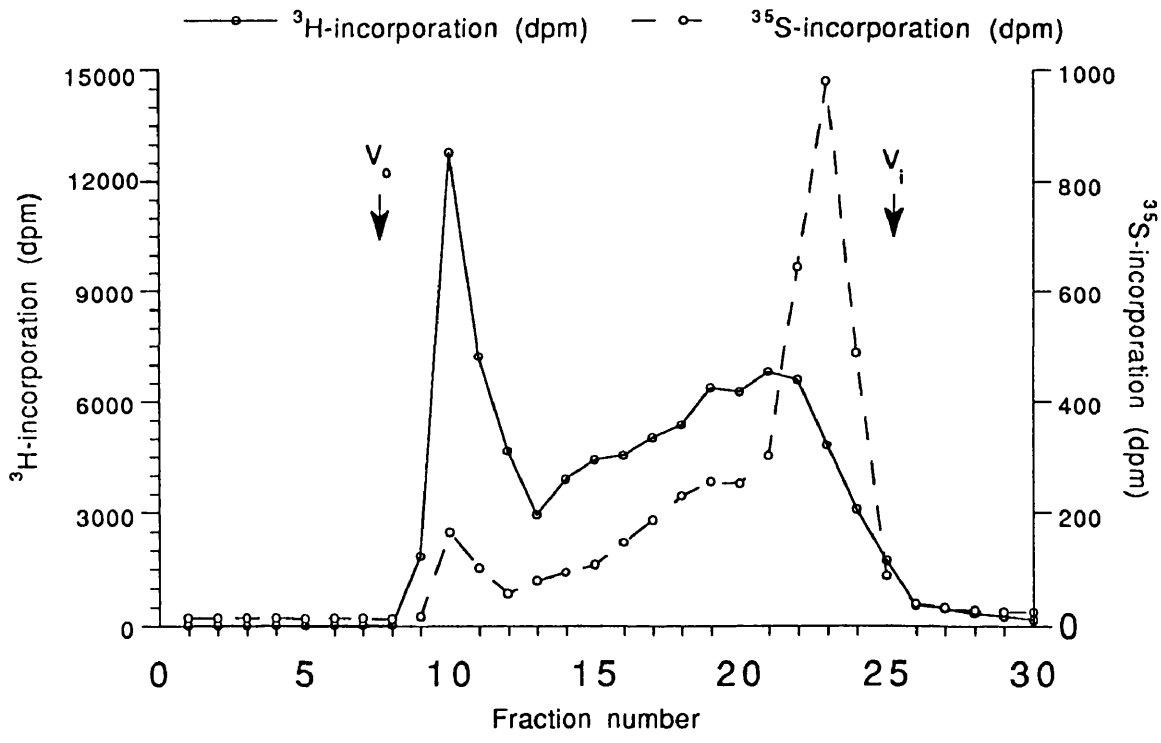
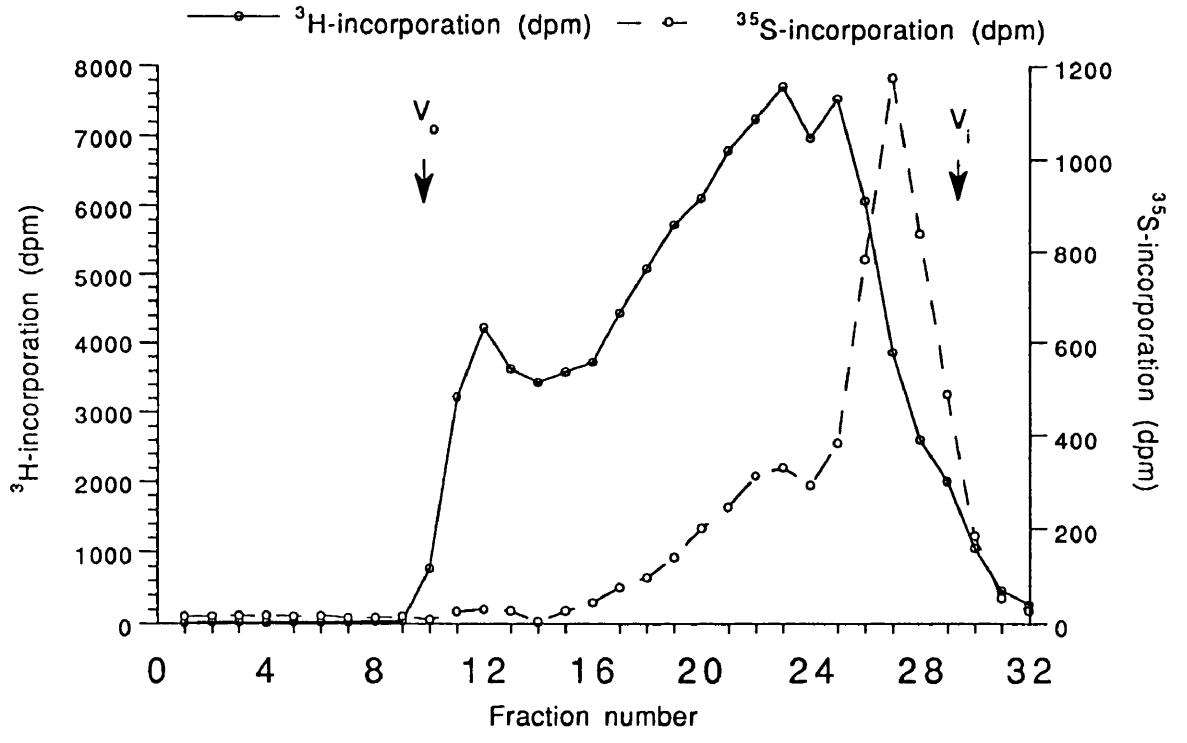


Fig.36d: Sephadex G50 GPC
Nitrous acid deamination of HS-GAG elution profile



4.4.2. Qualitative differences in GAG synthesis

The GAG synthesized by B16 melanoma cells of differing metastatic potentials were compared, and the effects of chemical modifiers of GAG biosynthesis assessed via IEC.

a) B16F1 versus B16F1M3 melanoma cells

The CS-GAG and HS-GAG synthesized by poorly metastatic B16F1 and moderately metastatic B16F1M3 melanoma cells possessed similar chemical properties. DEAE-Cellulose IEC failed to identify any significant qualitative differences in the charge densities of either the CS-GAG (fig.37) or the HS-GAG (fig.38) from the medium fraction, although comparison of the elution profiles appeared to indicate that the HS-GAG elution later.

Similar observations were noted for the trypsin-releasable GAG fractions. B16F1 and B16F1M3 melanoma cells synthesized CS-GAG species with similar charge density, although there was a wider spread of charge densities for B16F1 CS-GAG (fig.39). The HS-GAG synthesized by these melanomas were also of a similar charge density (fig.40).

No qualitative difference in the charge densities of the cell-associated CS-GAG (fig.41) and HS-GAG (fig.42) synthesized by B16F1 and B16F1M3 cells could be identified by DEAE-Cellulose IEC. The cell-associated GAG fractions however eluted closer to the end of the salt gradient, indicating the probability of a higher charge density.

HS-GAG and CS-GAG from the medium and trypsin-releasable fractions were further analysed on Mono-Q-Sepharose at low pH to measure the

Fig.37; DEAE-Cellulose IEC
Medium Fraction CS-GAG

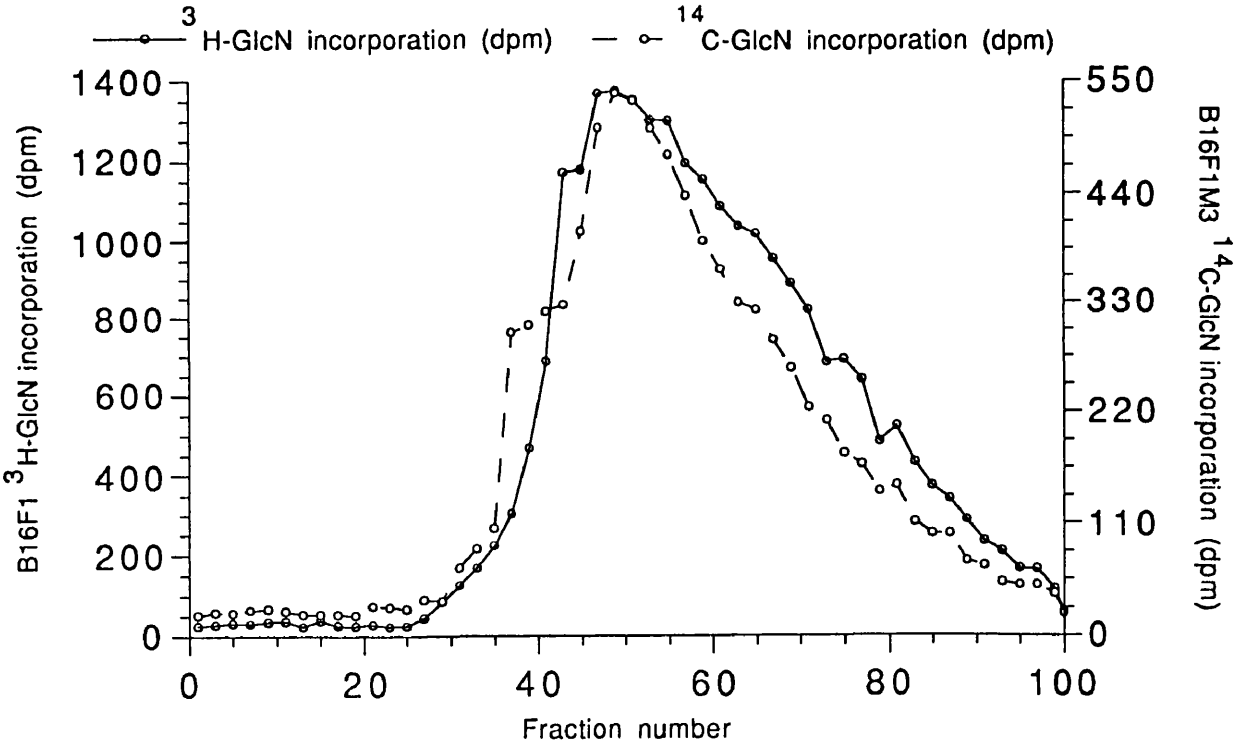


Fig.38; DEAE-Cellulose IEC
Medium fraction HS-GAG

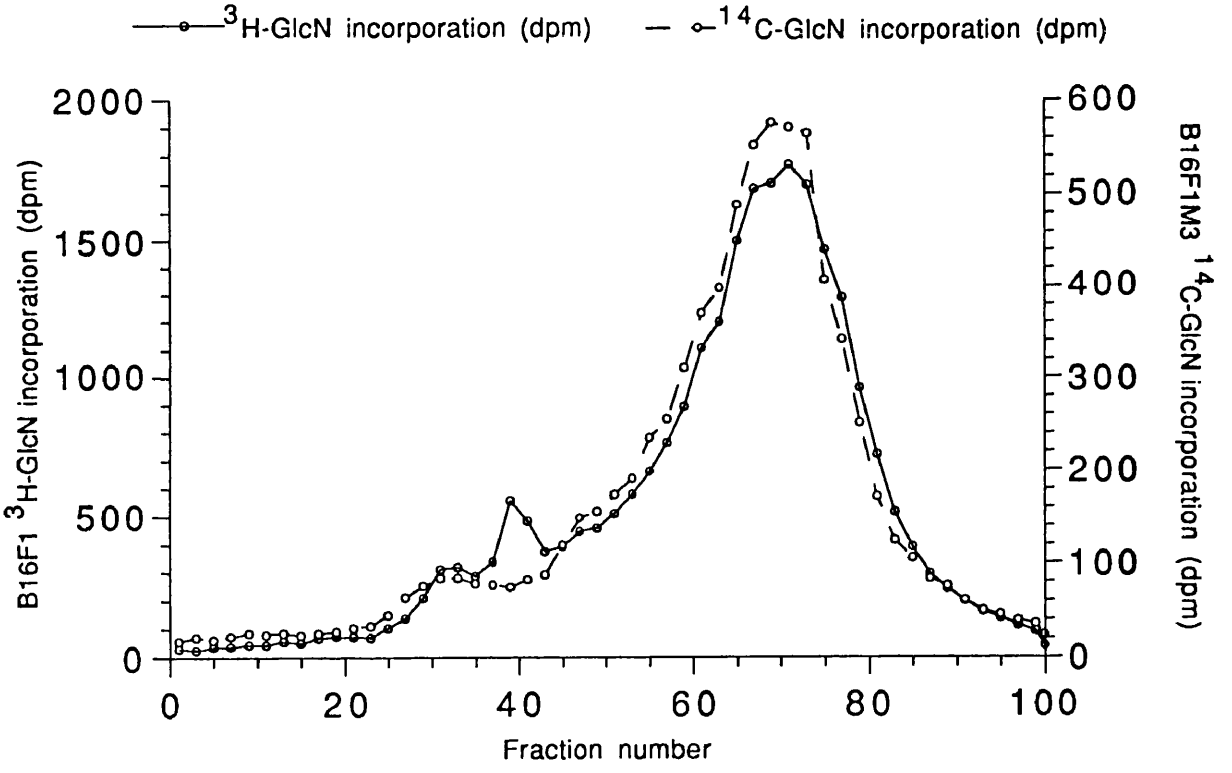


Fig.39; DEAE-Cellulose IEC
Trypsin Fraction CS-GAG

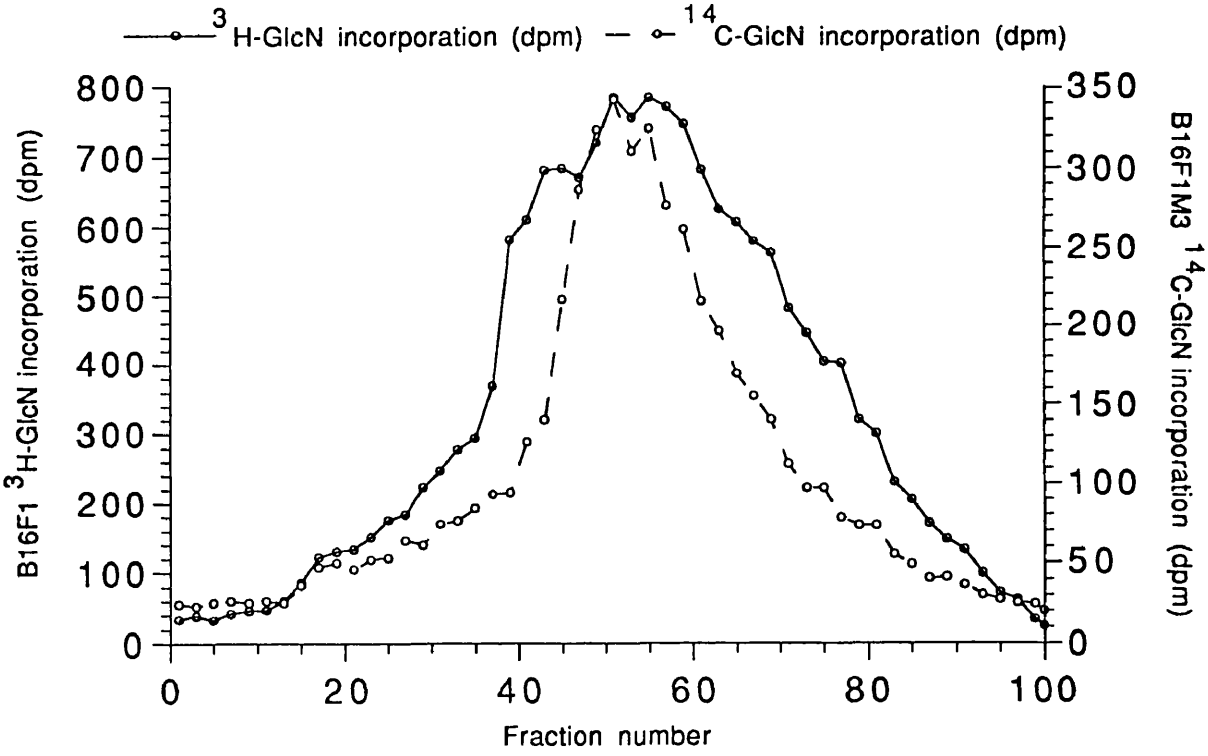


Fig.40; DEAE-Cellulose IEC
Trypsin fraction HS-GAG

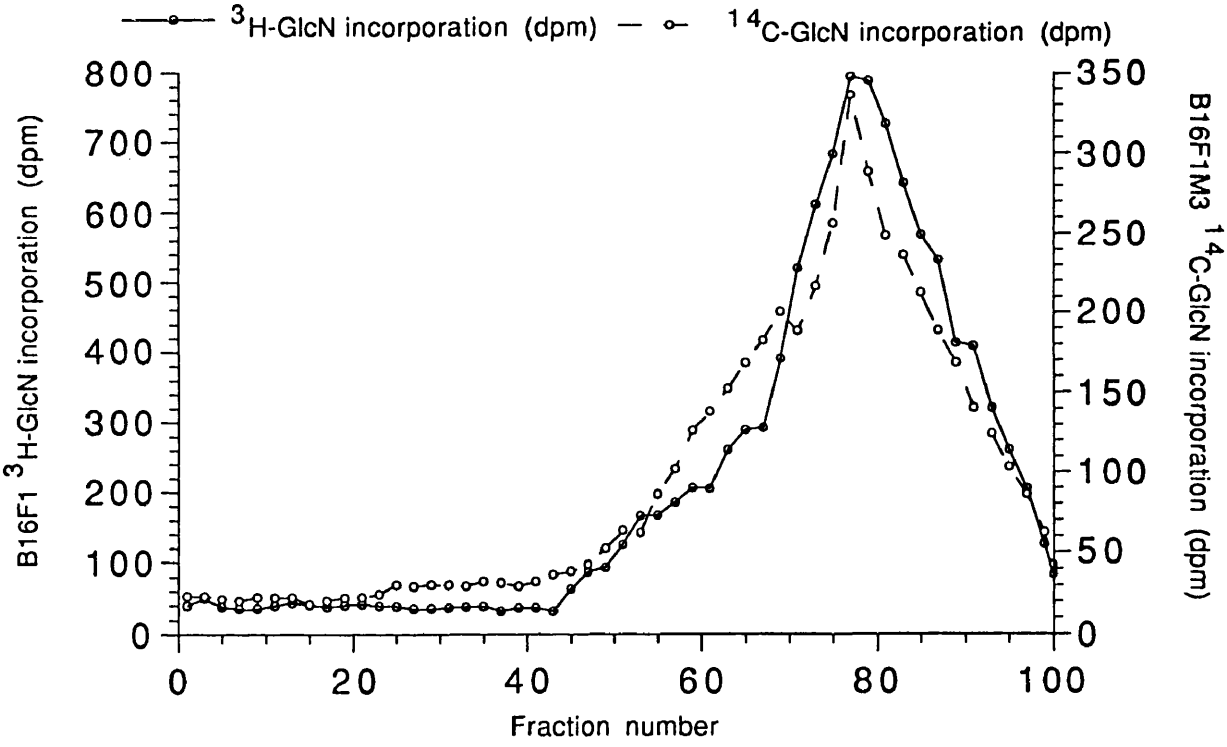


Fig.41; DEAE-Cellulose IEC
Cell Fraction CS-GAG

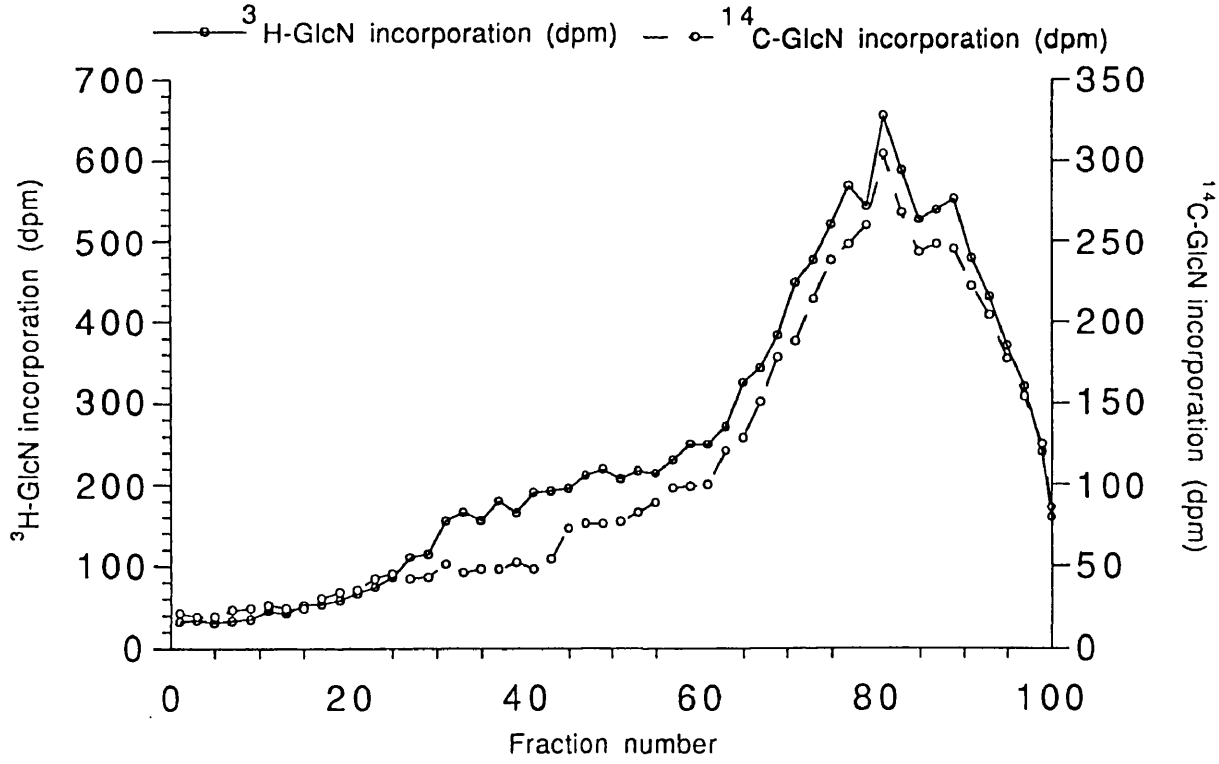
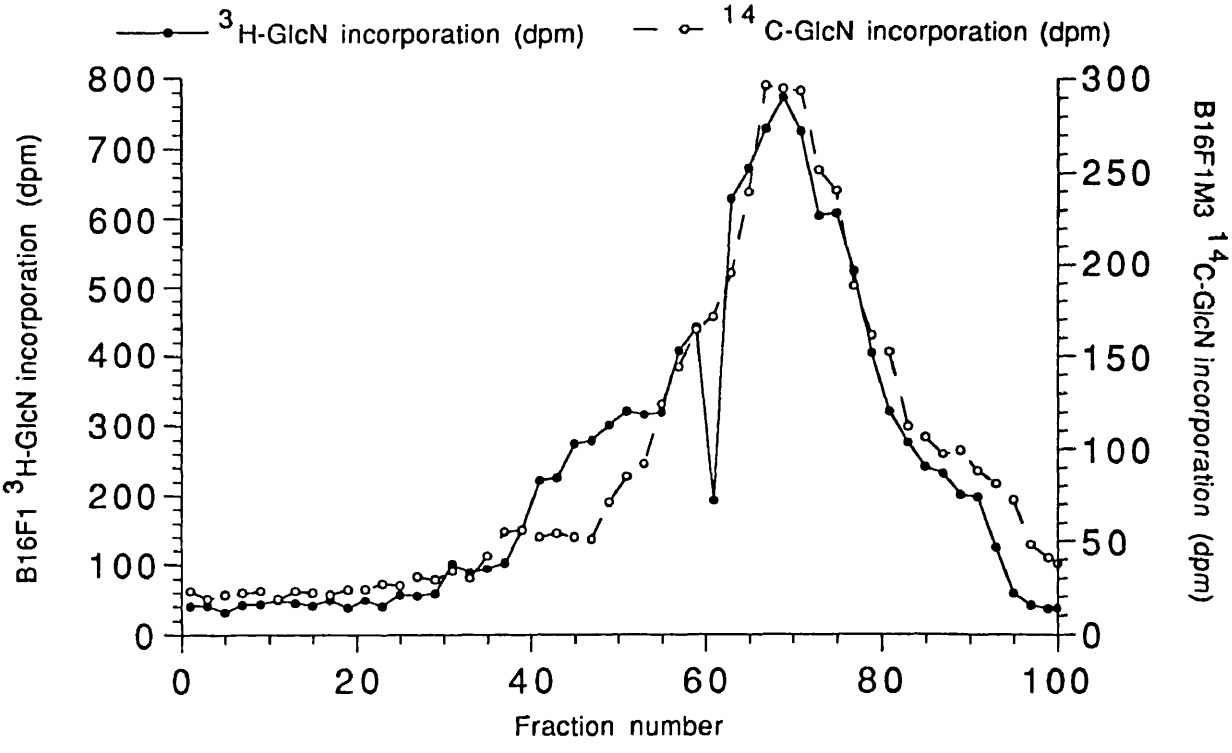


Fig.42; DEAE-Cellulose IEC
Cell fraction HS-GAG



degree of polymer sulphation. Mono-Q-Sepharose IEC suggested that in both the medium (fig.43) and trypsin-releasable fractions (fig.44), B16F1M3 melanoma cells synthesized CS-GAG with a lower degree of polymer sulphation than B16F1 melanoma cells. No difference in the degree of HS-GAG polymer sulphation was detected by Mono-Q-Sepharose IEC for the trypsin-releasable fractions (fig.45), although B16F1 cells appeared to synthesize a slightly more sulphated medium fraction HS-GAG (fig.46).

b) B16F1M3 ± 0.5%DMF

Incubation of B16F1M3 melanoma cells with 0.5% DMF resulted in profound changes in GAG biosynthesis. DMF induced the synthesis of B16F1M3 CS-GAG with a reduced charge density in both the medium (fig.47) and trypsin-releasable fractions (fig.48), with the reduction in charge density being more pronounced in the trypsin-releasable fraction. The CS-GAG present in the cell-associated fraction synthesized by DMF-treated cells also exhibited a large reduction in charge density (fig.49).

In contrast, DMF-treatment appeared to have the opposite effect upon the charge density of the medium fraction HS-GAG (fig.50), resulting in an increase in charge density as measured via DEAE-Cellulose IEC. DMF though appeared to have no obvious effect upon the charge density of the HS-GAG present in the trypsin-releasable fraction (fig.51). DMF-treated cells also exhibited a reduction in the charge density of the HS-GAG from the cell-associated fraction (fig.52).

The DMF-induced alterations in GAG biosynthesis were associated with changes in the degree of polymer sulphation as detected via Mono-Q-

Fig.43; Mono Q Sepharose IEC
Medium fraction CS-GAG

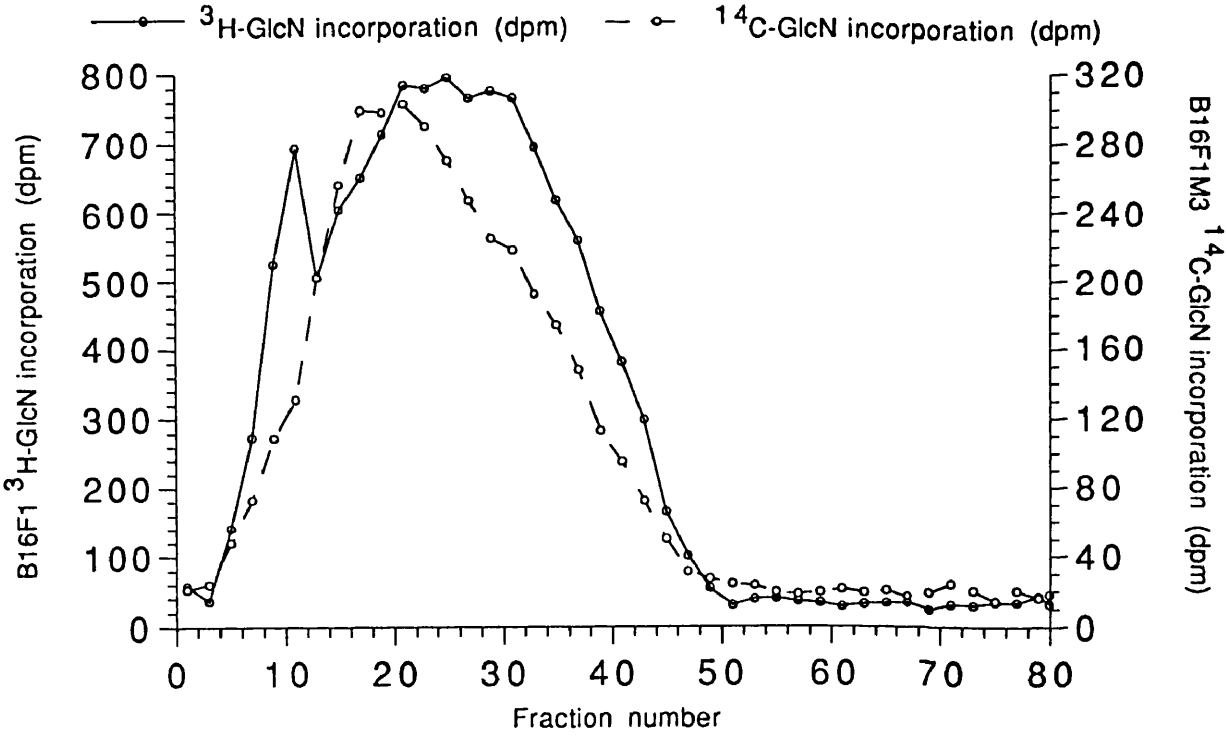


Fig.44; Mono Q Sepharose IEC
Trypsin fraction CS-GAG

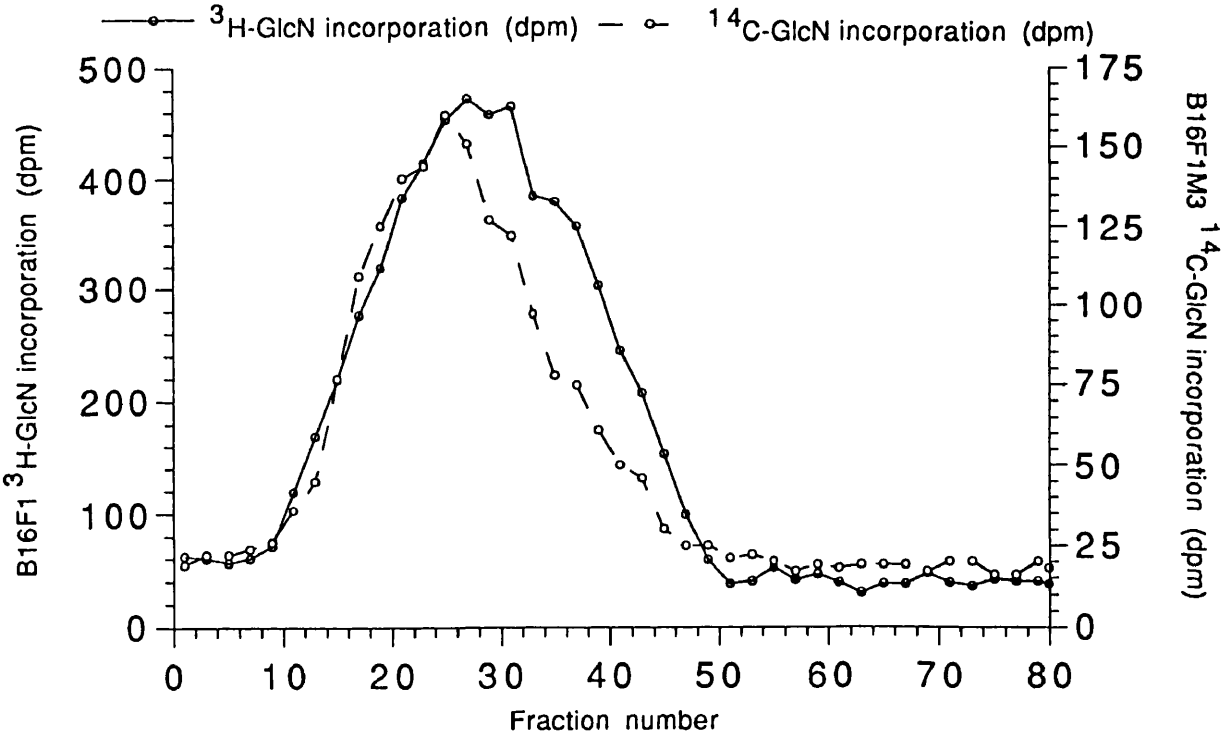


Fig.45; Mono Q Sepharose IEC
Trypsin fraction HS-GAG

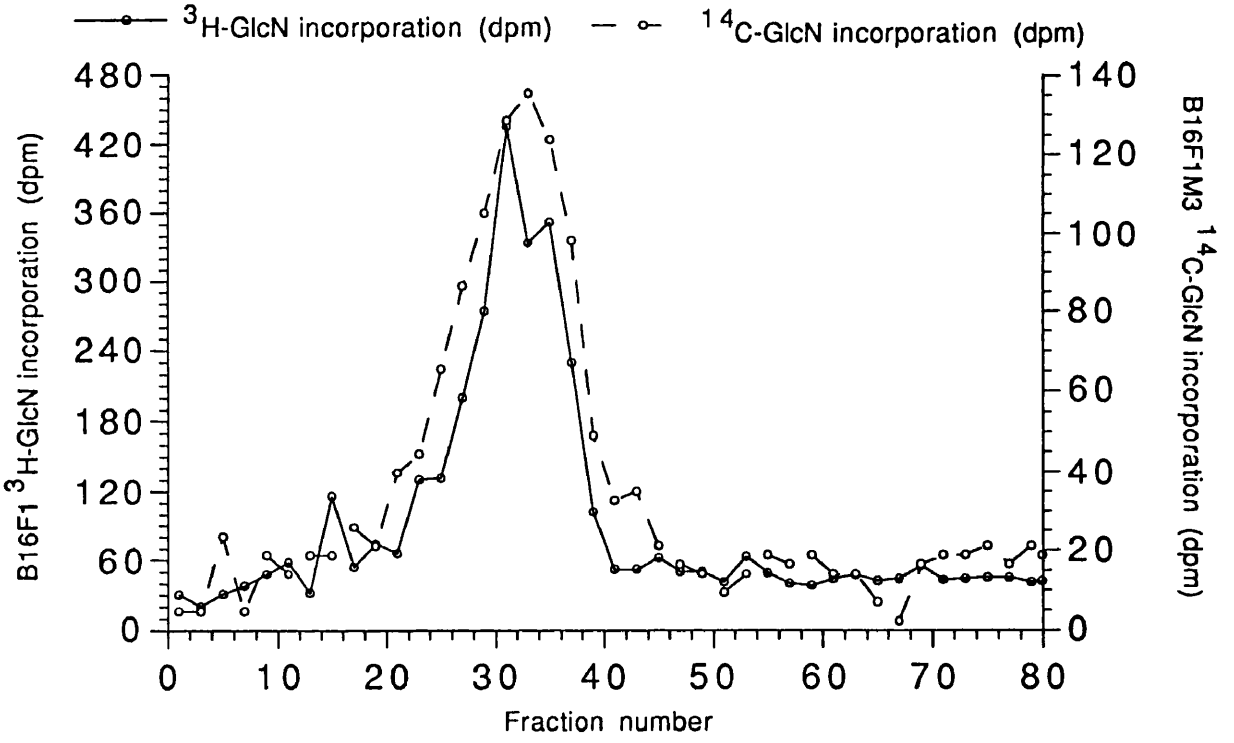


Fig.46; Mono Q Sepharose IEC
Medium fraction HS-GAG

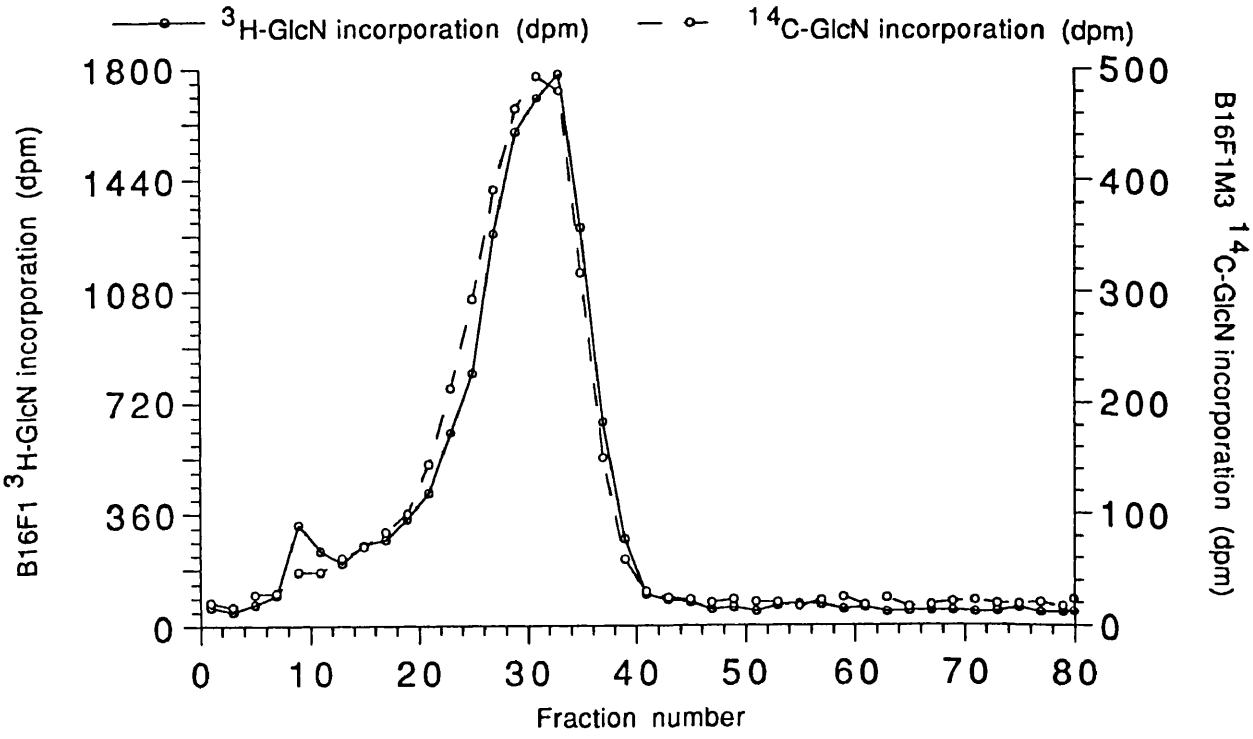


Fig.47; DEAE-Cellulose IEC
Medium fraction CS-GAG

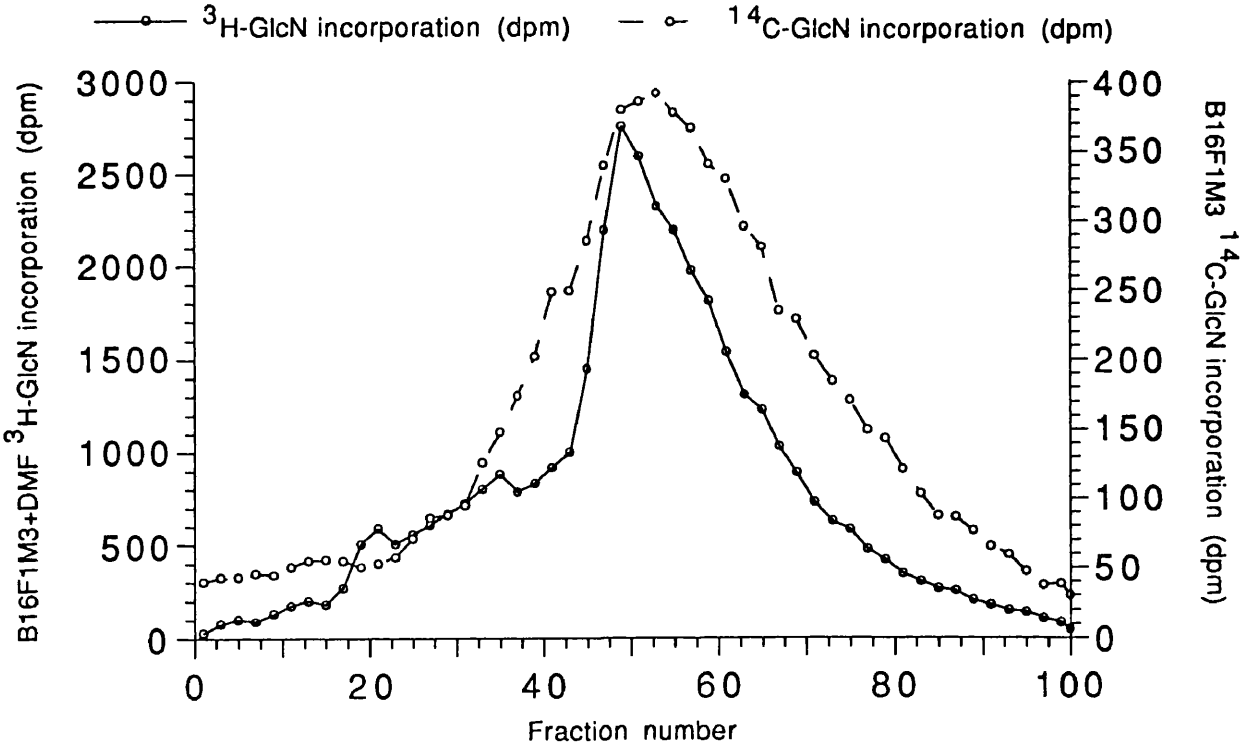


Fig.48; DEAE-Cellulose IEC
Trypsin fraction CS-GAG

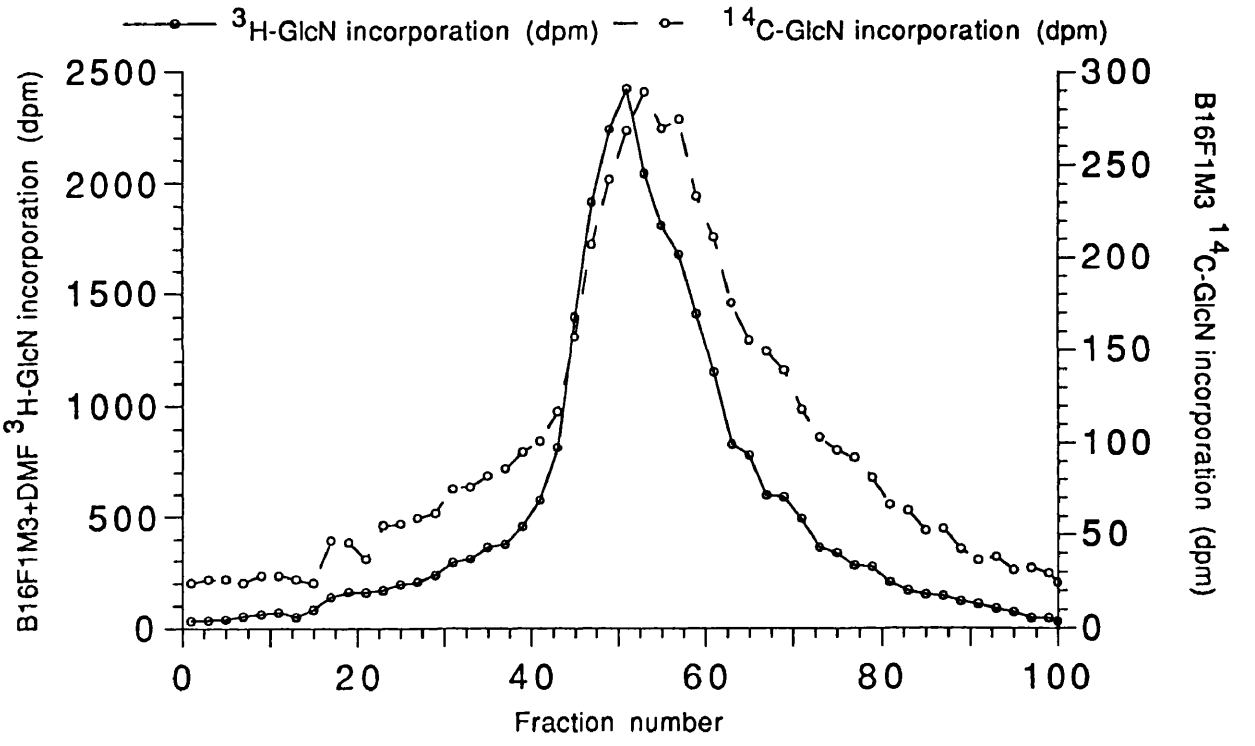


Fig.49; DEAE-Cellulose IEC
Cell fraction CS-GAG

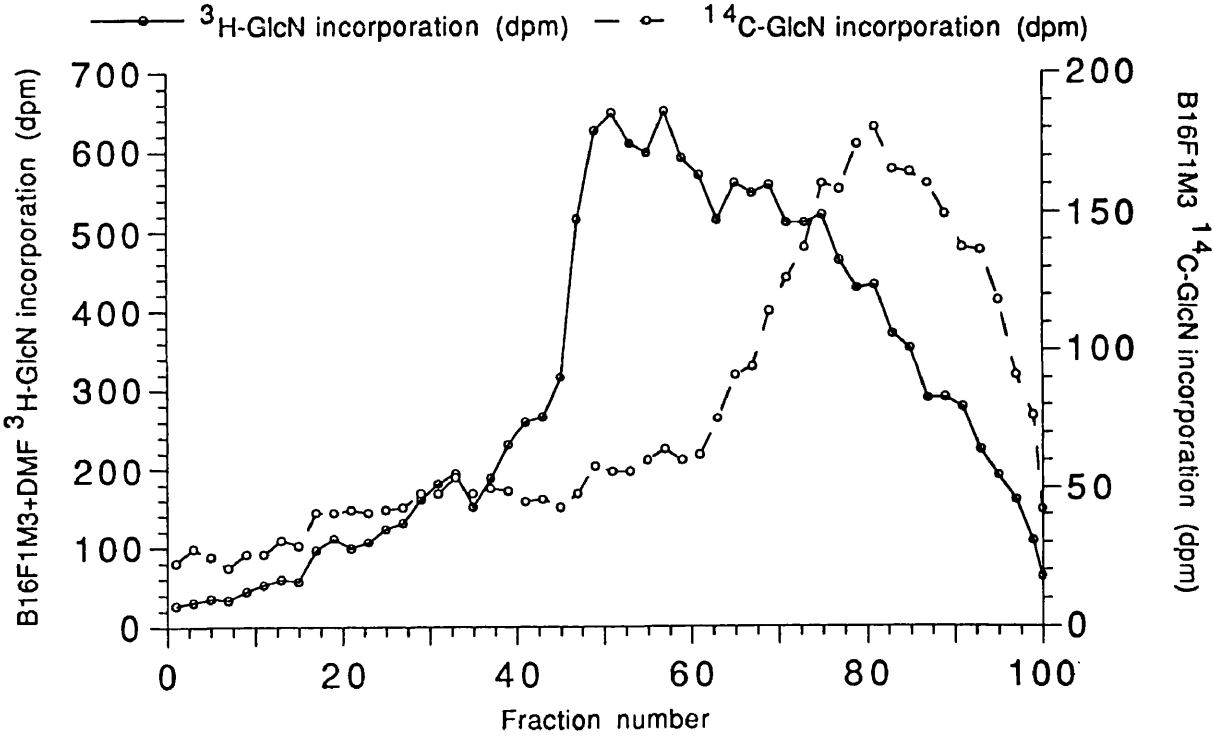


Fig.50; DEAE-Cellulose IEC
Medium fraction HS-GAG

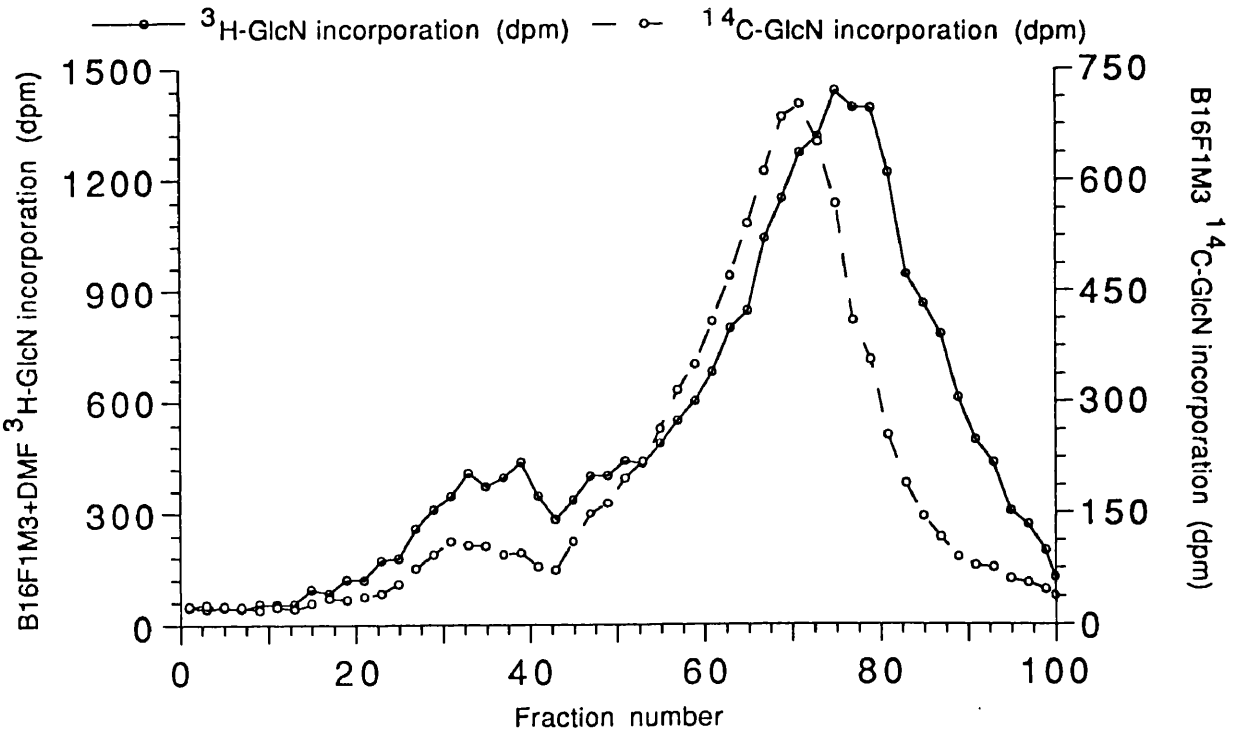


Fig.51; DEAE-Cellulose IEC
Trypsin fraction HS-GAG

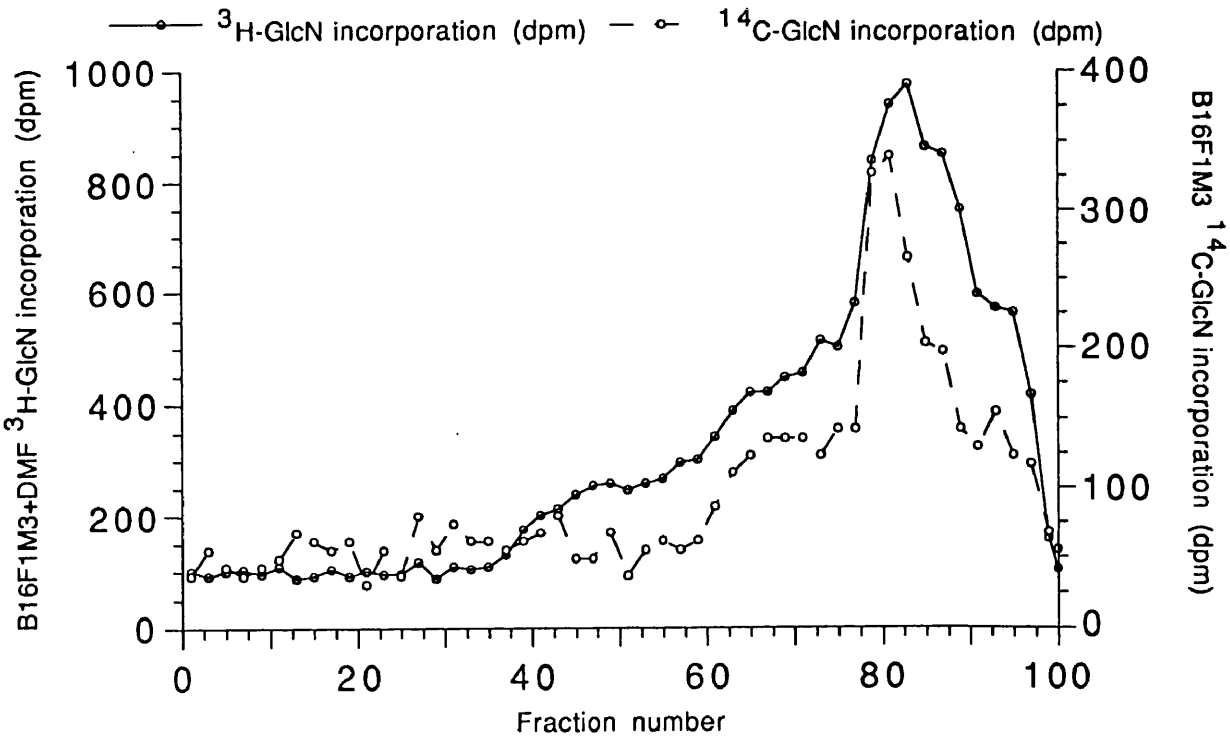
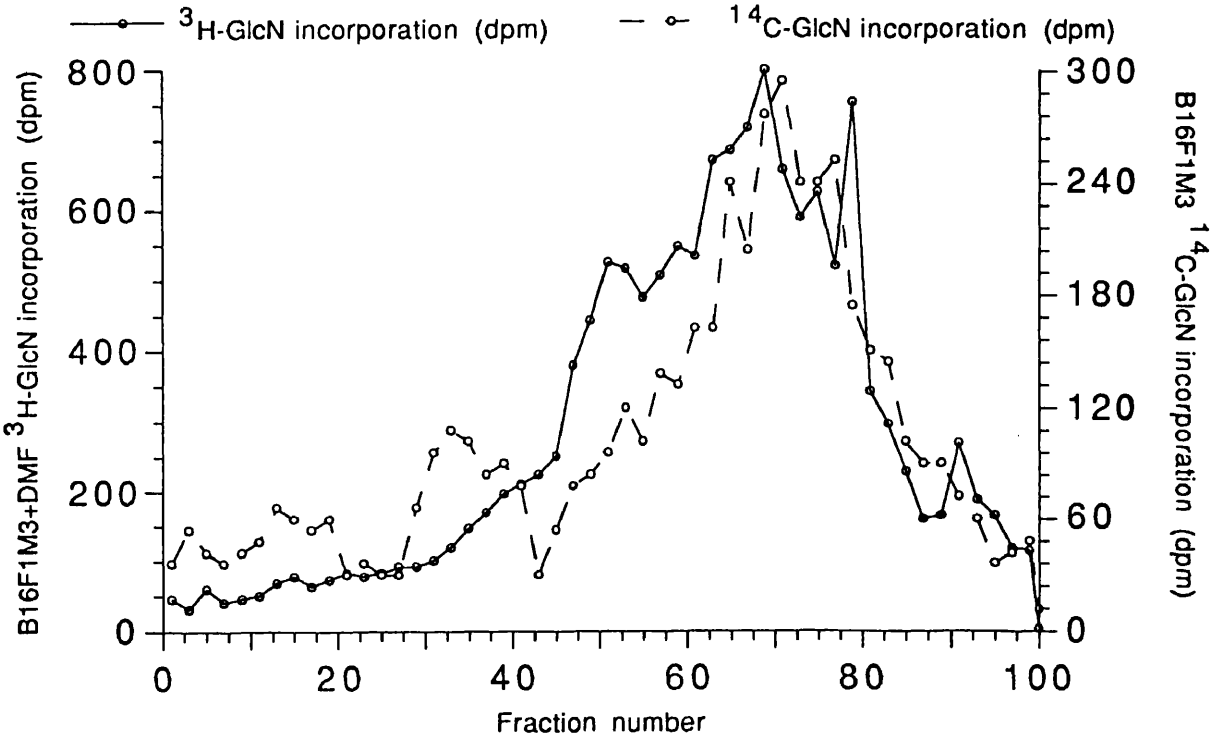


Fig.52; DEAE-Cellulose IEC
Cell fraction HS-GAG



Sepharose IEC analysis. When compared to those from control cultures, the medium and trypsin-releasable fractions from DMF-treated B16F1M3 melanoma cells contained undersulphated CS-GAG (fig.53 & fig.54 respectively) and oversulphated HS-GAG (fig.55 & fig.56 respectively), including the trypsin-releasable HS-GAG which showed no difference in charge desity as measured via DEAE-Cellulose IEC.

c) B16F1 versus B16F1M5

Preliminary DEAE-Cellulose IEC studies with B16F1 and B16F1M5 cells failed to demonstrate a qualitative difference in the charge density properties of the medium (fig.57) and trypsin-releasable (fig.58) HS-GAG fractions synthesized. As a consequence of the instability of the B16F1M5 phenotype, no further GAG analyses were performed with this cell line.

d) B16F1H5 versus B16F1D2

Poorly metastatic B16F1H5 and highly metastatic B16F1D2 melanoma cells were found to synthesize medium and trypsin-releasable CS-GAG with similar charge density properties (fig.59 & fig.60 respectively). Similarly, the trypsin-releasable HS-GAG synthesized by both clones also possessed similar charge density properties (fig.61), whereas B16F1H5 cells produced a more highly charged medium fraction HS-GAG than B16F1D2 cells (fig.62).

Mono-Q-Sepharose IEC failed to demonstrate a difference in the degree of polymer sulphation of the medium CS-GAG fraction (fig.63) or the trypsin-releasable HS-GAG fraction (fig.64), supporting the observations obtained using DEAE-Cellulose IEC (fig.59 & fig.61). Mono-Q-Sepharose IEC also failed to demonstrate a significant difference in the degree of

Fig.53; Mono Q Sepharose IEC
Medium fraction CS-GAG

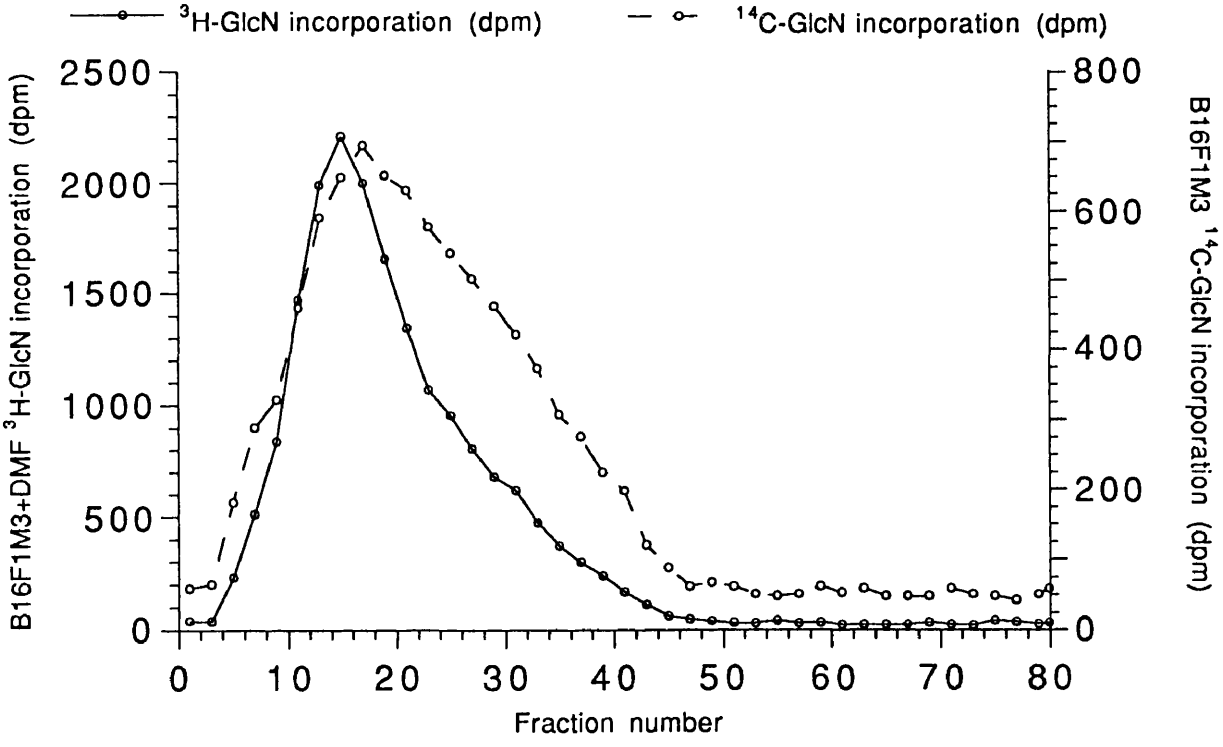


Fig.54; Mono Q Sepharose IEC
Trypsin fraction CS-GAG

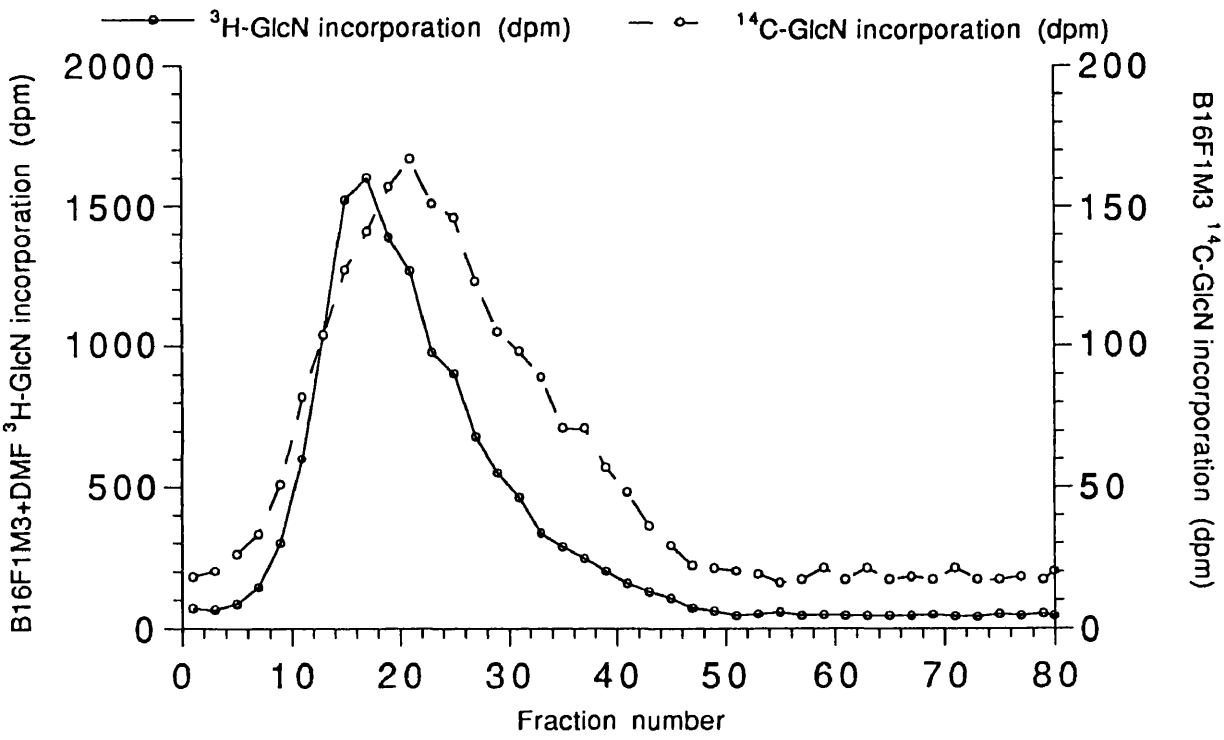


Fig.55; Mono Q Sepharose IEC
Medium fraction HS-GAG

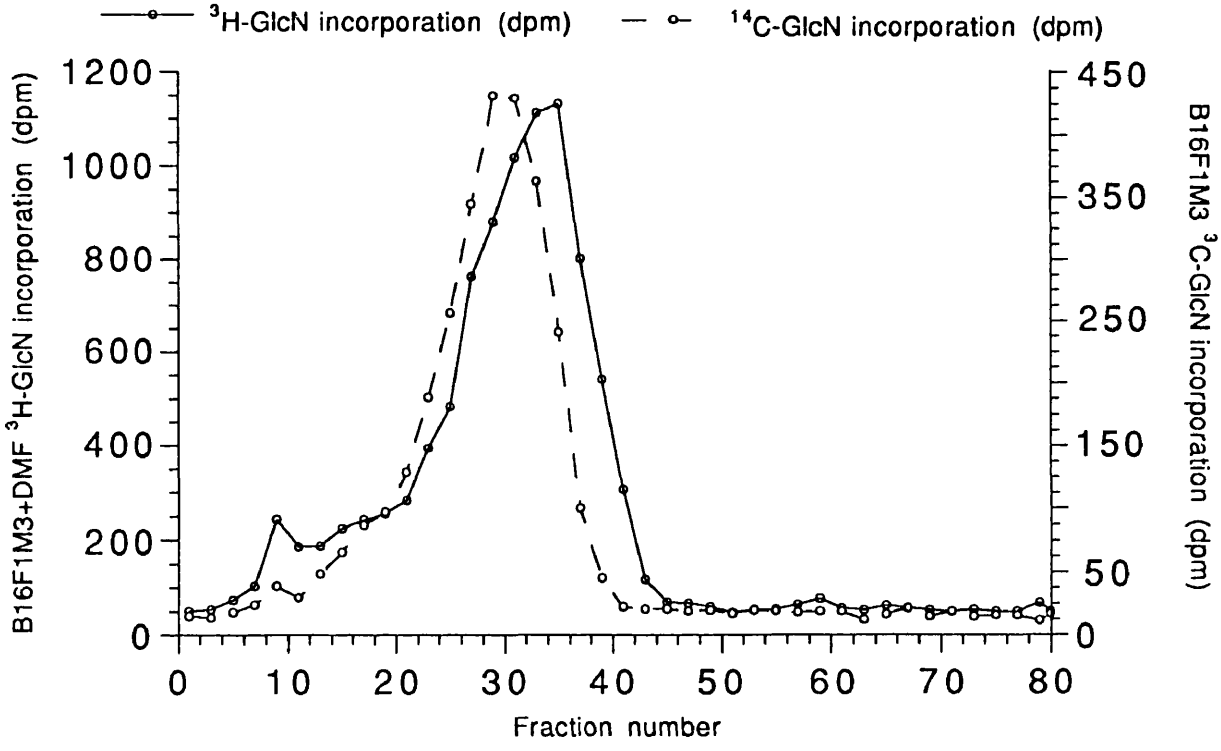


Fig.56; Mono Q Sepharose IEC
Trypsin fraction HS-GAG

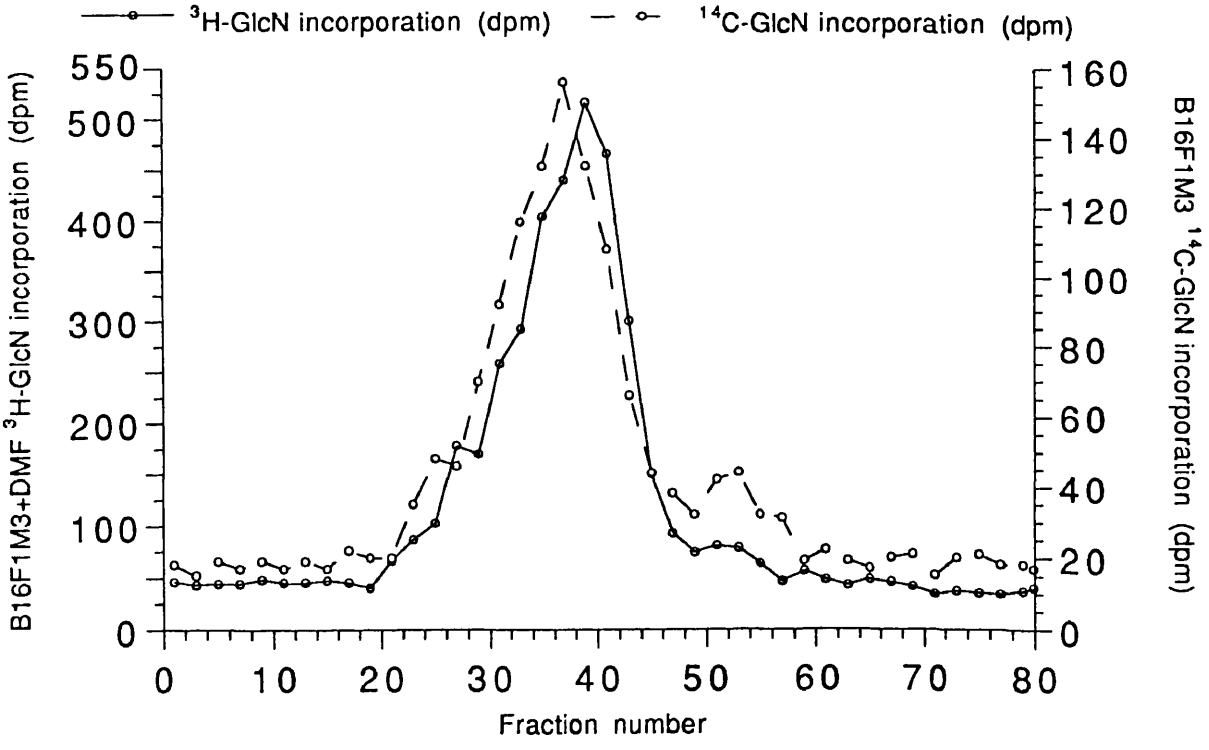


Fig.57; DEAE-Cellulose IEC
Mediun fraction HS-GAG

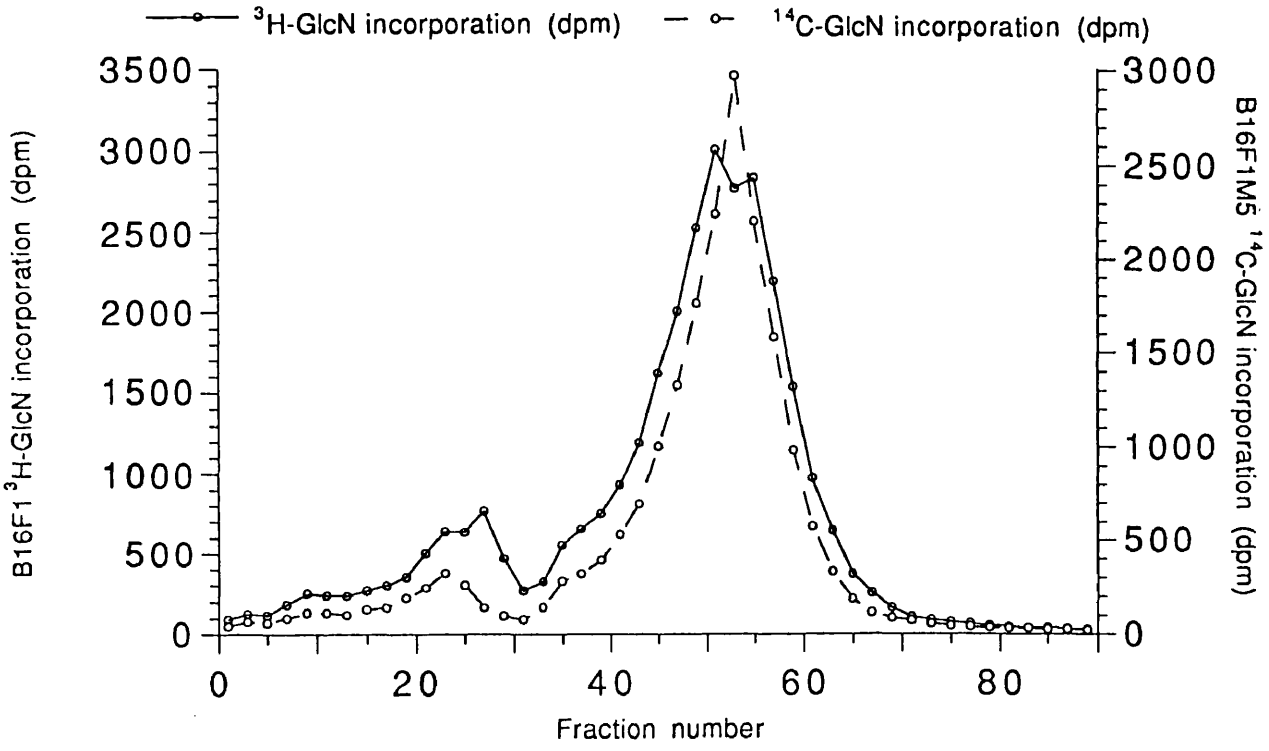


Fig.58; DEAE-Cellulose IEC
Trypsin fraction HS-GAG

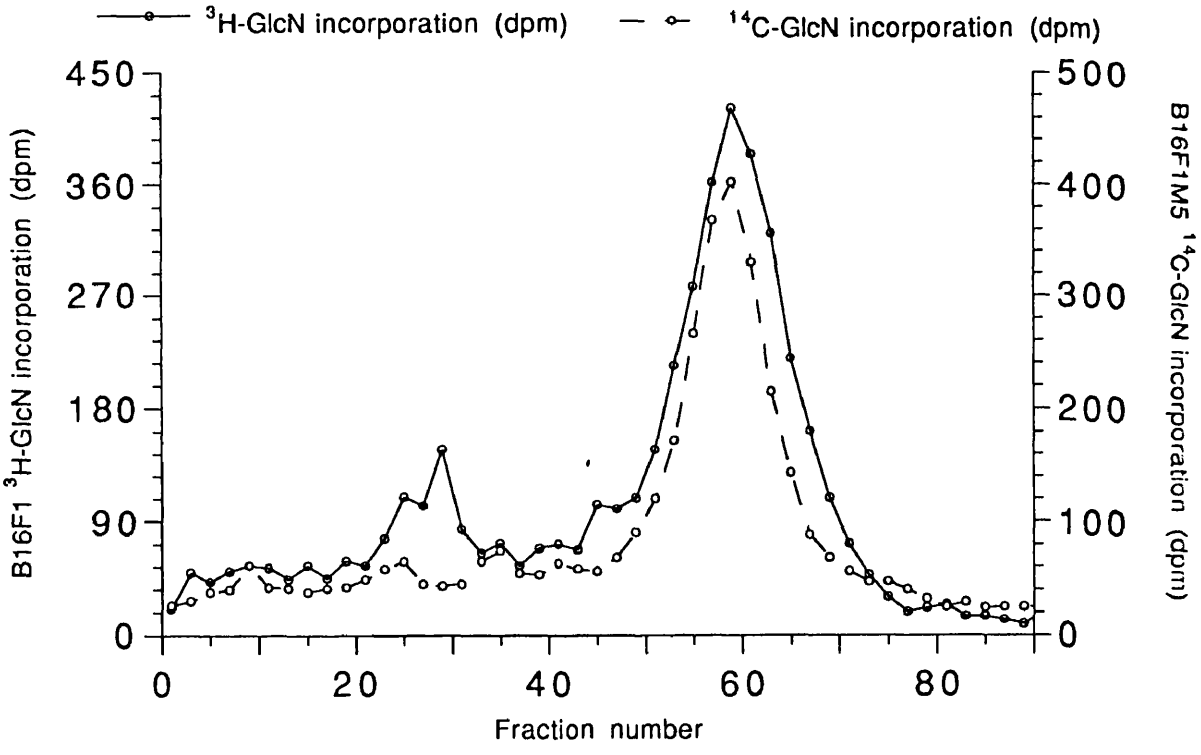


Fig.59; DEAE-Cellulose IEC
Medium fraction CS-GAG

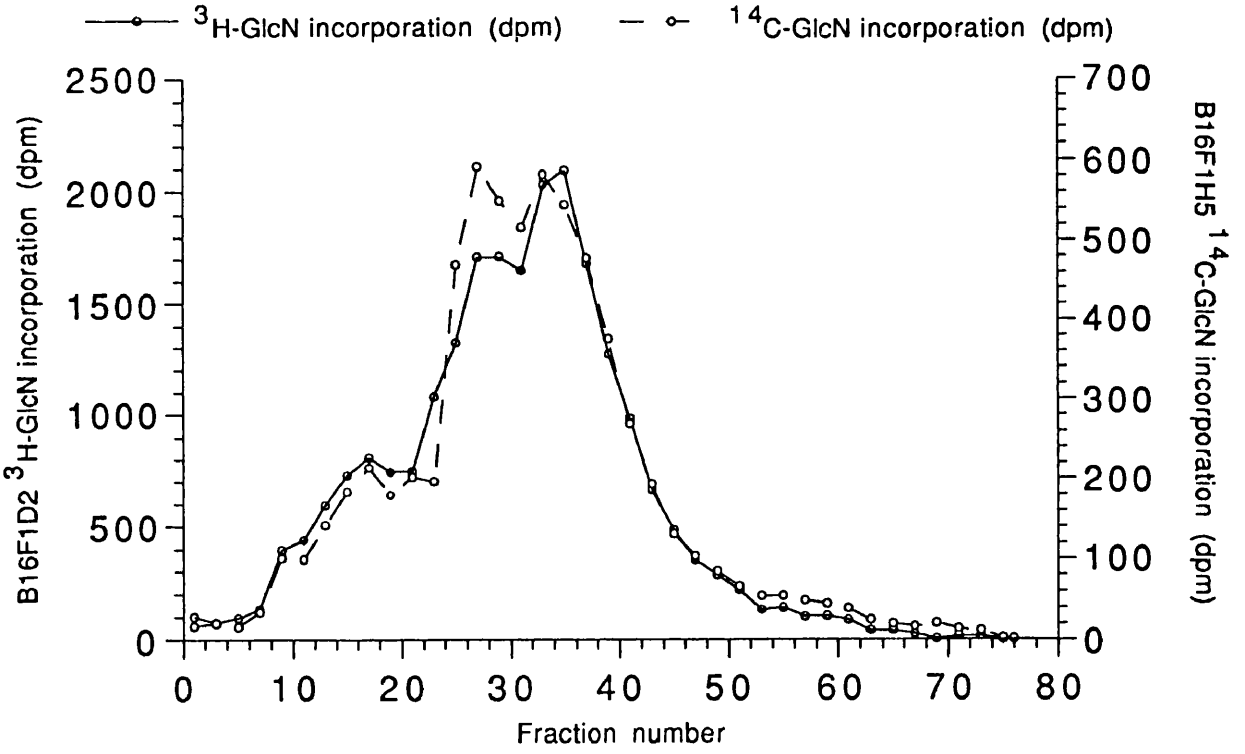


Fig.60; DEAE-Cellulose IEC
Trypsin fraction CS-GAG

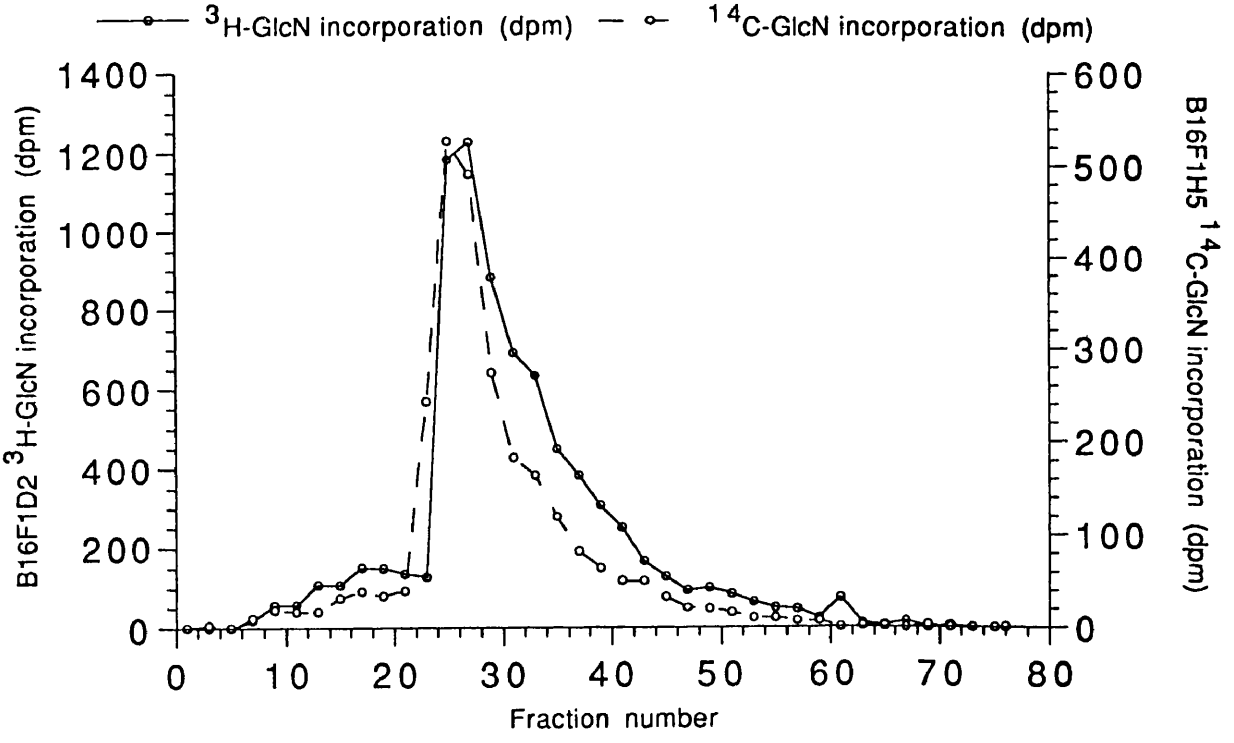


Fig.61; DEAE-Cellulose IEC
Trypsin fraction HS-GAG

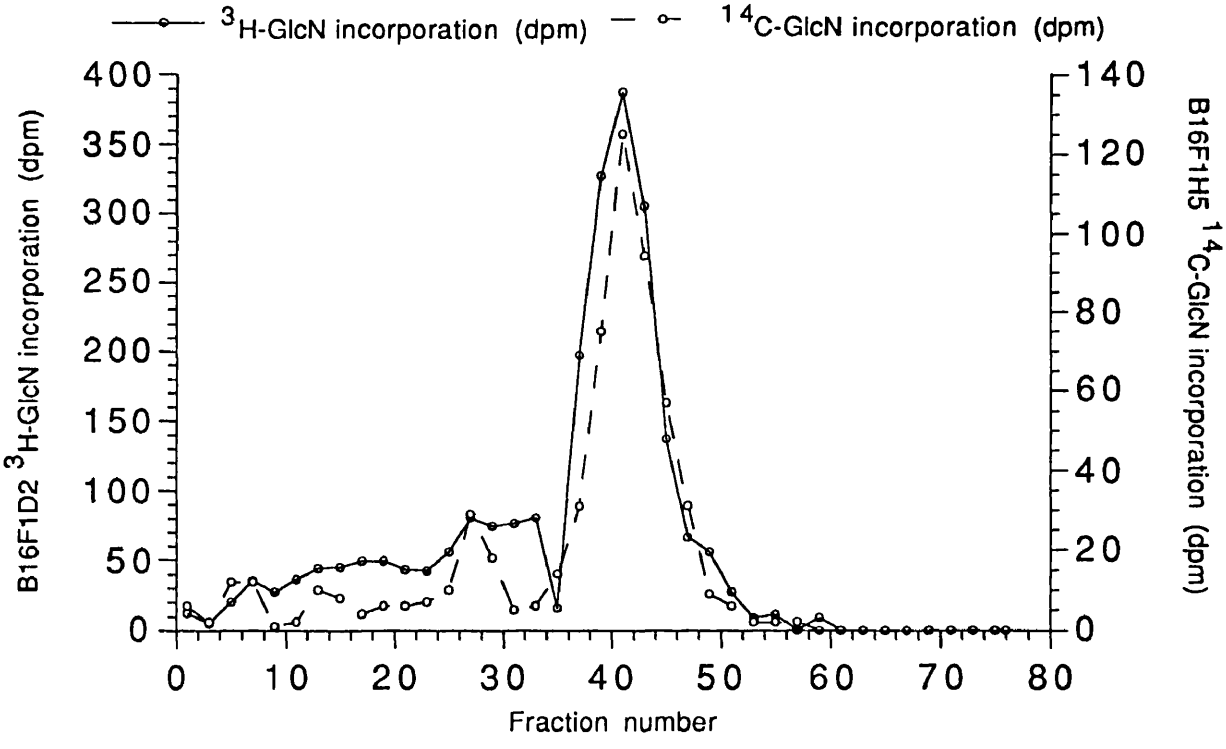
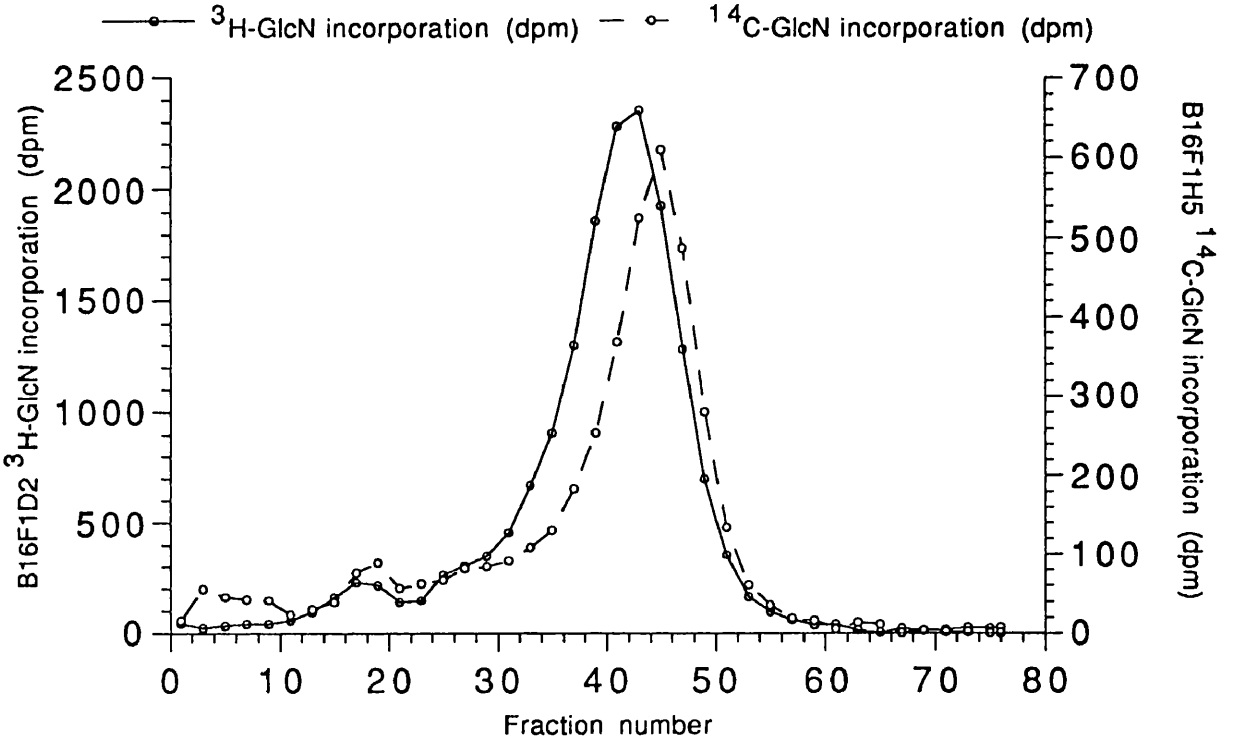


Fig.62; DEAE-Cellulose IEC
Medium fraction HS-GAG



polymer sulphation of the trypsin-releasable CS-GAG (fig.65), suggesting that the increase in charge density of B16F1D2 trypsin-releasable CS-GAG (fig.60) is not related to an increase in the degree of polymer sulphation. Mono-Q-Sepharose also indicated that although B16F1H5 cells synthesized a predominantly more sulphated medium HS-GAG than B16F1D2 cells, B16F1D2 cells synthesized HS-GAG with a wider range of sulphation levels (fig.66).

e) B16F1M3 ± 50mM sodium chlorate

Sodium chlorate inhibits GAG sulphation by competing with GAG for the sulphate donor phosphoadenosylphosphosulphate (PAPS) (Burnell & Roy, 1978; Baeuerle & Huttner, 1986). The presence of 50mM sodium chlorate in culture induced B16F1M3 cells to synthesize both medium fraction and trypsin-releasable fraction HS-GAG with reduced charge density (fig.67 & fig.68 respectively). The trypsin-releasable fraction demonstrated the most pronounced reduction in charge density. In agreement with other reports, sodium chlorate also induced a largely reduced charge density in CS-GAG (fig.69).

f) B16F1M3 ± 20µg/ml heparin

Preincubation of cells with heparin has been reported to alter the degree of HS-GAG polymer sulphation (Nader *et al.*, 1989). The qualitative effect of heparin on B16F1M3 melanoma cell GAG synthesis was therefore analysed using Mono-Q-Sepharose IEC. It was observed that heparin did not affect the degree of CS-GAG polymer sulphation in the medium and trypsin-releasable fractions nor the trypsin-releasable HS-GAG fraction. However heparin was observed to induce the synthesis of oversulphated medium fraction HS-GAG (fig.70).

Fig.63; Mono Q Sepharose IEC
Medium fraction CS-GAG

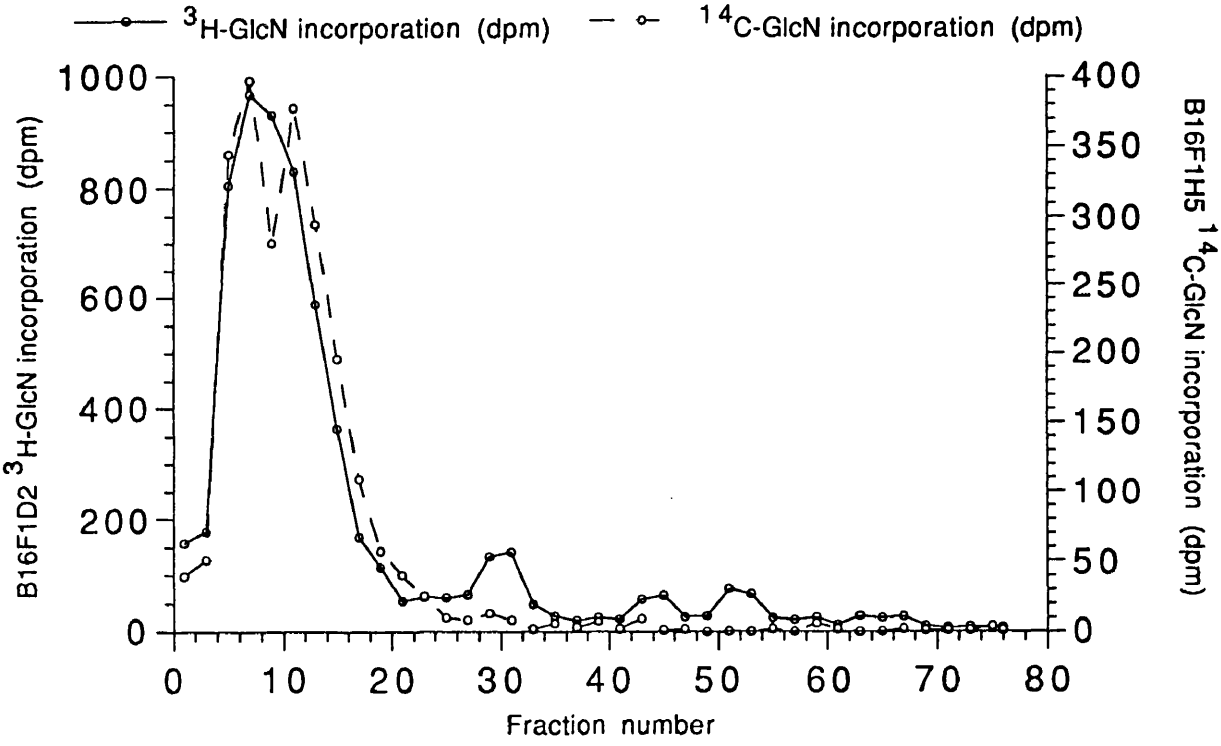


Fig.64; Mono-Q-Sepharose IEC
Trypsin fraction HS-GAG

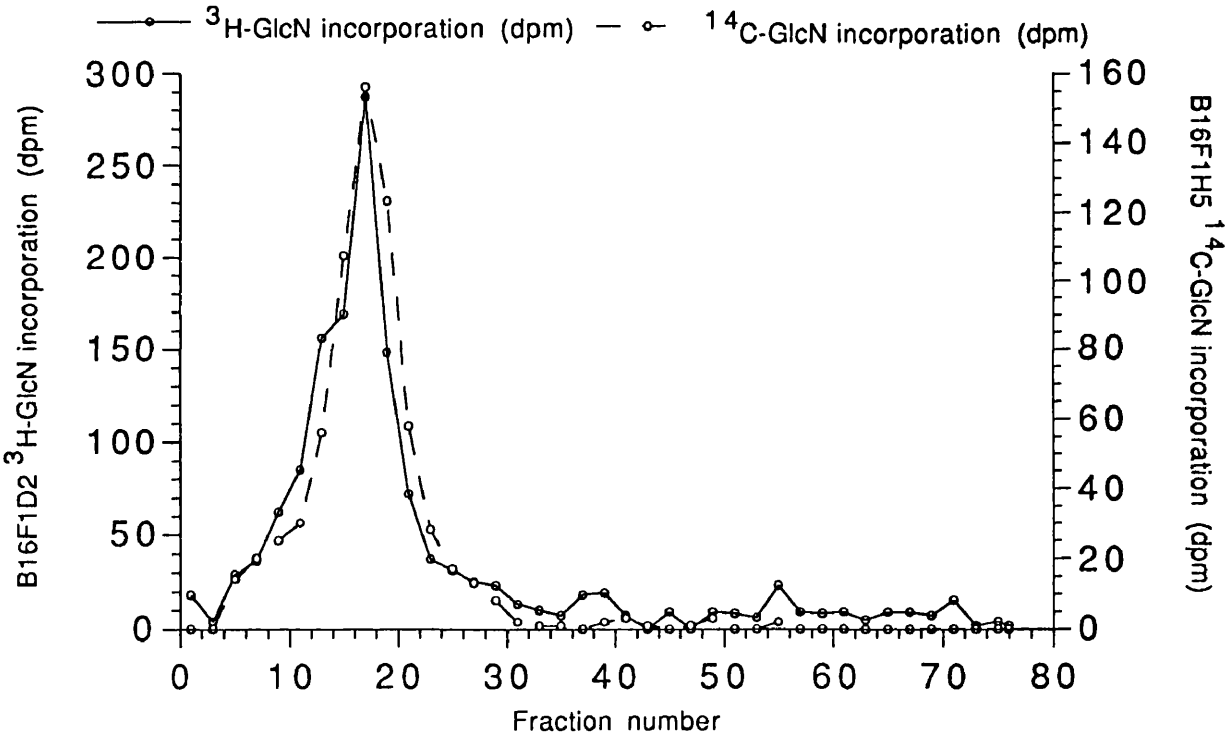


Fig.65; Mono-Q-Sepharose IEC
Trypsin fraction CS-GAG

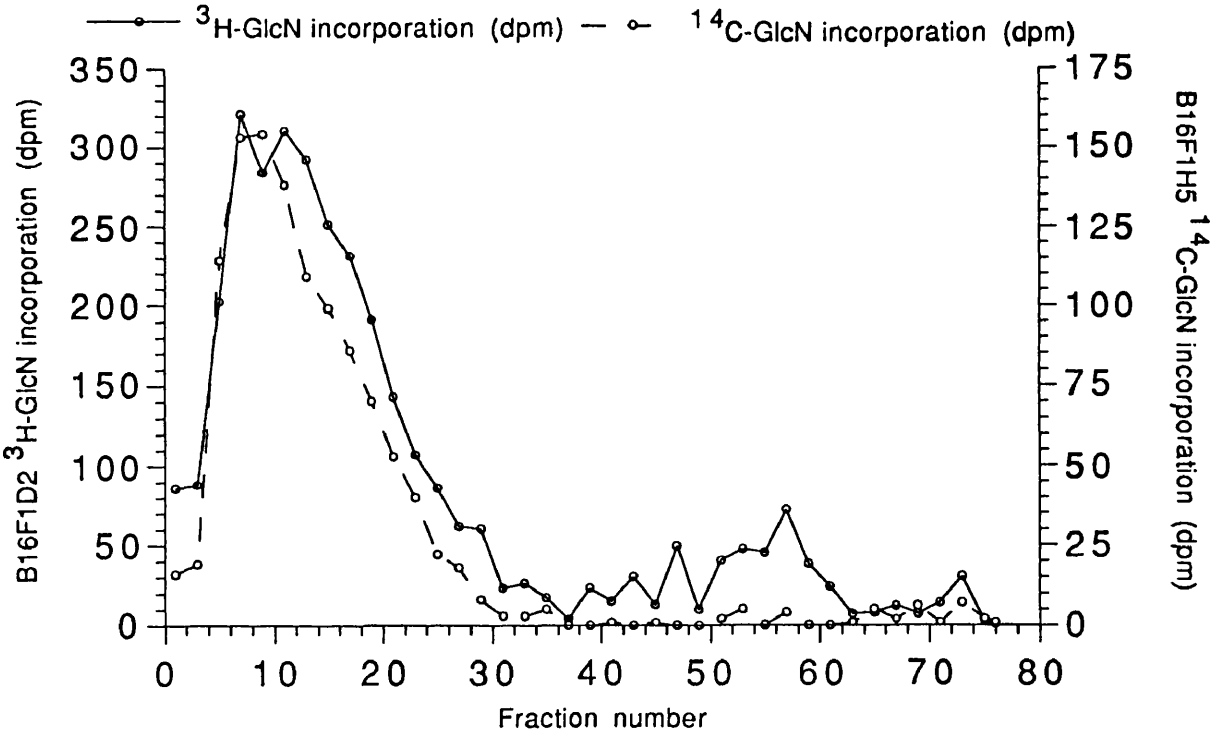


Fig.66; Mono-Q-Sepharose IEC
Medium fraction HS-GAG

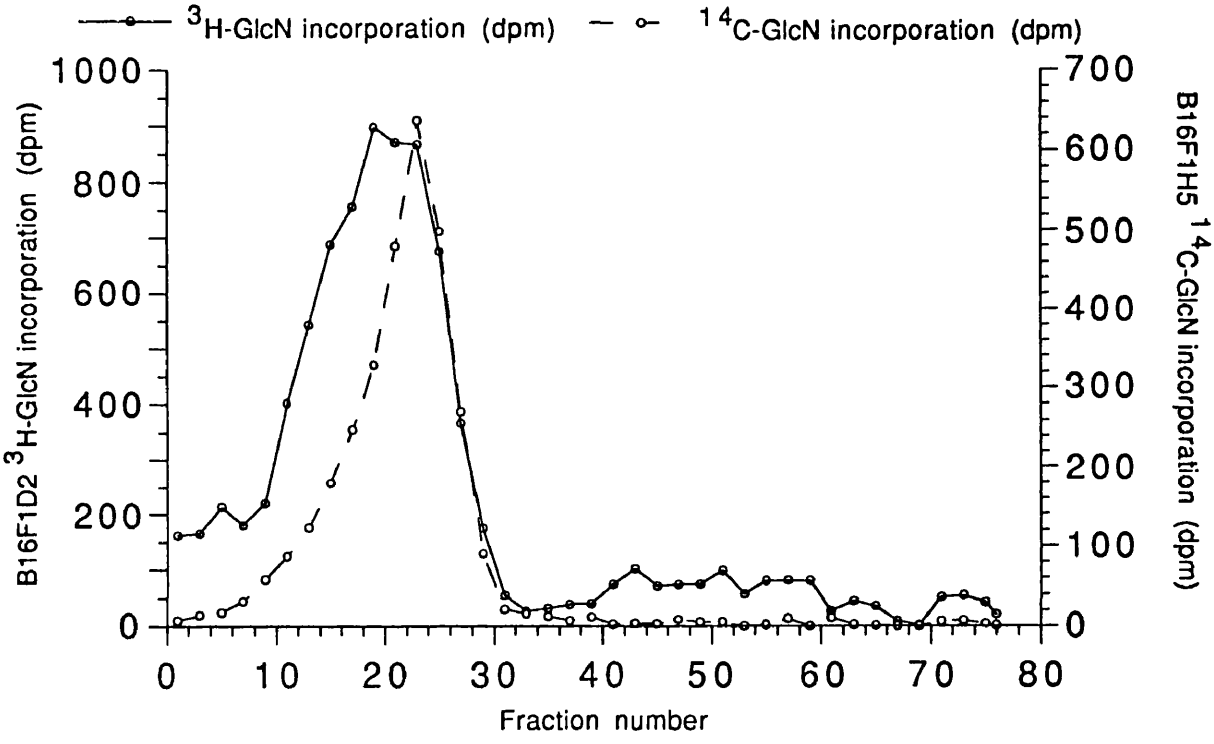


Fig.67; DEAE-Cellulose IEC
Medium fraction HS-GAG

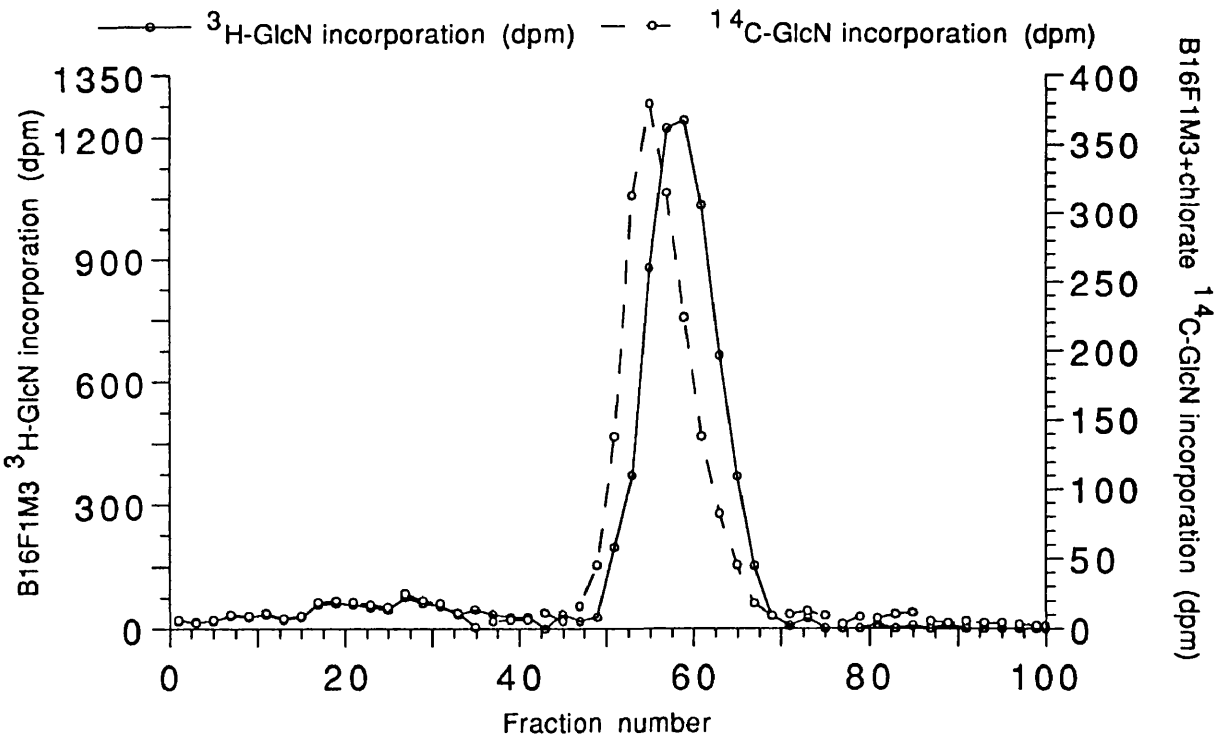


Fig.68; DEAE-Cellulose IEC
Cell-Lysate fraction HS-GAG

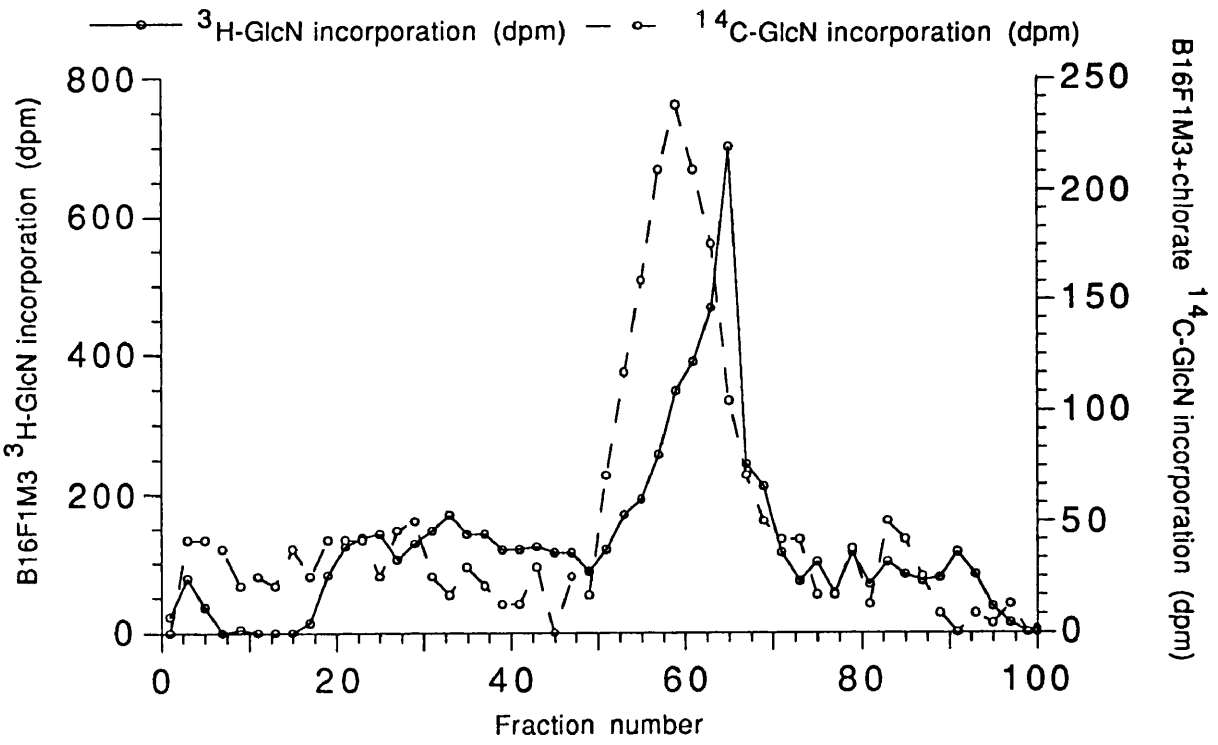


Fig.69; DEAE-Cellulose IEC
Medium fraction CS-GAG

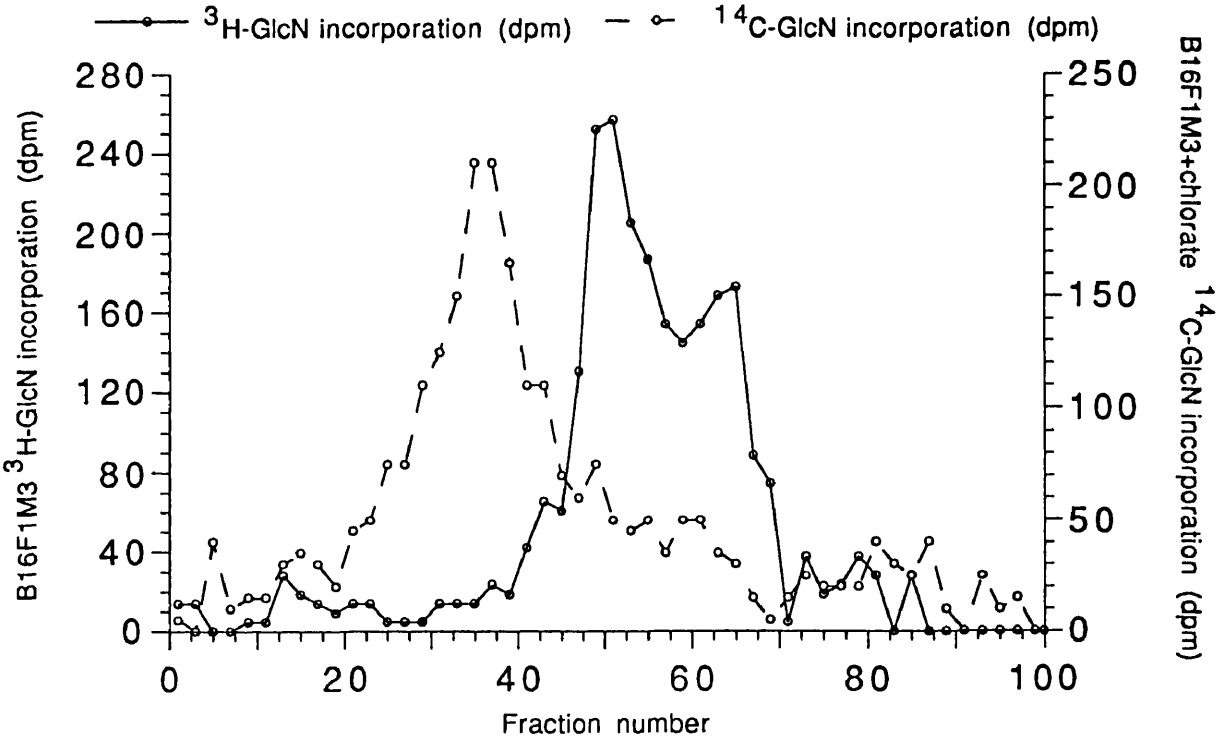
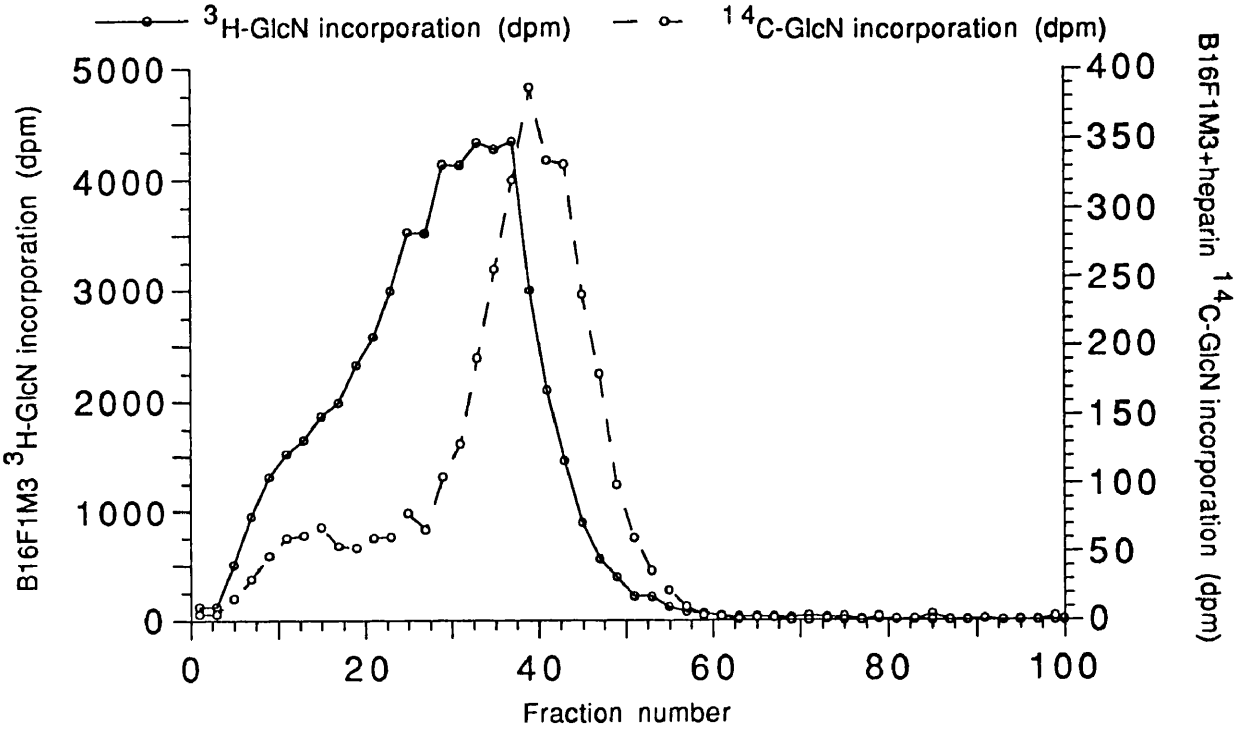


Fig.70; Mono Q Sepharose IEC
Medium fraction HS-GAG



The previous IEC analyses of B16F1M3 GAG fractions have suggested that the cell surface contains more highly charged HS-GAG species than those present in the culture medium. Heparin is able to displace endogenous GAG from the cell surface (Kaji & Sakuragawa, 1990), and therefore control cells were briefly exposed to heparin prior to the harvesting of the cell fractions in order to assess whether heparin displaced "oversulphated" HS-GAG from the cell surface. Subsequently it was observed that no difference in the degree of polymer sulphation could be detected in either the CS-GAG or HS-GAG from the medium or trypsin-releasable fractions, and both the medium and trypsin-releasable HS-GAG had similar levels of sulphation (not shown). This suggests that heparin therefore stimulates B16F1M3 cells to synthesize medium fraction HS-GAG with an increased degree of polymer sulphation.

g) B16F1D2 ± 0.5mM ß-D-xyloside treatment

The GAG derived from the medium-associated and cell-lysate fractions derived from the proteoglycan from control and ß-D-xyloside treated B16F1D2 cells were analysed via IEC. ß-D-xyloside was observed to influence GAG synthesis; the CS-GAG associated with the cell surface of ß-D-xyloside-treated cells (fig.71) and the medium-associated HS-GAG (fig.72) had a lower charge density than those of control cells, though this reduction was less pronounced for the HS-GAG. In contrast, pretreatment with ß-D-xyloside-treatment had no effect on the charge density measured for either the medium-associated CS-GAG (fig.73) or the cell surface HS-GAG (fig.74).

Fig.71; DEAE-Cellulose IEC
Cell lysate fraction CS-GAG

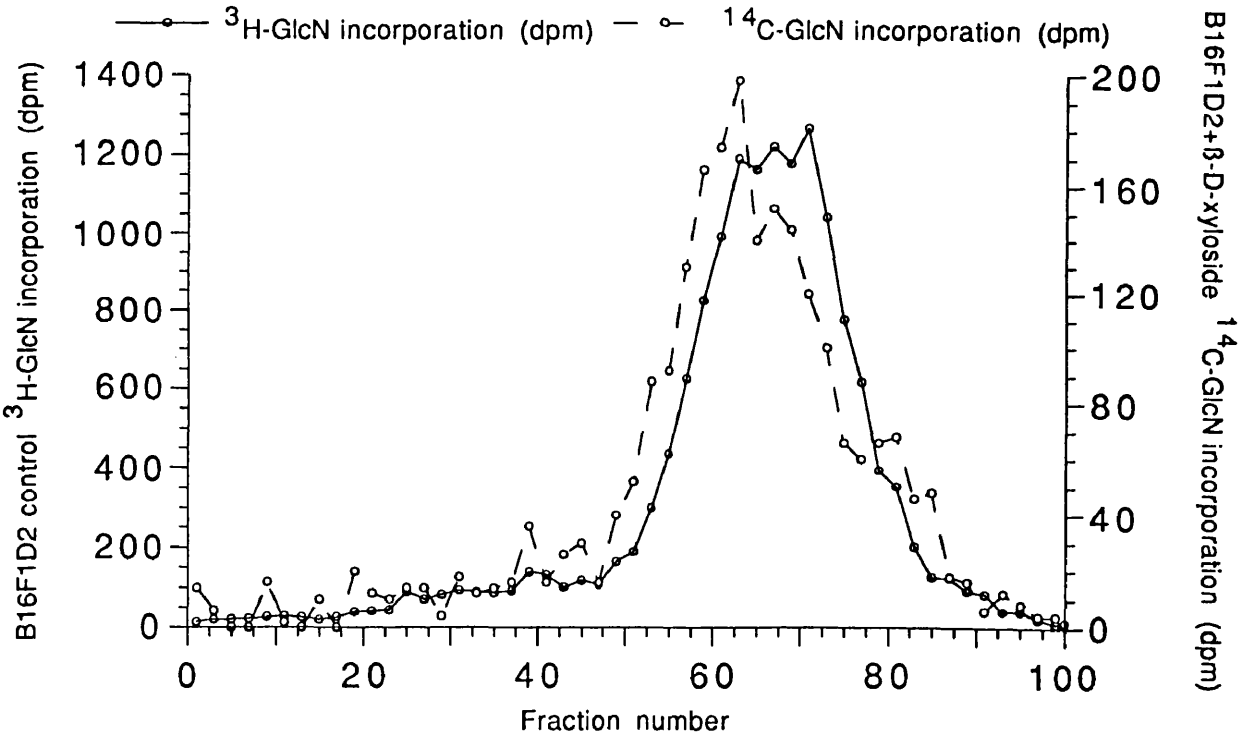


Fig.72; DEAE-Cellulose IEC
Medium fraction HS-GAG

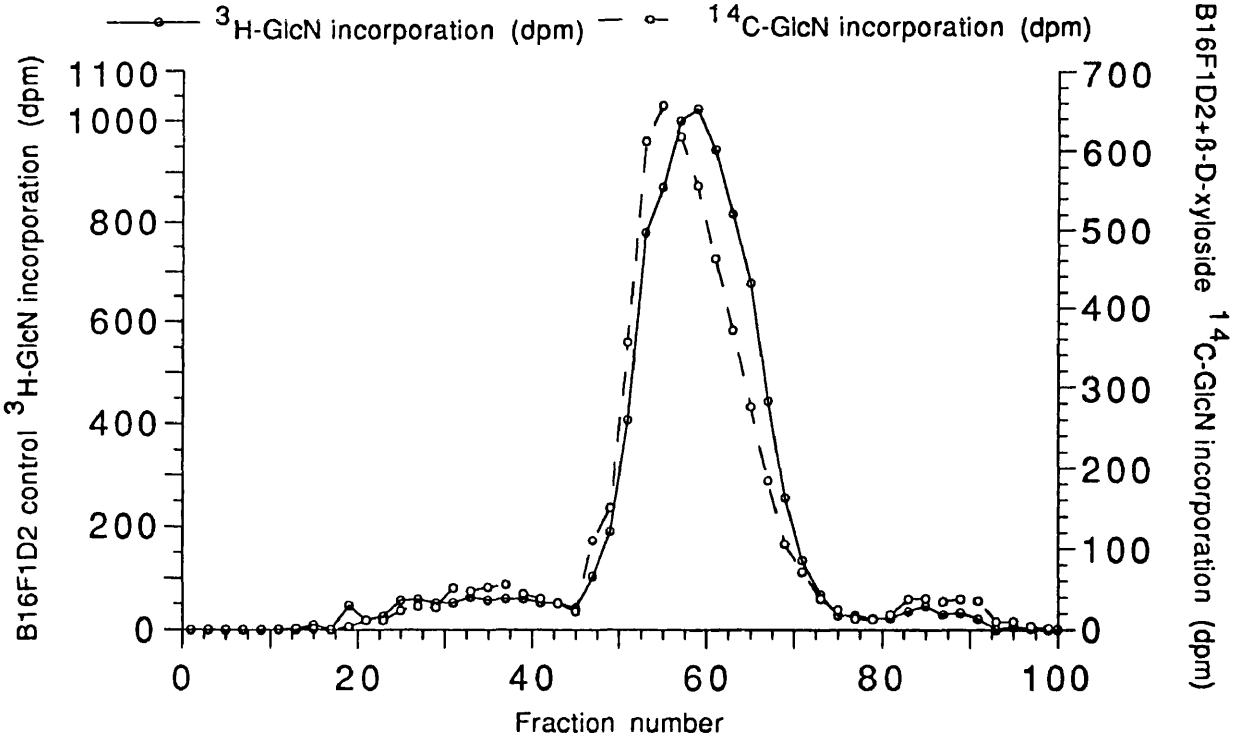


Fig.73; DEAE-Cellulose IEC
Medium fraction CS-GAG

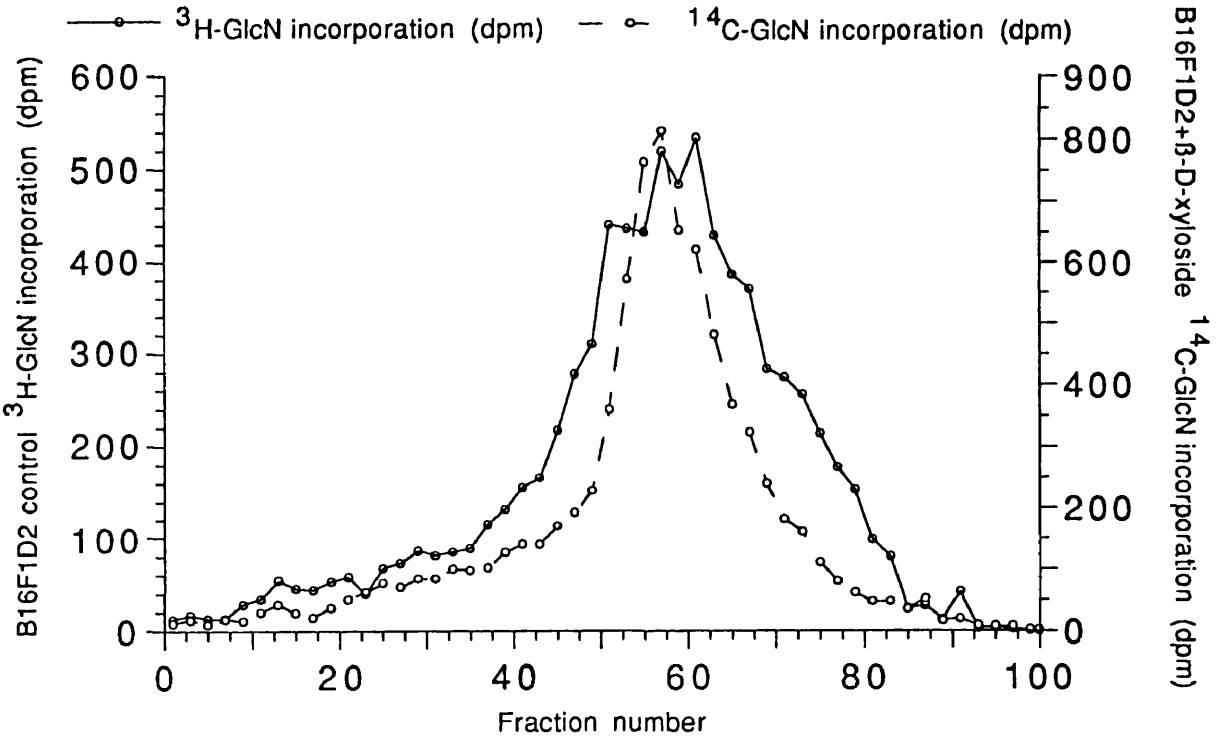
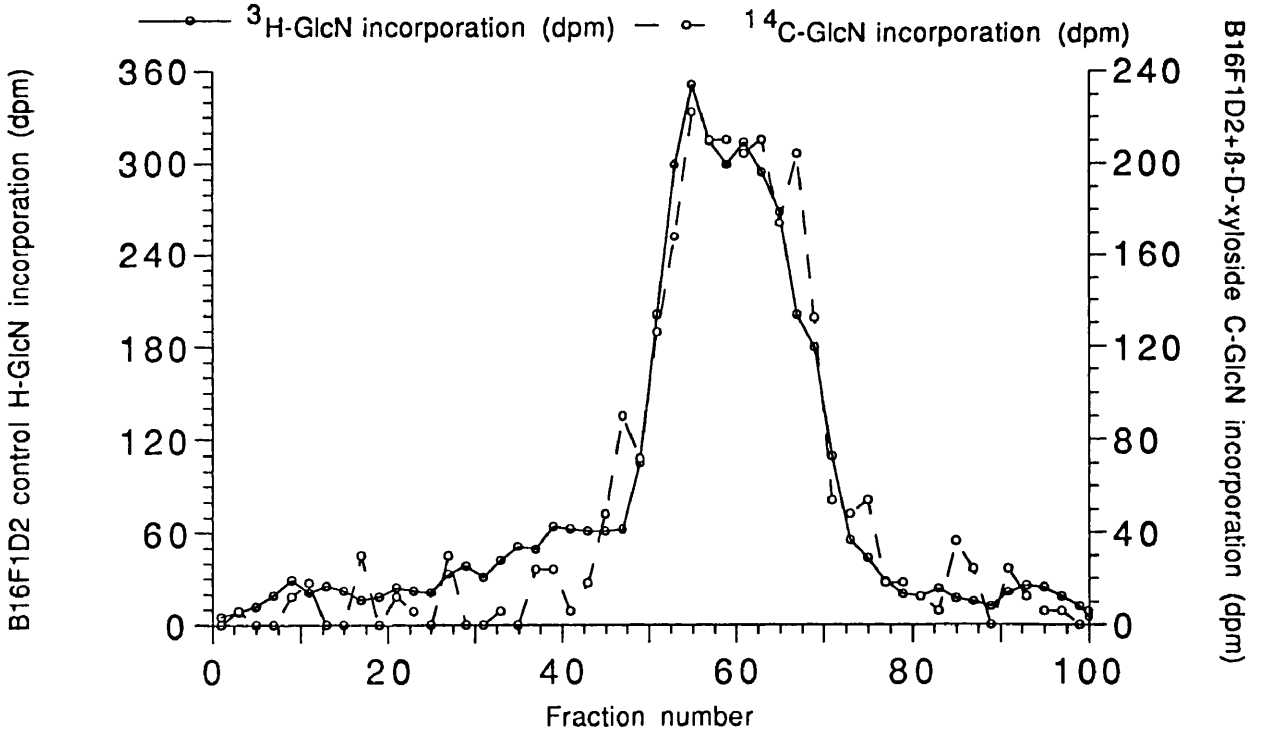


Fig.74; DEAE-Cellulose IEC
Cell lysate fraction HS-GAG



4.4.3. Qualitative differences in proteoglycan synthesis

Qualitative Sepharose CL4B GPC was performed on the proteoglycans and GAG synthesized by the B16 melanoma cell clones, B16F1H5 and B16F1D2. Furthermore, due to the significant effect of ß-D-xyloside on lung colonization and the mode of action of this agent, the proteoglycans from ß-D-xyloside-treated cells were also analysed in this manner.

Two distinct proteoglycan fractions were isolated and analysed; the medium-associated and cell-lysate fractions. A measure of proteoglycan and GAG size (K_{av}) was determined from their point of elution from the GPC column where the peak was highest:

$$K_{av} = (V_e - V_o)/(V_t - V_o)$$

V_o: void volume

V_t: total included volume

V_e: volume in which molecule of interest elutes

For a 90cm Sepharose CL4B GPC column the V_o volume was typically 25ml and the V_t volume 75ml.

a) B16F1H5 vs B16F1D2

Sepharose CL4B GPC analysis of the medium-associated proteoglycan fraction demonstrated that both cell lines possessed similar elution profiles (fig.75). The elution profile for each cell line comprised two major regions. For B16F1D2 cells the first region contained two separate peaks with K_{av} of approximately (~) 0.32 and 0.45. The smaller second region contained a peak with an K_{av} ~ 0.59 which was also observed for B16F1H5 cells. The first region of B16F1H5 cells contained a peak

with a K_{av} ~0.42 at its highest point and a third distinct peak (K_{av} ~0.85) eluting close to the total included volume (V_t). The resultant loss of most of the first region in both the medium-associated (fig.77 & fig.78) and cell-lysate (fig.79 & fig.80) fractions upon pronase digestion indicated that the first peak represented the proteoglycan fraction and the second peak free GAG.

The cell-lysate proteoglycan fraction elution profiles were comprised of two regions that differed markedly between the two cell lines (fig.76). The first peak (K_{av} ~0.39) was the predominant form in B16F1D2 cells with the second peak (K_{av} ~0.75) being the minor form. This pattern was reversed for the B16F1H5 cells where the first peak (K_{av} ~0.49) was less distinct and the second peak (K_{av} ~0.72) was the major form.

Overall, the medium-associated fractions from B16F1H5 and B16F1D2 melanoma cells contained proteoglycans and GAG of similar size, but the B16F1D2 cells possessed a larger cell-lysate fraction proteoglycan pool. These fractions were subsequently digested with chondroitinase ABC or nitrous acid to leave intact HSPG or CSPG fractions respectively. The GPC elution point for GAG chains was determined by digesting proteoglycans with pronase.

B16F1D2 cells synthesized larger cell-lysate HSPG (fig.81; K_{av} ~0.50) than that synthesized by B16F1H5 cells (K_{av} ~0.57). Additionally, the cell-lysate fraction from both cell lines contained free HS-GAG chains identifiable as a distinct second peak (K_{av} ~0.78). Similarly, the

Fig.75; Sepharose CL4B GPC
Medium fraction PG/GAG

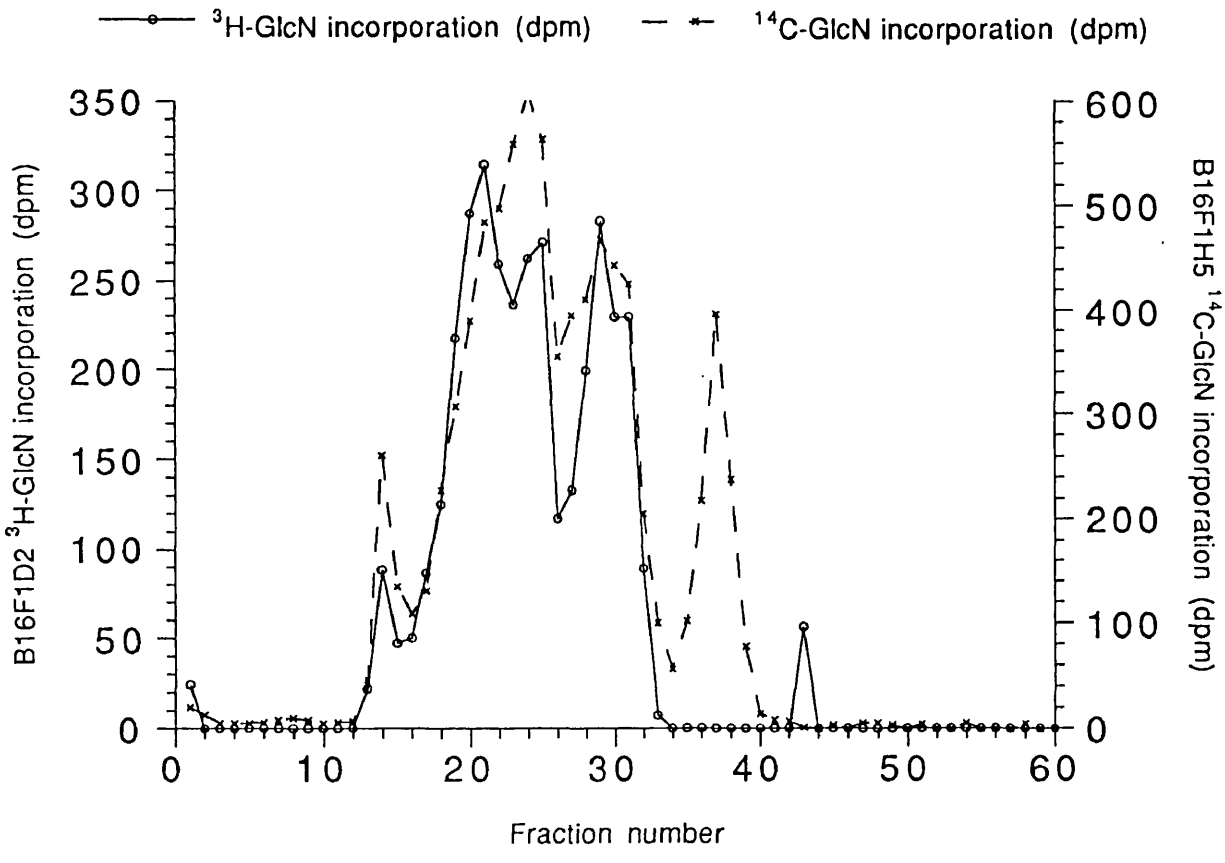


Fig.76; Sepharose CL4B GPC
Cell lysate fraction PG/GAG

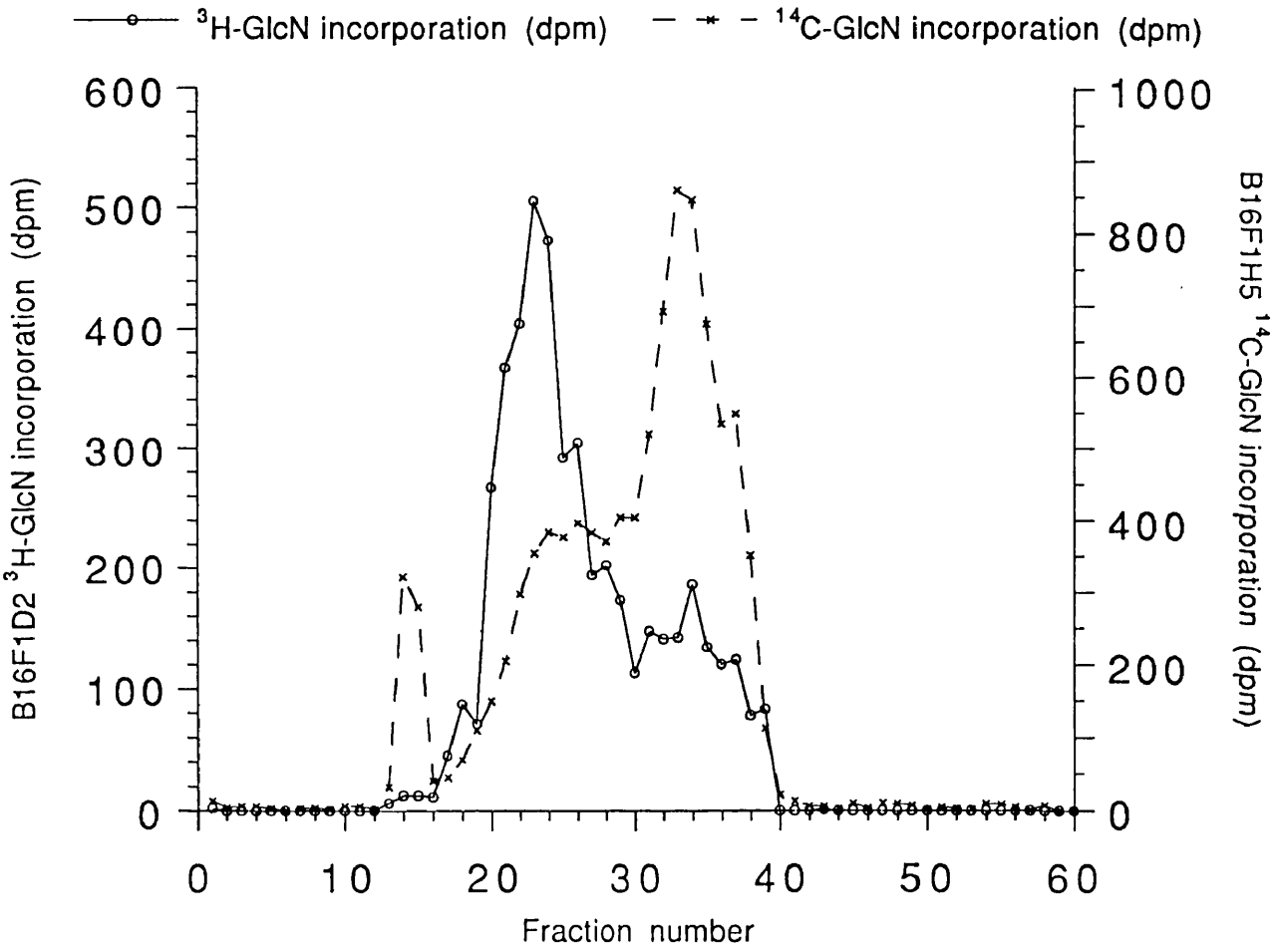


Fig.77; Sepharose CL4B GPC
Medium fraction CSPG/GAG/Pronase

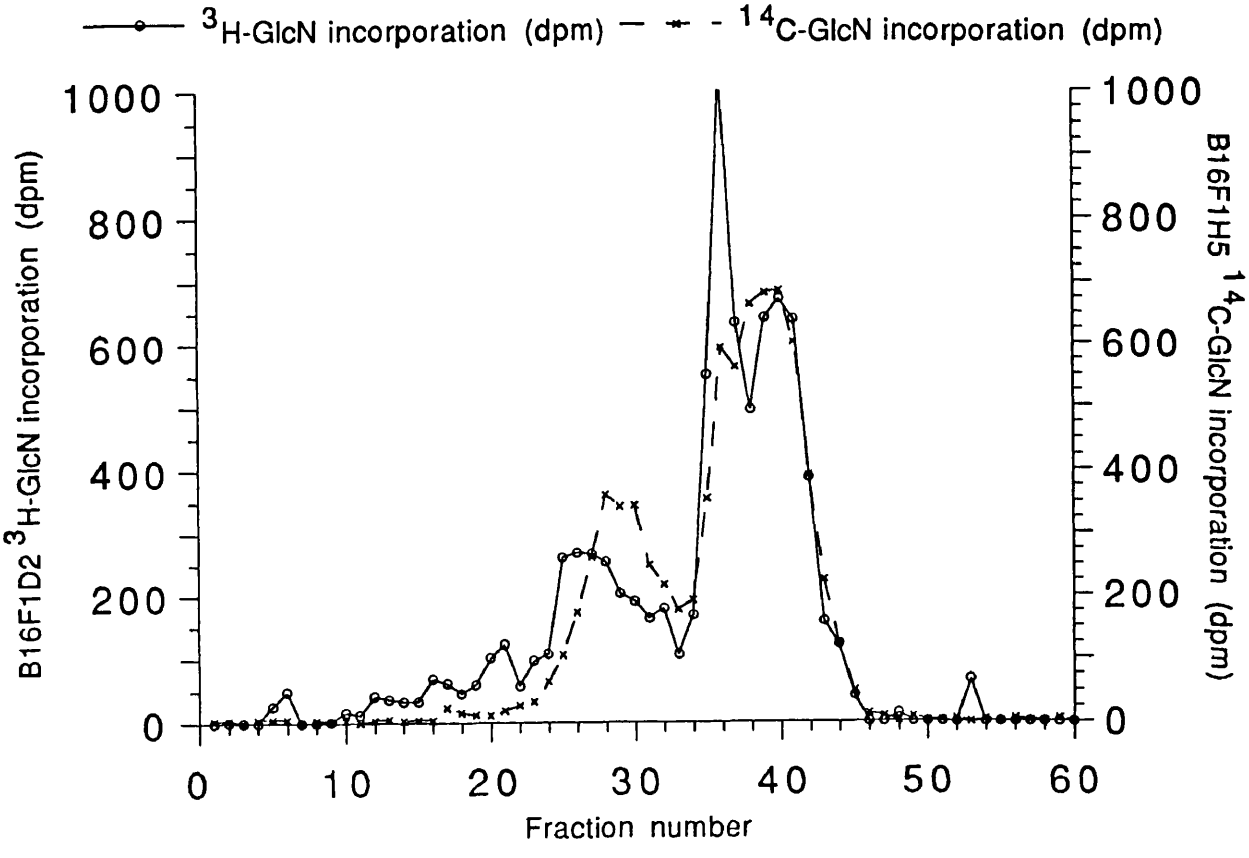


Fig.78;Sepharose CL4B GPC
Medium fraction HSPG/GAG/Pronase

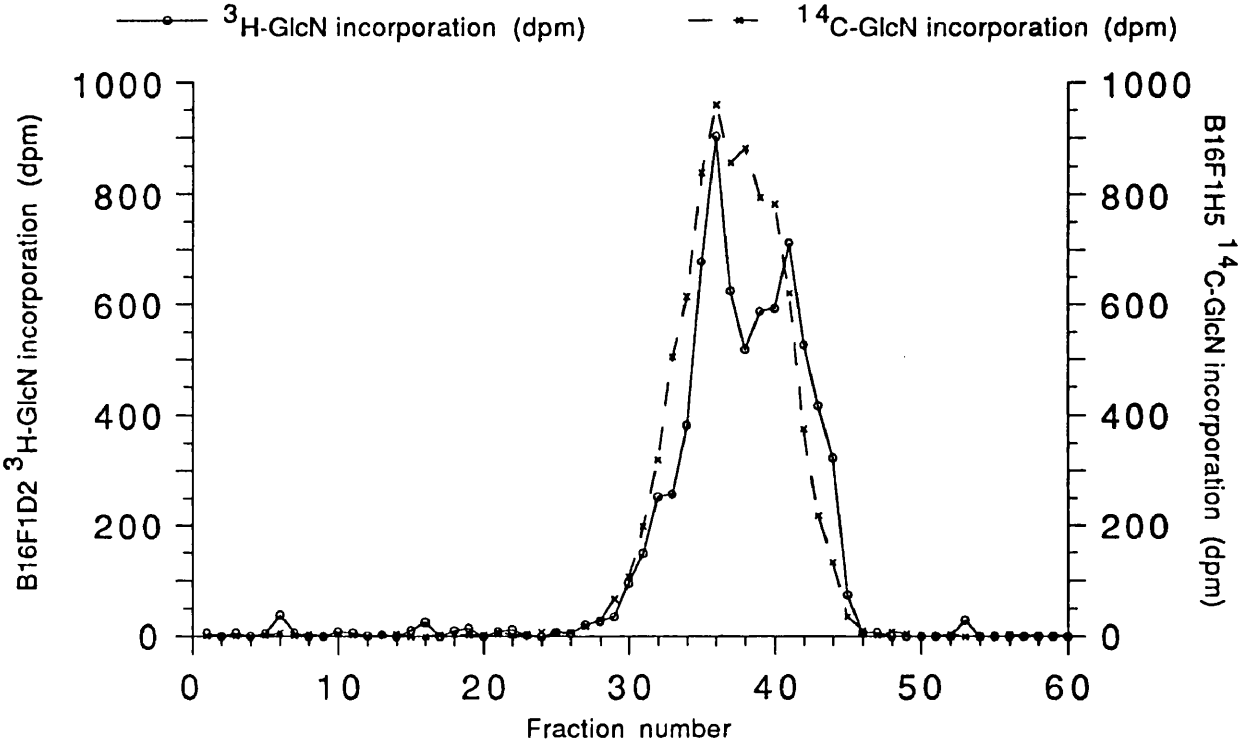


Fig.79; Sepharose CL4B GPC
Cell lysate fraction CSPG/GAG/Pronase

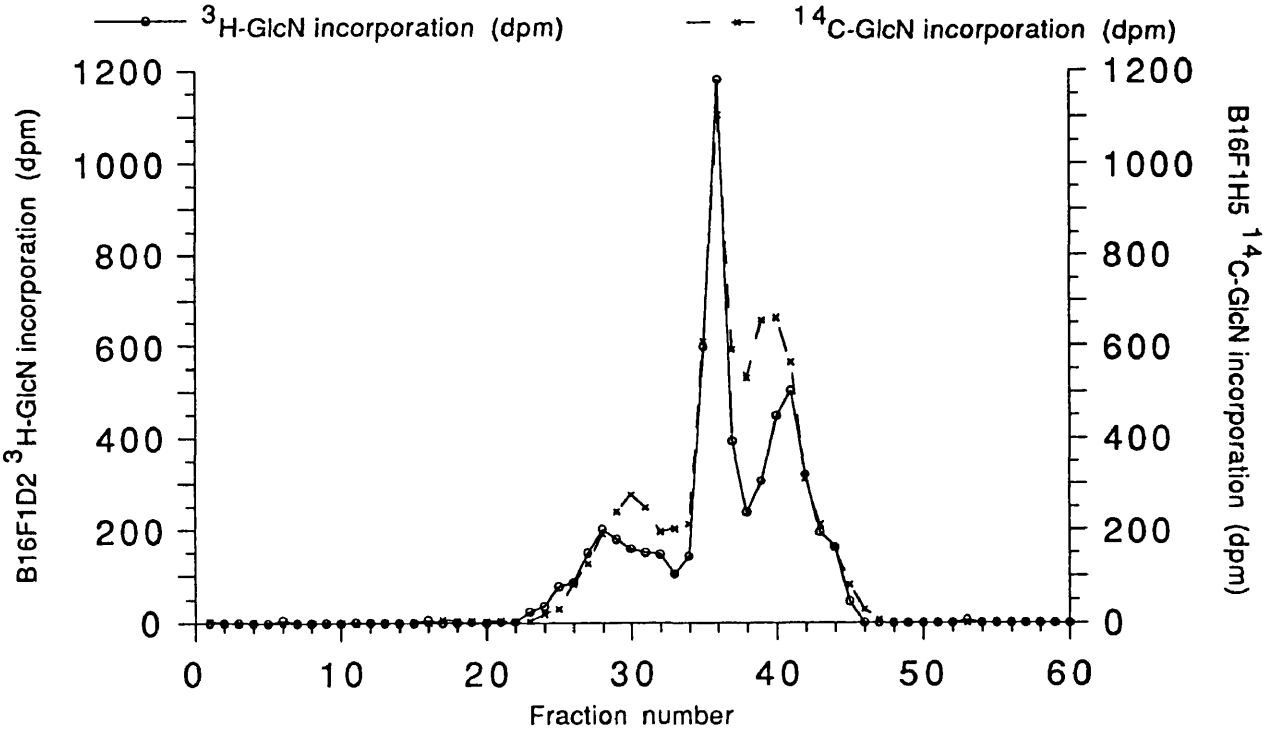


Fig.80; Sepharose CL4B GPC
Cell lysate fraction HSPG/GAG/Pronase

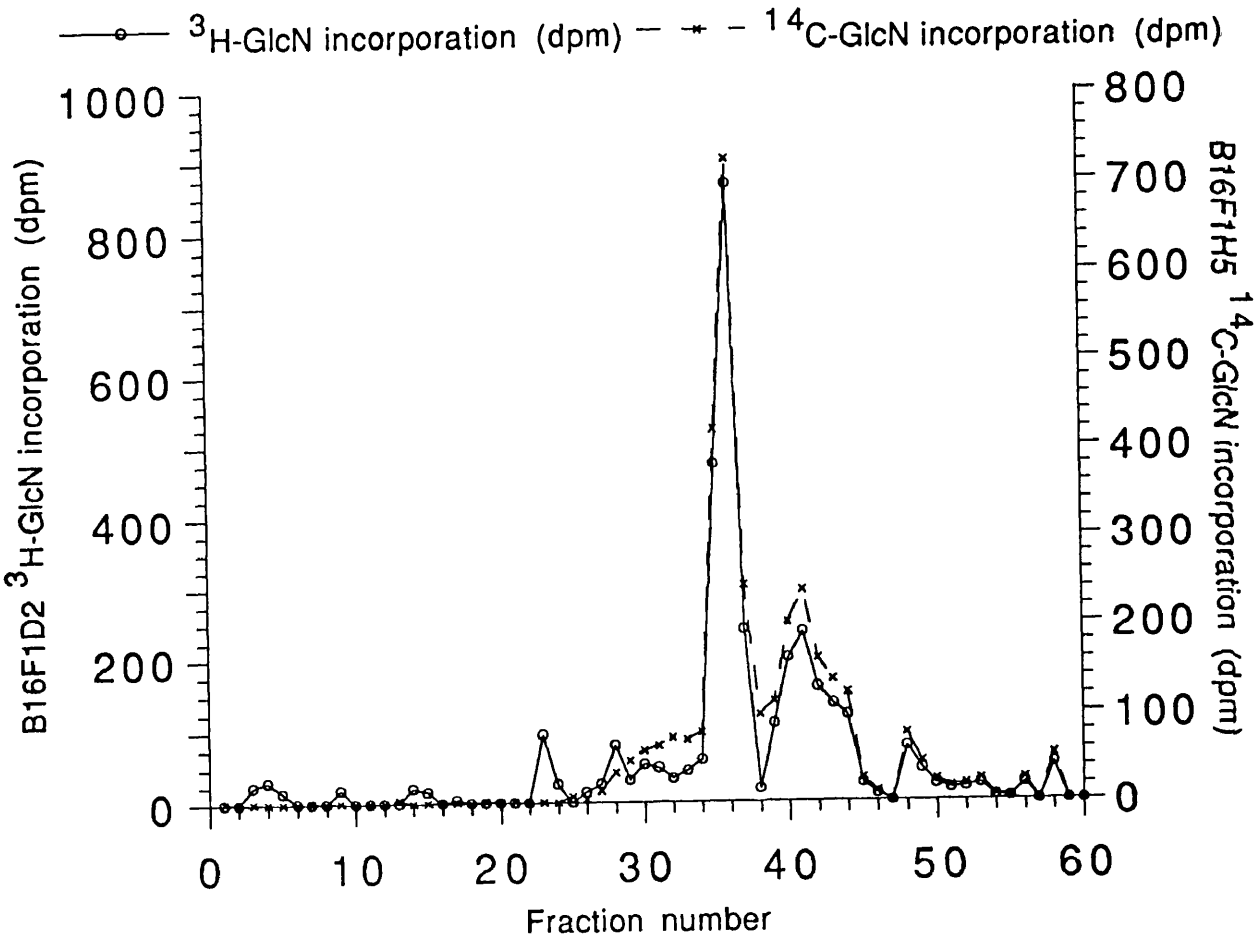


Fig.81; Sepharose CL4B GPC
Cell lysate fraction HSPG/GAG

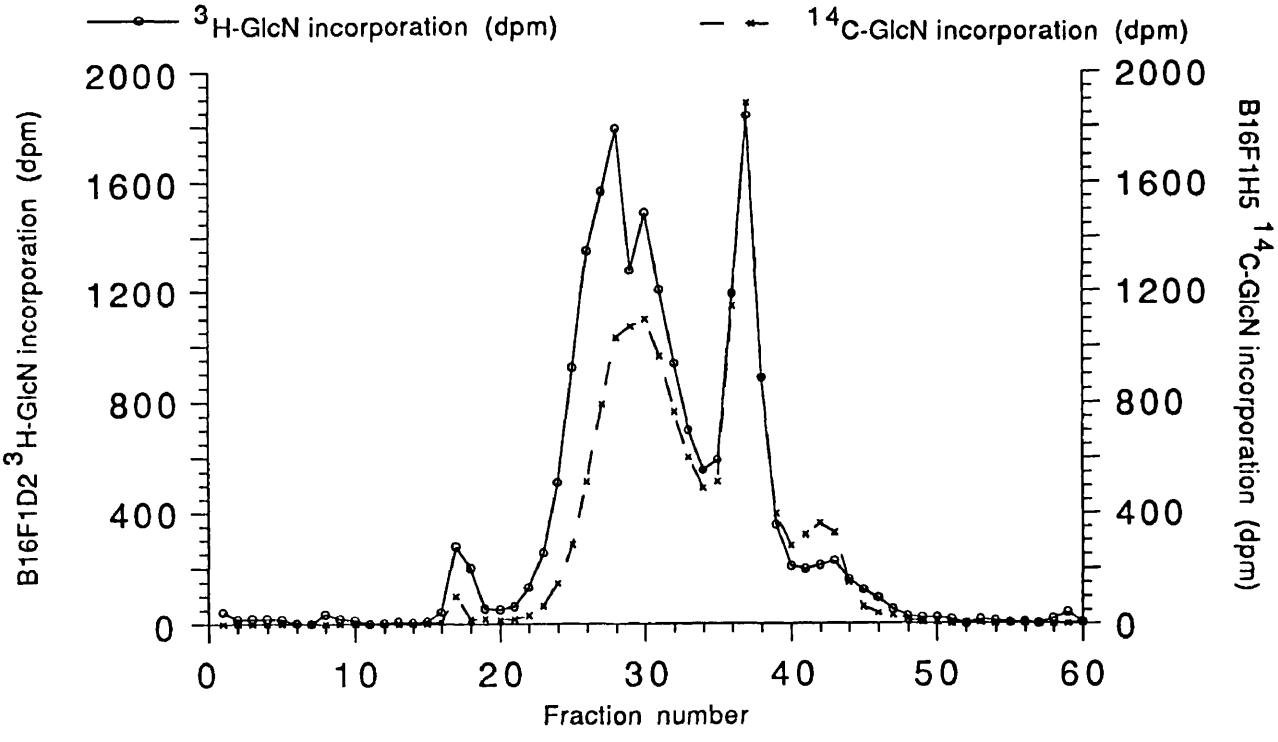


Fig.82; Sepharose CL4B GPC
Cell lysate fraction CSPG/GAG

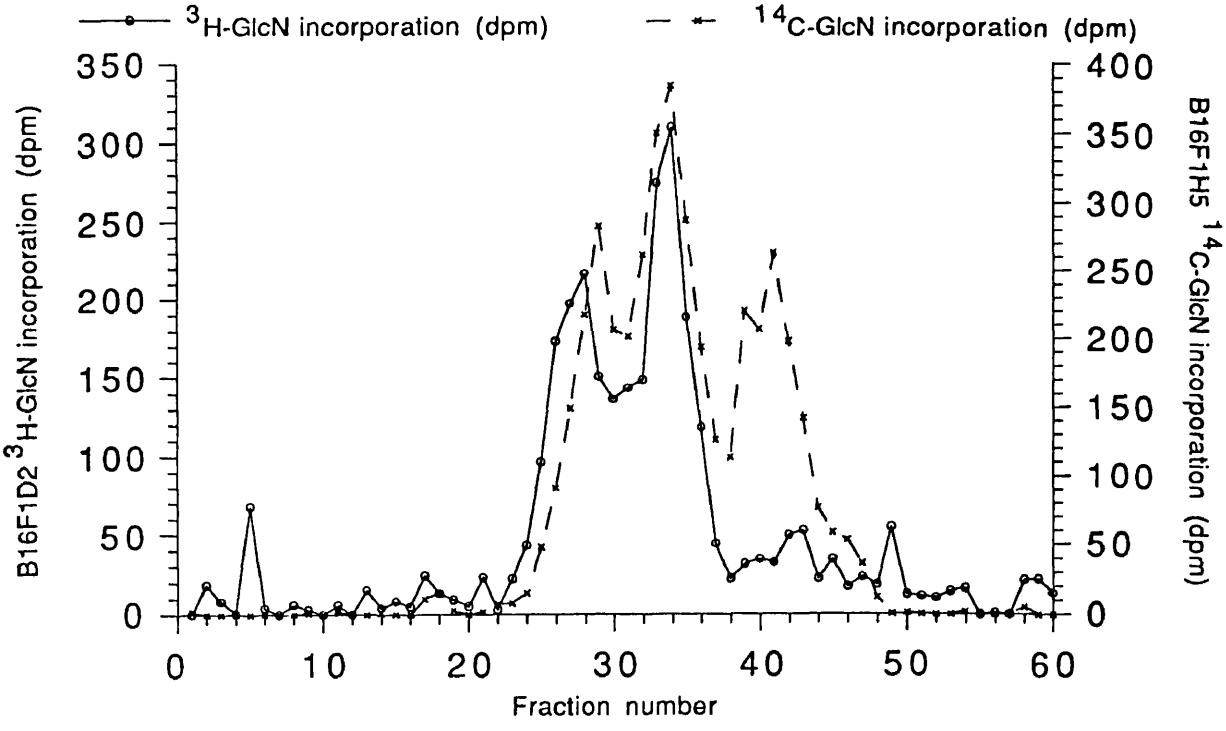


Fig.83; Sepharose CL4B GPC
Medium fraction HSPG/GAG

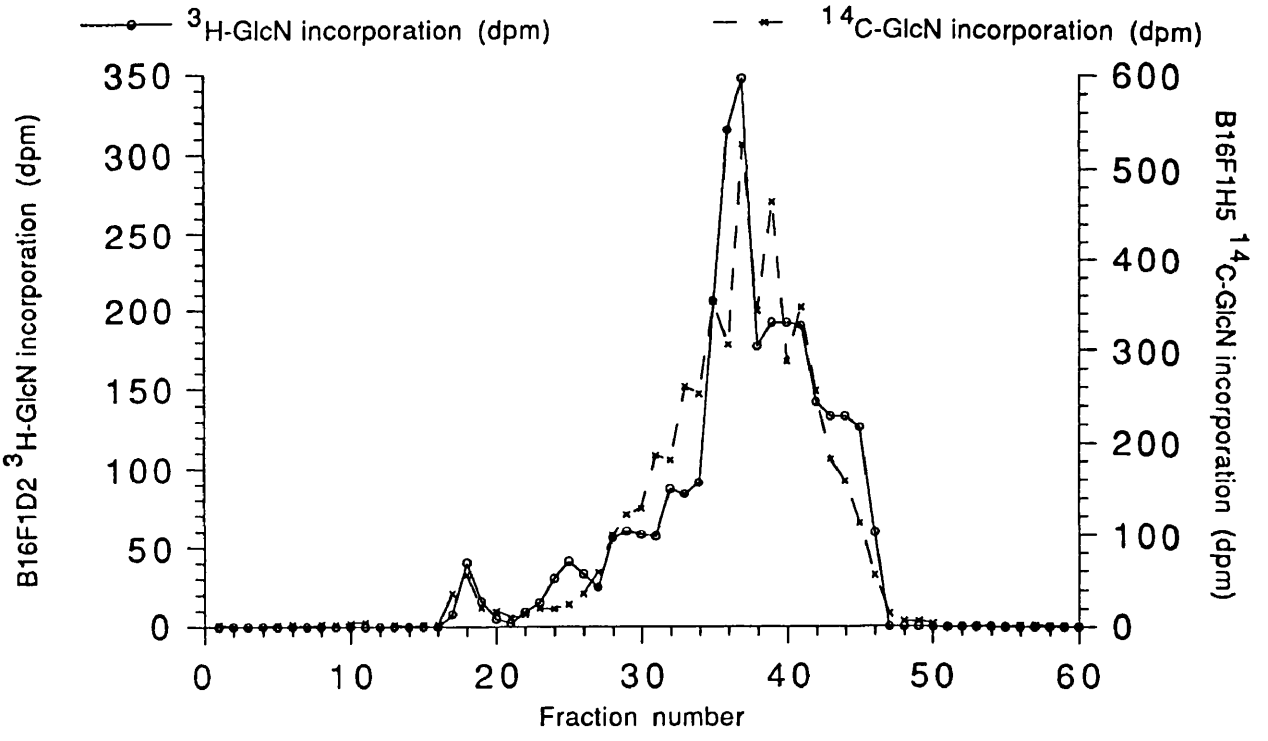
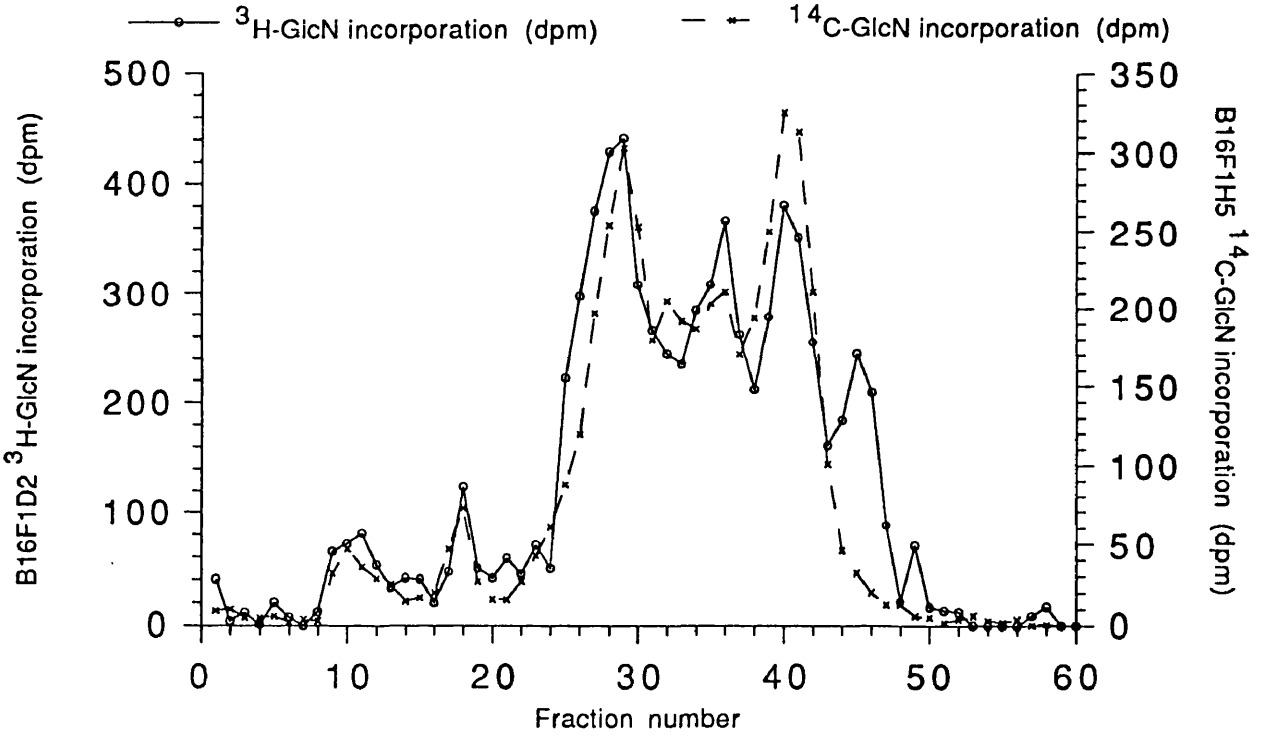


Fig.84; Sepharose CL4B GPC
Medium fraction CSPG/GAG



cell-lysate fraction CSPG (fig.82) from B16F1D2 cells was apparently larger (K_{av} ~0.50) than that of B16F1H5 cells (K_{av} ~0.54). Interestingly, these peaks were not completely lost upon pronase digestion (fig.79). The cell-lysate fraction (fig.82) also contained distinct free CS-GAG peaks and for B16F1H5 cells, an additional third peak eluting close to V_t.

HSPG was not present in the medium-associated fraction of either cell line. Rather, a peak that appeared to correspond to free HS-GAG chains (fig.83) and which did not alter significantly upon pronase digestion (fig.78) was present. The region corresponding to the medium-associated CSPG revealed that there was no significant difference in the size of the CSPG synthesized by either cell line (fig.84). A similar observation was noted for the CS-GAG in this fraction although the radioactivity eluting close to V_t was marginally smaller for B16F1H5 cells (fig.84). Pronase digestion did not eliminate all of the radioactivity associated with either the medium-associated or cell-lysate CSPG peaks (fig.77 & fig.79), suggesting that either these peaks represent very high molecular weight CS-GAG or pronase resistant CSPG since both were sensitive to digestion with chondroitinase ABC (fig.78 & fig.80).

The medium-associated pronase-resistant chondroitin sulphate species synthesized by B16F1H5 melanoma cells and eluting in the proteoglycan region (fig.77), had an apparently smaller molecular weight (K_{av} ~0.54) than that synthesized by B16F1D2 cells (K_{av} ~0.46). A similar difference was also observed in the cell-lysate chondroitin fraction (fig.79); the large chondroitin sulphate species synthesized by B16F1H5 cells was apparently smaller (K_{av} ~0.57) than that synthesized by B16F1D2 cells

(K_{av} ~0.50). There were no significant differences in the sizes of the free CS-GAG chains (fig.77 & fig.79) and HS-GAG chains (fig.78 & fig.80) synthesized by both cell lines.

In brief, B16F1D2 melanoma cells appeared to synthesize larger cell-lysate CSPG and HSPG species than B16F1H5 cells. Neither cell line synthesized a medium fraction HSPG. Free GAG chains were present in both the medium-associated and cell-lysate fractions which also contained a pronase-resistant, chondroitinase ABC-sensitive peak eluting in the proteoglycan region of the Sepharose CL4B GPC column. The ratios of proteoglycan to GAG also differed; in the cell-lysate fraction B16F1D2 cells synthesized proteoglycan as the predominant GAG form whereas free GAG chain was the predominant form for B16F1H5 cells. The medium fraction ratio of proteoglycan to GAG were similar except that B16F1H5 cells possessed a distinct third peak that eluted close to V_t.

b) B16F1D2 ± 0.5mM ß-D-xyloside

Sepharose CL4B GPC proteoglycan analysis of the medium-associated fraction revealed the presence of two distinct peaks in control cells, whereas cells treated with ß-D-xyloside possessed only the second of the two peaks (fig.85). Pronase digestion resulted in the virtual elimination of this first peak in control cells (fig.87) indicating that this corresponded to proteoglycans (K_{av} ~0.45). The second peak was pronase-resistant and corresponded to free GAG chains (K_{av} ~0.70). ß-D-xyloside treatment therefore resulted in the elimination of the proteoglycan peak from the medium-associated fraction of B16F1D2 cells. The medium-associated fraction also contained a pronase-resistant peak (fig.87) with an elution

point (K_{av} ~0.5) similar to that observed in the medium-associated and cell-lysate chondroitins in fig.77 and fig.79.

There were no significant differences in the GPC elution profile obtained for the cell-lysate fraction from control and ß-D-xyloside treated cells. Both elution profiles contained two distinct peaks corresponding to proteoglycan (K_{av} ~0.57) and GAG (K_{av} ~0.72) fractions (fig.86). Pronase digestion resulted in a shift in this proteoglycan peak (fig.88). The results indicate that B16F1D2 melanoma cells cultured in the presence of ß-D-xyloside maintain a similar cell surface proteoglycan profile as that observed for control cells. Indirect comparison of the cell-lysate proteoglycan profiles for B16F1D2 cells in fig.76 and fig.86 reveal differences in the two constituent peaks elution profiles, which may reflect the presence of DMSO in the culture medium of the control and ß-D-xyloside-treated cells.

CSPG was the only proteoglycan present in the medium of the B16F1D2 cells (fig.89). The medium-associated fraction from both control and ß-D-xyloside-treated cells also contained free CS-GAG and HS-GAG chains (fig.89 & fig.90 respectively). Sepharose CL4B GPC analysis again failed to detect the presence of HSPG in the medium-associated heparan sulphate fraction of control cells (fig.90). Free HS-GAG chains were also present in the cell-lysate fraction of both the control and ß-D-xyloside-treated cells (fig.92).

The cell-associated fraction from control and treated cells contained both CSPG (fig.91), HSPG (fig.92), and free CS-GAG and HS-GAG. The cell-lysate CSPG and HSPG were of similar size (K_{av} ~0.57) as were the GAG species

Fig.85; Sepharose CL4B GPC
Medium fraction PG/GAG

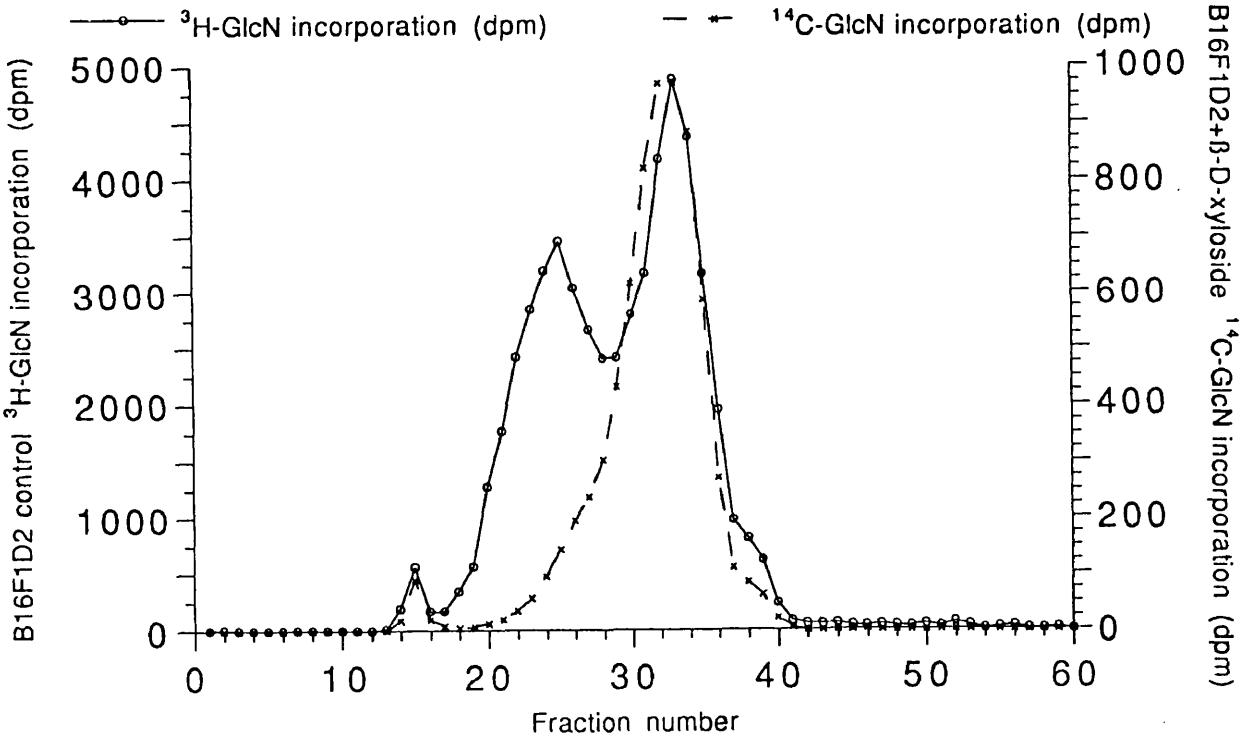


Fig.86; Sepharose CL4B GPC
Cell lysate fraction PG/GAG

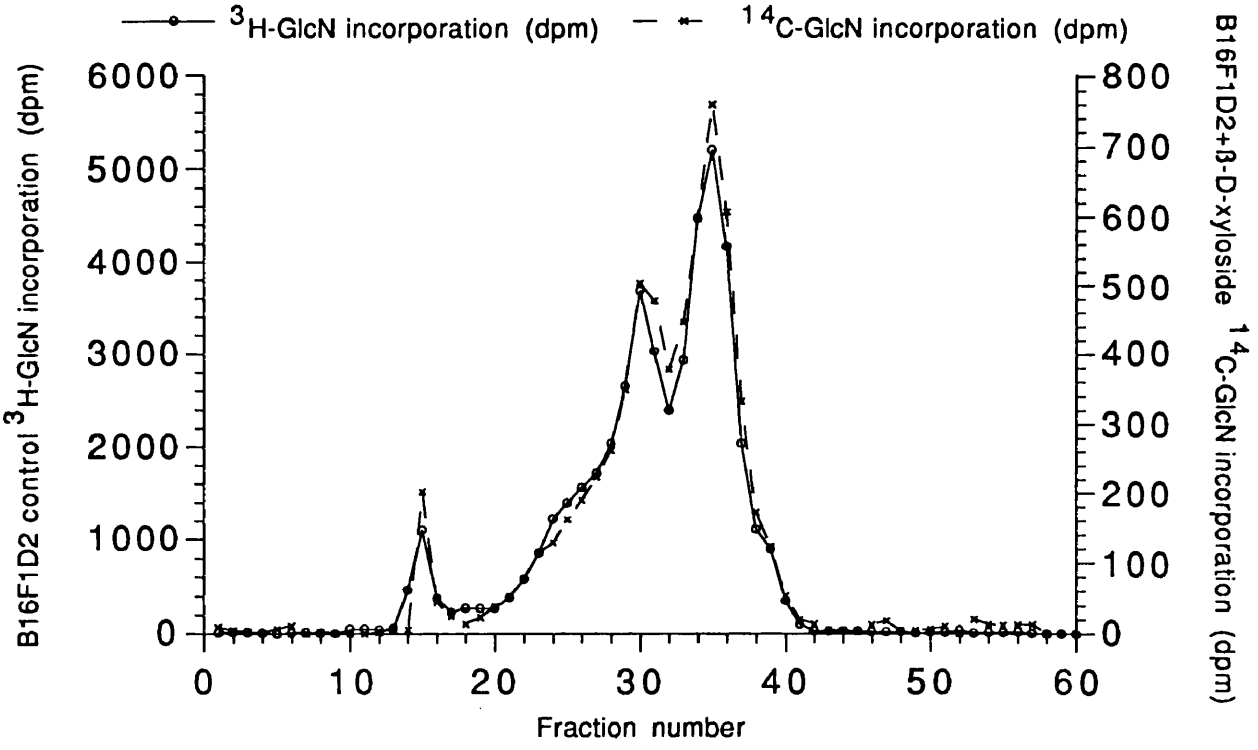


Fig.87; Sepharose CL4B GPC
Medium fraction PG/GAG/Pronase

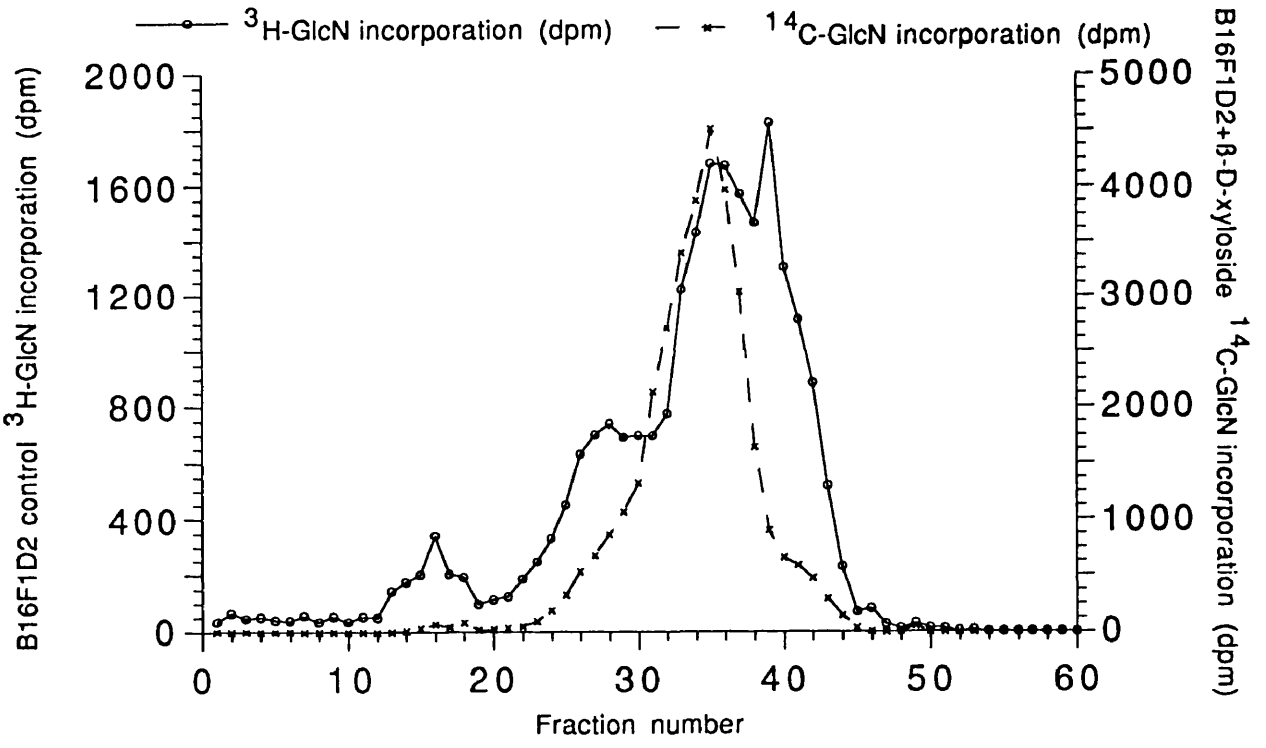


Fig.88; sepharose CL4B GPC
Cell lysate fraction PG/GAG/Pronase

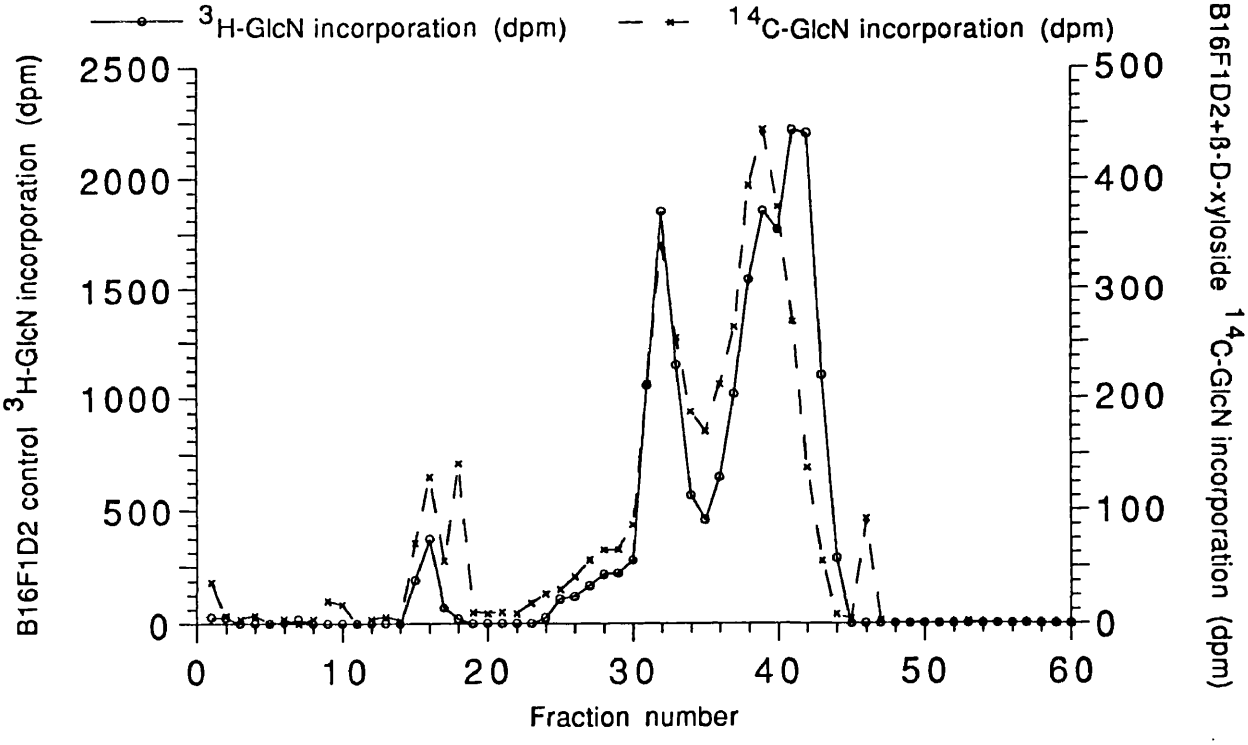


Fig.89; Sepharose CL4B GPC
Medium fraction CSPG/GAG

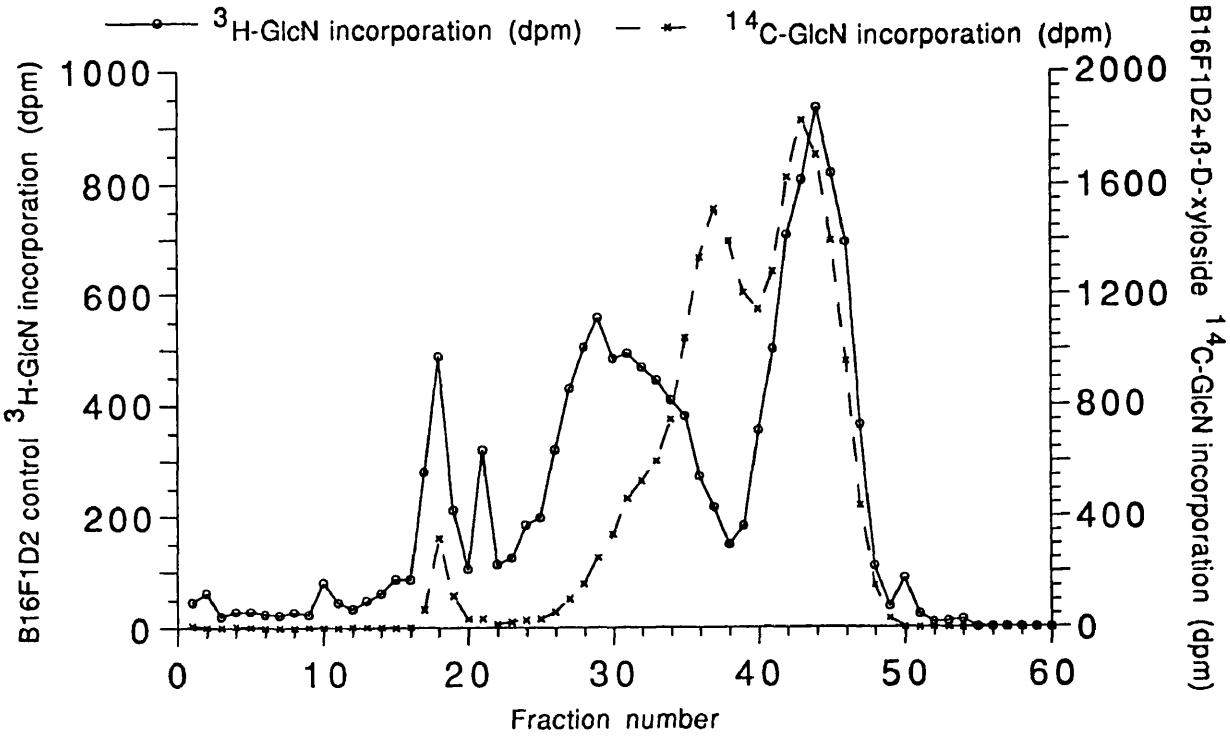


Fig.90; Sepharose CL4B GPC
Medium fraction HSPG/GAG

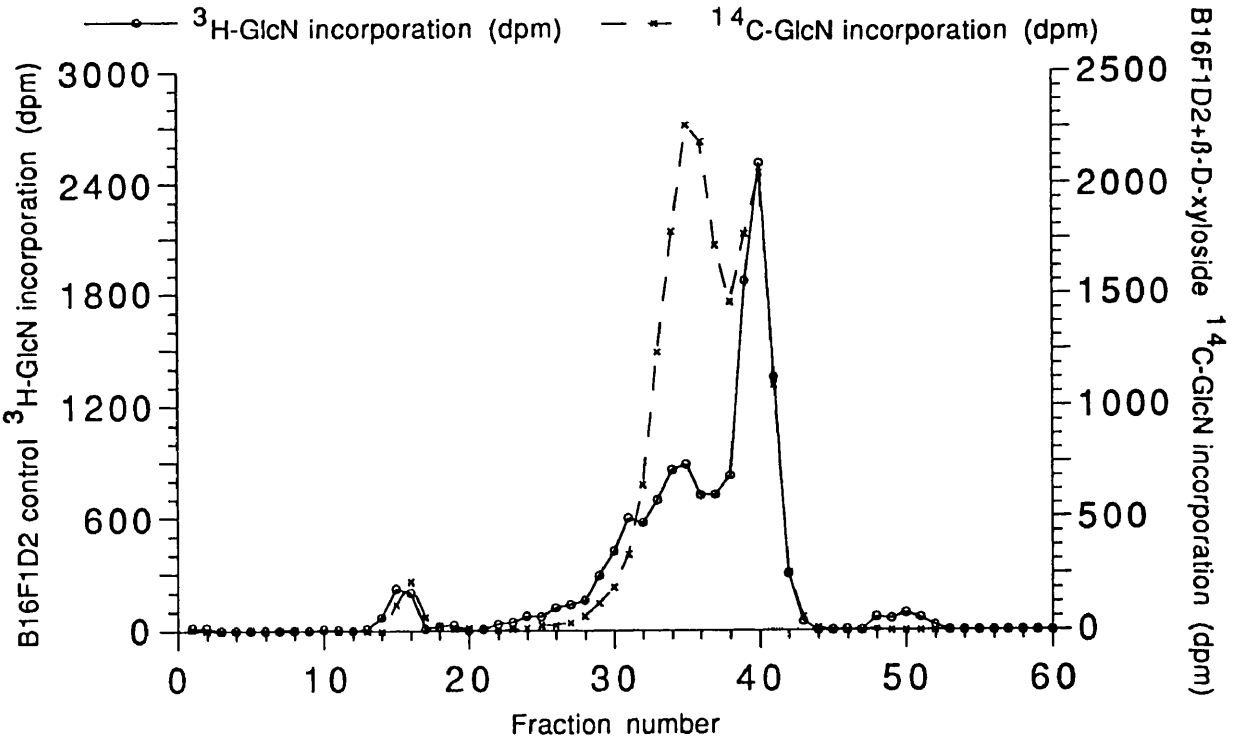


Fig.91; Sepharose CL4B GPC
Cell lysate fraction CSPG/GAG

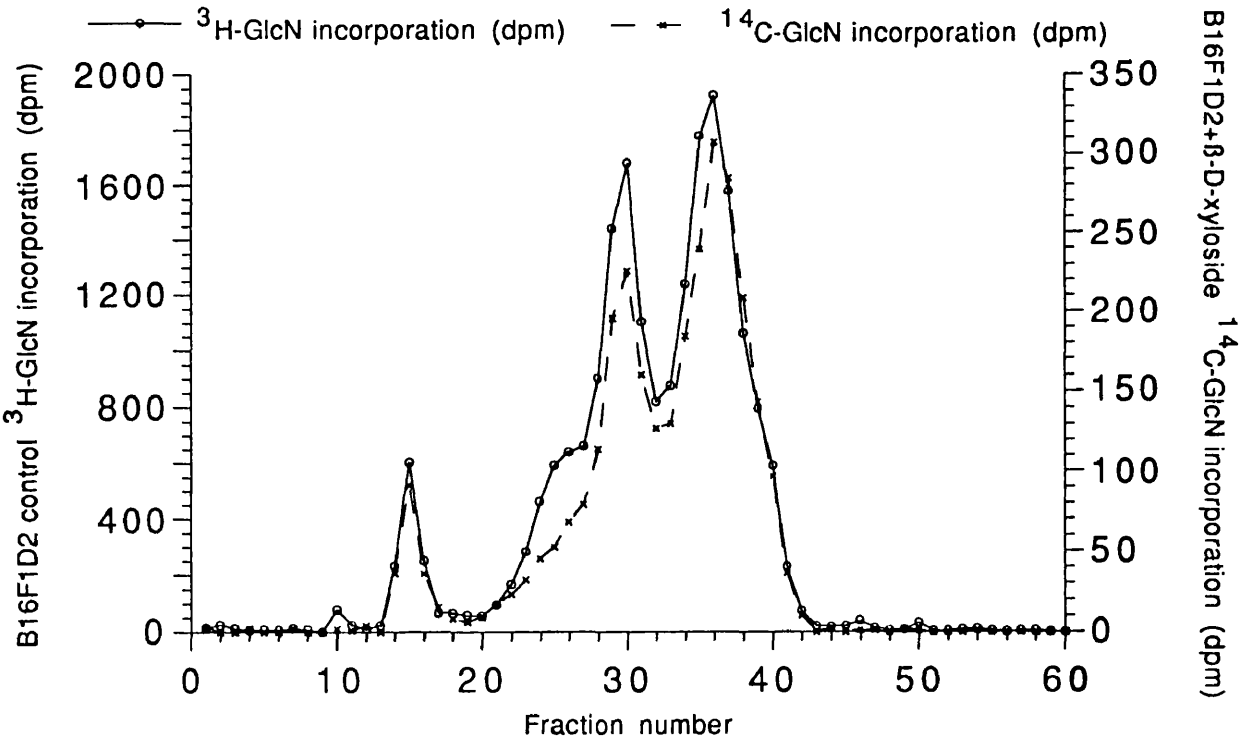
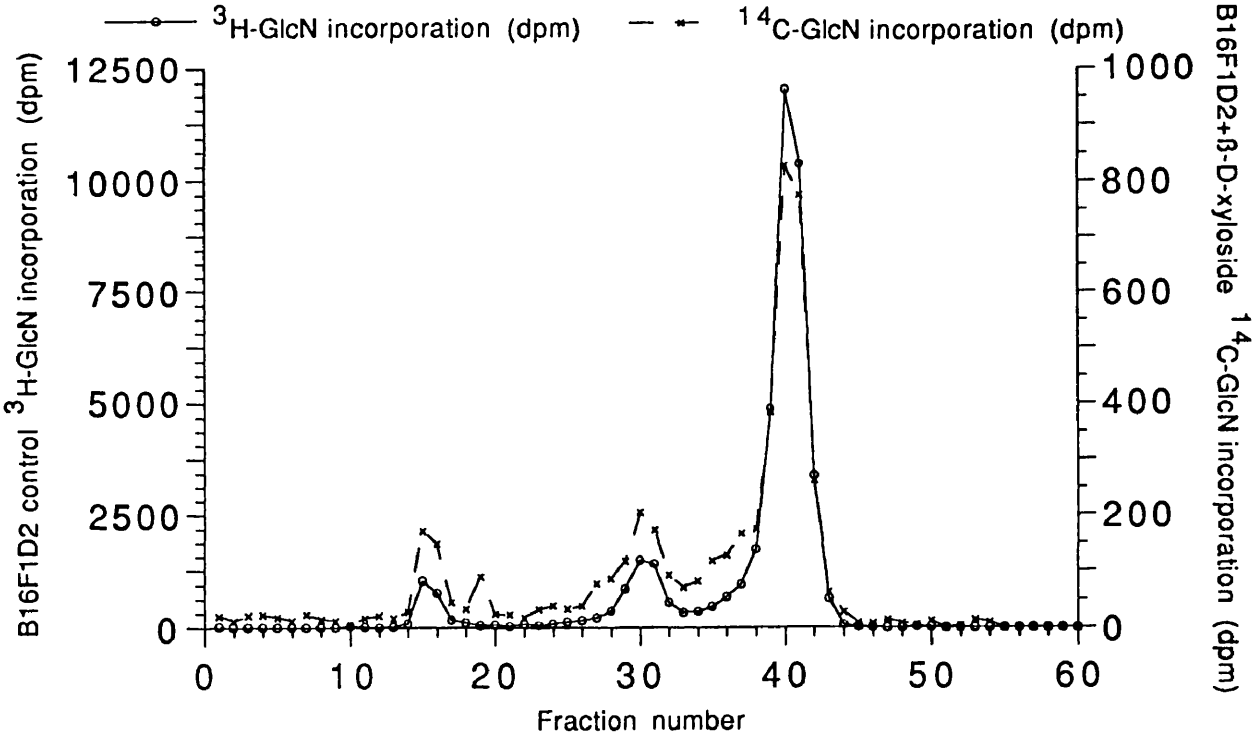


Fig.92; sepharose CL4B GPC
Cell lysate fraction HSPG/GAG



synthesized (K_{av}0.75). Interestingly, ß-D-xyloside-treated cells possessed a similar cell-lysate proteoglycan elution profile as that observed for control cells for both chondroitins and heparans (fig.91 and fig.92).

4.4.4. Quantitative differences in GAG synthesis

Quantitative changes in GAG synthesis may influence the structure and function of the pericellular matrix. Consequently quantitative differences in the GAG synthesized by the B16 melanomas were subjected to analysis. Metabolic labelling of cell medium, trypsin-releasable and cell-associated GAG with ^{3}H-GlcN was used to estimate the relative proportions of HS-GAG and CS-GAG synthesized, and the total amounts in each fraction. Metabolic labelling with ^{35}S permitted the estimation of the ^{3}H/^{35}S ratio and the degree of N-sulphation of HS-GAG. The n value used for statistical analysis refers to the number of fractions analysed.

a) B16F1 vs B16F1M3 ± 0.5%DMF

The relative proportions of CS-GAG and HS-GAG in the medium and trypsin-releasable fractions of B16F1, B16F1M3 and DMF-treated melanoma cells were measured using Sephadex G50 GPC separation of ^{3}H-GlcN-labelled GAG species. GAG fractions were degraded with chondroitinase ABC and separated by GPC into intact and degraded fractions thereby permitting calculation of the relevant proportions of radioactivity in each peak (fig.36b). Intact GAG eluted close to V_o as a distinct peak and degraded GAG close to V_t. The V_o volume contained the HS-GAG and was confirmed by nitrous acid deamination at low pH (fig.36d) resulting in a peak shift towards V_t. Because of the non-uniform distribution of N-sulphate groups along the HS-GAG chains, deamination of

HS-GAG generates a series of degradation products with a range of differing molecular weights which do not all elute near to V_t.

The relative proportions of HS-GAG and CS-GAG in the medium fractions of the melanoma cell lines, B16F1, B16F1M3, and DMF-treated B16F1M3 cells did not differ significantly (Table.2), although significant differences were detected in the trypsin-releasable GAG fractions: B16F1 and B16F1M3 cells possessed significantly different proportions of HS-GAG and CS-GAG ($0.01>P$). Incubation of B16F1M3 cells in the presence of DMF significantly altered the proportions of HS-GAG and CS-GAG to values comparable for those obtained for B16F1 cells. DMF induced a significant increase in the level of HS-GAG and a concomitant decrease in that of CS-GAG ($0.05>P$).

Table.2 % Glycosaminoglycan composition (mean±SEM; n=3)

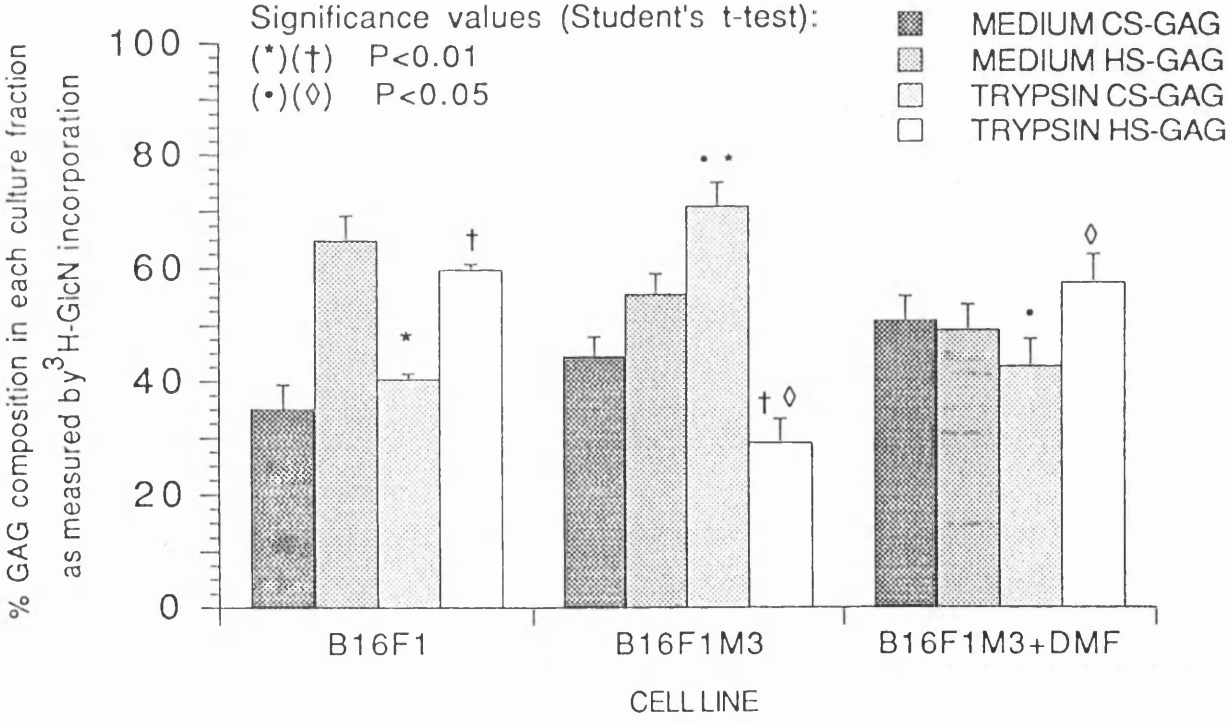
	MEDIUM		TRYPSIN	
	CS-GAG	HS-GAG	CS-GAG	HS-GAG
B16F1	35.1±4.3	64.9±4.3	40.3±1.1a	59.7±1.1b
B16F1M3	44.5±3.5	55.5±3.5	70.8±4.2ac	29.2±4.2bd
B16F1M3+DMF	50.8±4.4	49.2±4.4	42.6±4.9c	57.4±4.9d

Significance values (Student's t-test):

(a)(b): 0.01>P

(c)(d): 0.05>P

Table.2: % Composition of GAG in each culture fraction isolated (mean ± SEM; n=3)



The total quantity of radioactivity associated with the medium GAG fraction was similar for both B16F1 and B16F1M3 melanoma cells (Table.3a and Table.3b). The incubation of B16F1M3 cells in 0.5%DMF stimulated the level of ^{3}H-GlcN incorporated into medium GAG by approximately 20% to 15.95x10^{5}dpm/10^{5} cells, although this increase was not statistically significant ($P>0.05$). The level of radioactivity associated with the trypsin-releasable fraction indicated that B16F1M3 cells possessed 20% more cell surface GAG (3.72x10^{5}dpm/10^{5} cells) than B16F1 cells (3.12x10^{5}dpm/10^{5} cells), and 0.5%DMF stimulated this value by a further 60% to 5.91x10^{5}dpm/10^{5} cells. DMF-treated B16F1M3 cells possessed approximately 90% more cell-surface associated GAG than B16F1 cells. The poorly metastatic B16F1 and moderately metastatic B16F1M3 melanoma synthesized similar levels of total extracellular GAG (medium-associated and trypsin-releasable), and DMF induced a 30% stimulation of this extracellular level by B16F1M3 cells. A pattern emerged relating the level of trypsin-releasable GAG to metastatic potential. B16F1 cells possessed the lowest level of radioactivity and highly metastatic DMF-treated cells the highest level. The level of radioactivity associated with the trypsin-releasable GAG fraction of moderately metastatic B16F1M3 was intermediate (Table.3b).

Table.3a Quantity of Glycosaminoglycan as Measured by ^{3}H-GlcN Incorporation

^{3}H-GlcN incorporation (dpm x10^{-5}) per 10^{5} cells

(mean±SEM; n=3)

	MEDIUM		TRYPSIN	
	CS-GAG	HS-GAG	CS-GAG	HS-GAG
B16F1	4.85±0.91	8.65±0.11a	1.28±0.002bc	1.84±0.13de
B16F1M3	6.40±0.57	6.71±0.64a	2.65±0.30b	1.07±0.01df
B16F1M3+DMF	7.98±1.40	7.97±0.72	2.18±0.01c	3.73±0.04ef

Significance values (Student's t-test):

(a)(b): 0.05>P

(d): 0.01>P

(c)(e)(f): 0.001>P

Table.3a: Quantity of GAG present in cell culture fractions (^{3}H-GlcN incorporation)
(mean ± SEM; n=3)

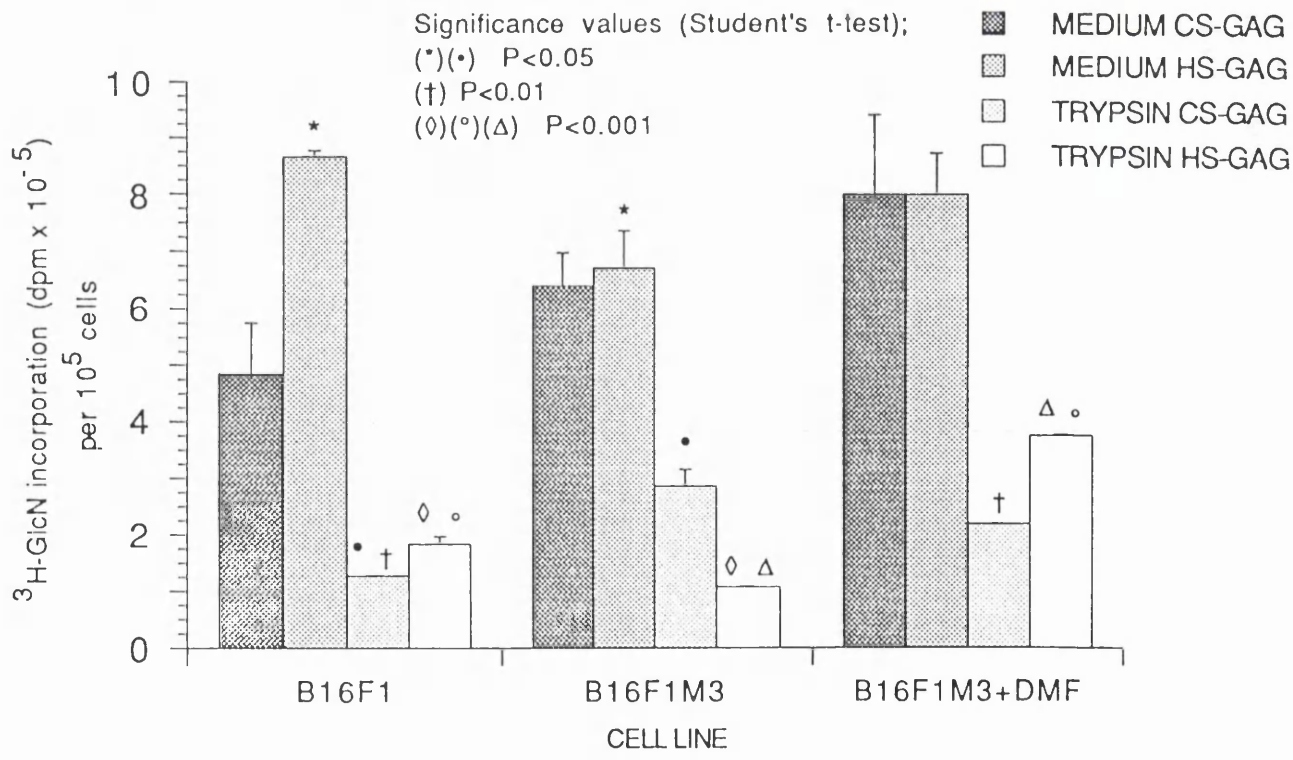


Table.3b Total Glycosaminoglycan as Measured by ^{3}H-GlcN Incorporation

^{3}H-GlcN incorporation (dpm x10^{-5}) per 10^{5} cells

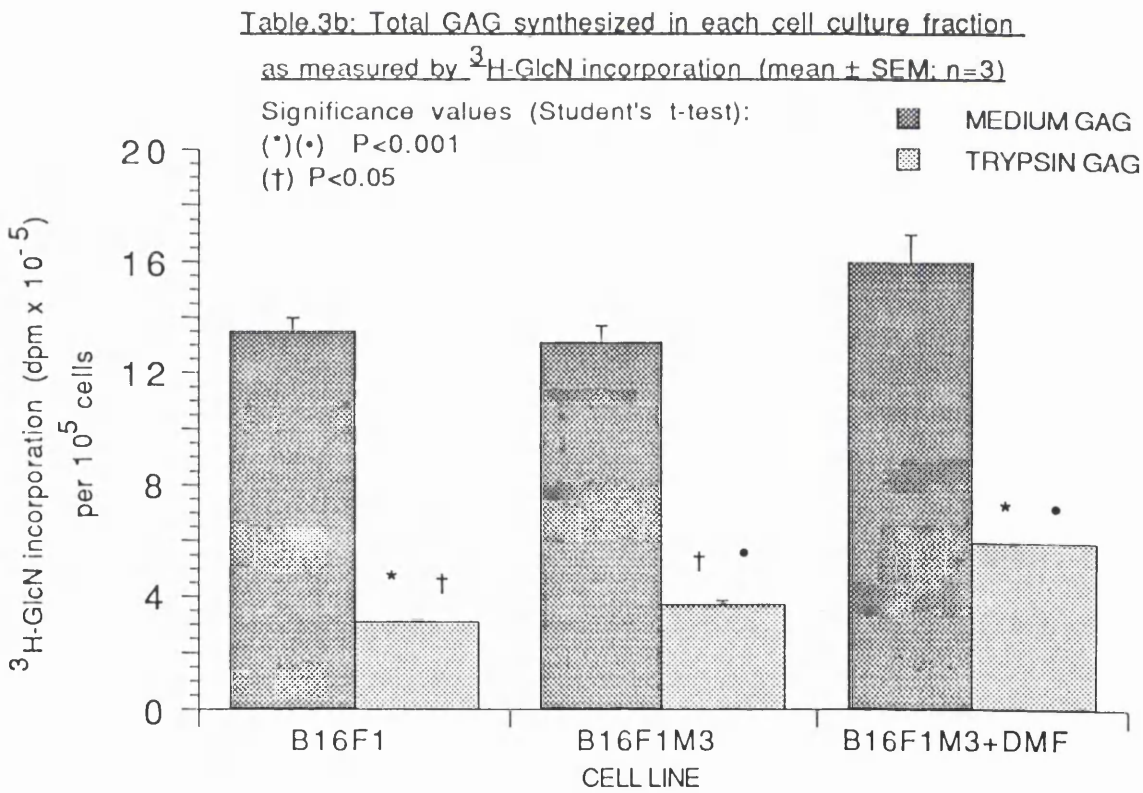
(mean±SEM; n=3)

	MEDIUM	TRYPSIN
B16F1	13.50±0.48	3.12±0.06ac
B16F1M3	13.11±0.57	3.72±0.15bc
B16F1M3+DMF	15.95±1.00	5.91±0.02ab

Significance values (Student's t-test):

(a)(b): 0.001>P

(c): 0.05>P



The level of $^{35}SO_4$ incorporated into CS- and HS-GAG (Table.3c) were too low to estimate the $^{3}H/^{35}S$ ratio or the degree of N-sulphation present in HS-GAG. The degree of ^{35}S-incorporation is therefore can best estimated from the Mono-Q-Sepharose IEC results obtained for the cell lines.

Table.3c $^{35}SO_4$ Incorporation into Glycosaminoglycan

$^{35}SO_4$-incorporation (dpm x10^{-3}) per 10^6 cells

(mean±SEM; n=3)

	MEDIUM		TRYPSIN	
	CS-GAG	HS-GAG	CS-GAG	HS-GAG
B16F1	2.26±0.12f	10.44±0.12g	0.70±0.07b	0.90±0.07eh
B16F1M3	*1.58±0.21	9.85±0.21a	0.26±0.02bc	0.58±0.02de
B16F1M3+DMF	1.64±0.15f	8.51±0.15ag	0.50±0.05c	1.32±0.05dh

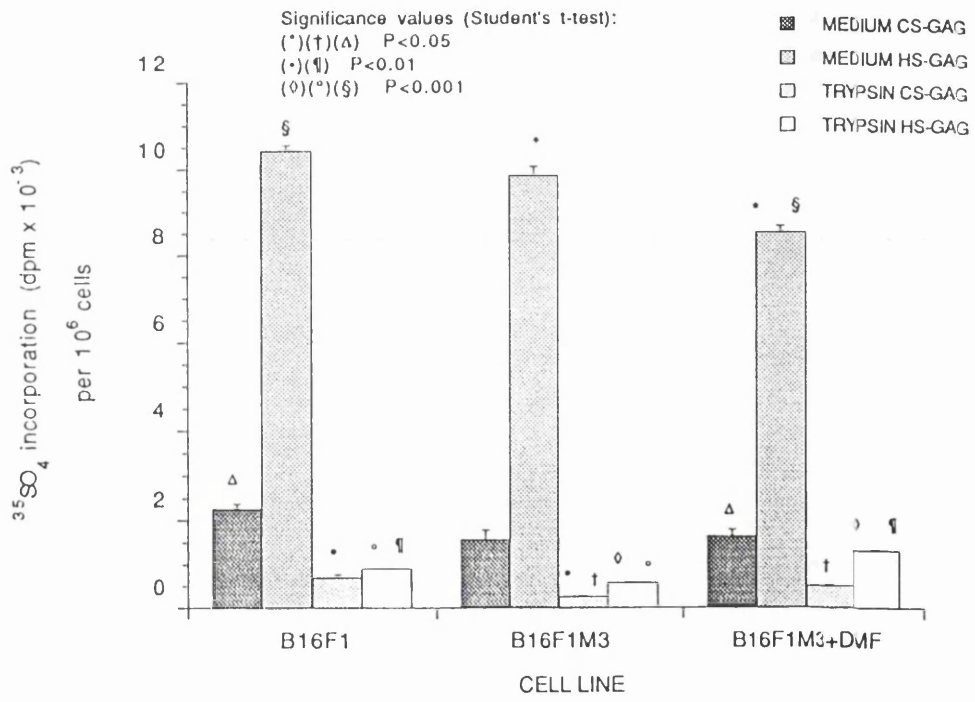
Significance values (Student's t-test):

(a)(c)(f): 0.05>P

(b)(h): 0.01>P

(d)(e)(g): 0.001>P *n=2

Table.3c: ^{35}Sulphate incorporation into GAG
(mean ± SEM; n=3 except * n=2)



b) B16F1H5 vs B16F1D2

B16F1H5 and B16F1D2 melanoma cells possessed similar HS:CS ratios for their trypsin-releasable and cell-associated fractions, but there was a significant increase in the level of HS-GAG in the medium-associated fraction (0.01>P), becoming nearly exclusive for B16F1D2 cells (Table.4). B16F1D2 cells incorporated twice as much radioactivity into GAG as B16F1H5 cells (Table.5a, Table.5b). The cell surface of B16F1D2 cells contained 36% more GAG than that of B16F1H5 cells; B16F1D2 cells also possessed 197% more cell-associated GAG and 94% more medium fraction GAG (Table.5b).

Table.4 % Glycosaminoglycan composition (mean±SEM; n=3)

	B16F1H5		B16F1D2	
	CS-GAG	HS-GAG	CS-GAG	HS-GAG
MEDIUM	42.7±0.3a	57.3±0.3b	14.8±5.1a	85.2±5.1b
TRYPSIN	51.1±8.6	48.9±8.6	53.0±2.8	47.0±2.8
CELL	48.4±5.7	51.6±5.7	51.7±1.8	48.3±1.8

Significance values (Student's t-test):

(a)(b): 0.01>P

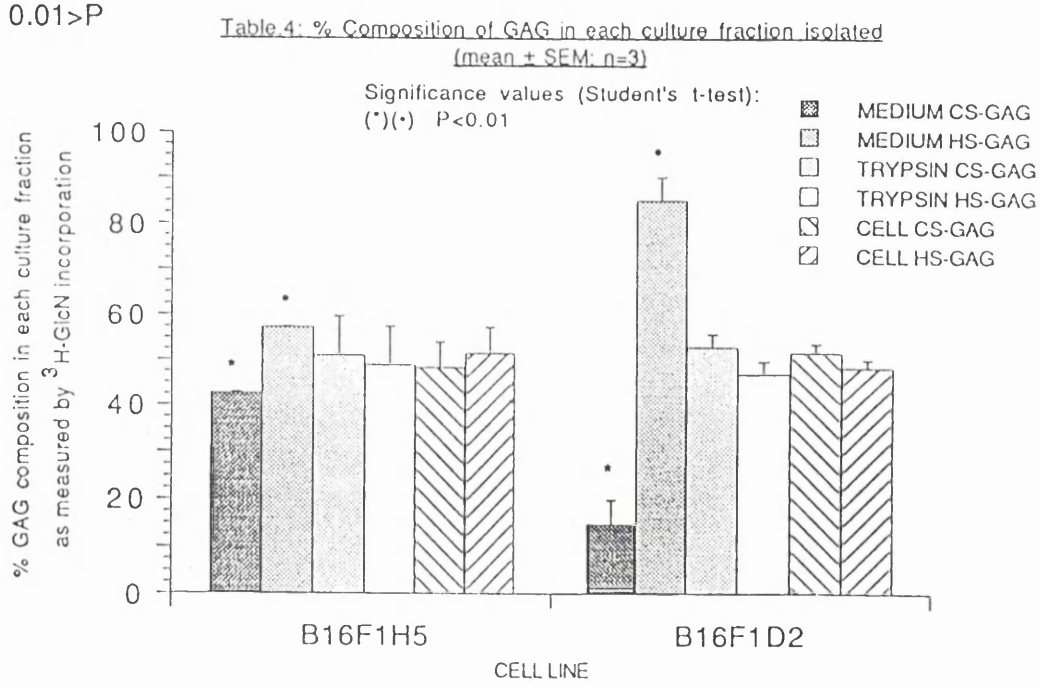


Table.5a Quantity of Glycosaminoglycan as Measured by ^{3}H-GlcN Incorporation

^{3}H-GlcN incorporation (dpm x10^{-5}) per 10^{5} cells (mean±SEM; n=3)]

	B16F1H5		B16F1D2	
	CS-GAG	HS-GAG	CS-GAG	HS-GAG
MEDIUM	2.56±0.02	3.44±0.02a	1.72±0.59	9.89±0.59a
TRYPSIN	1.02±0.17	0.97±0.17	1.44±0.08	1.27±0.08
CELL	0.93±0.11b	0.99±0.11c	2.95±0.10b	2.75±0.10c

Significance values (Student's t-test):

(a)(b)(c): 0.001>P

Table.5a: Quantity of GAG present in cell culture fractions (^{3}H-GlcN incorporation) (mean ± SEM; n=3)

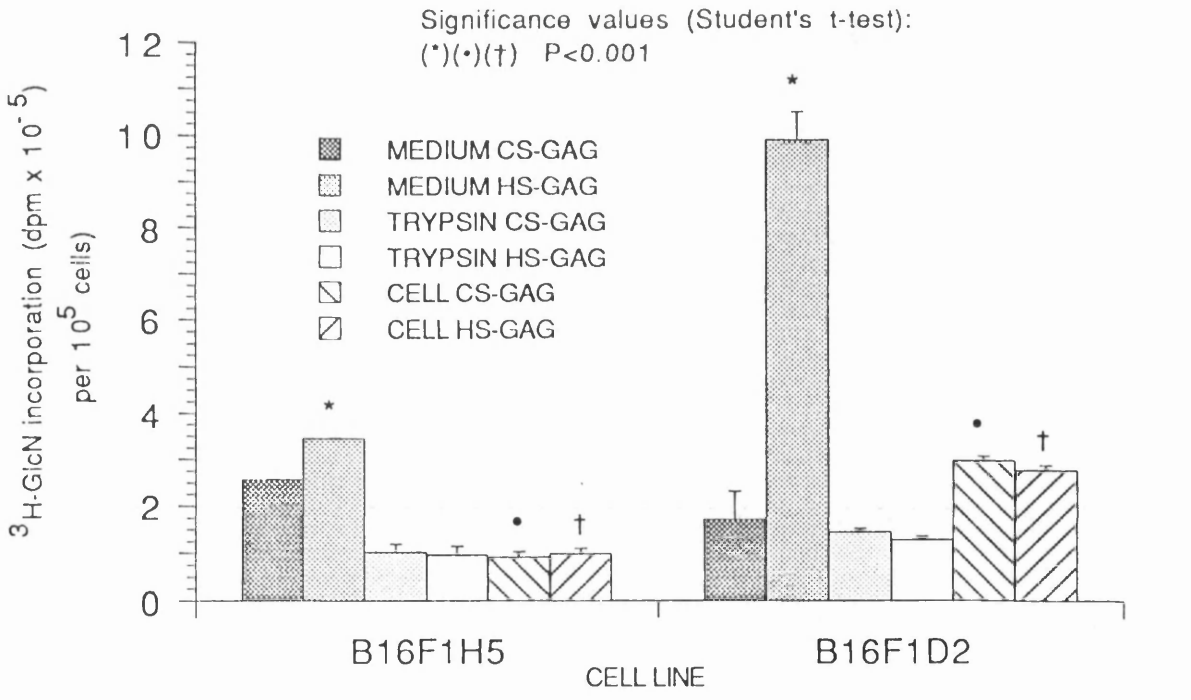


Table.5b Total Glycosaminoglycan as Measured by ^{3}H-GlcN Incorporation

^{3}H-GlcN incorporation (dpm x 10^{-5}) per 10^{5} cells

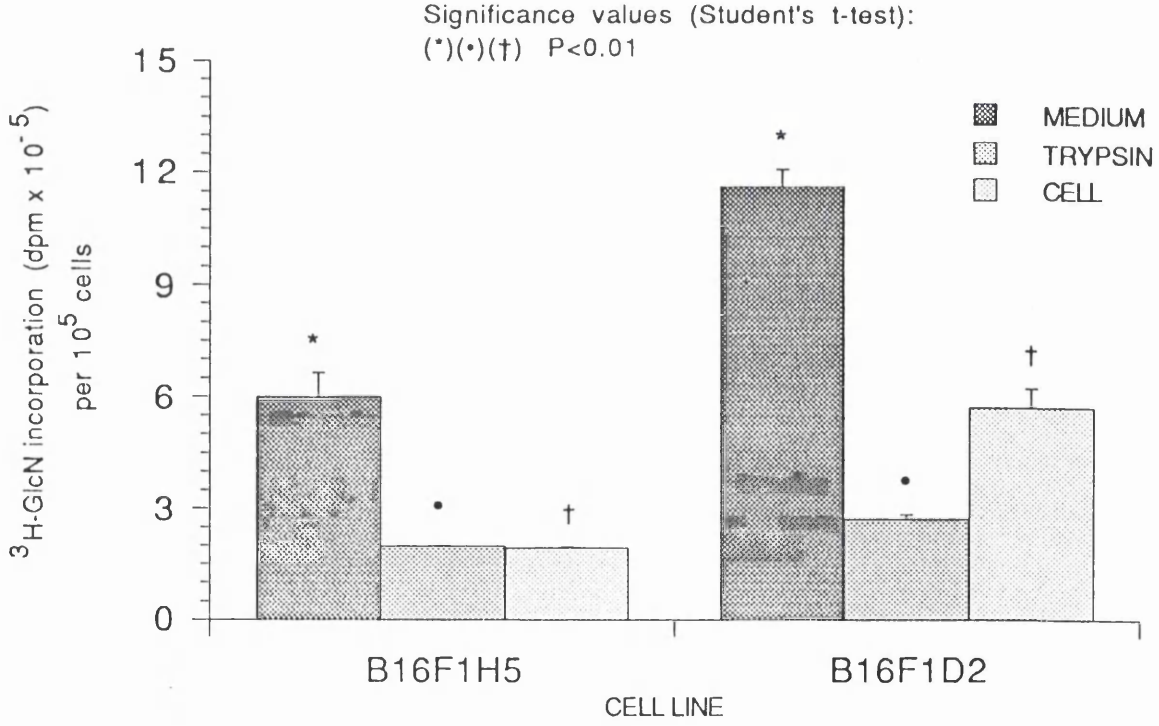
(mean±SEM; n=3)]

	B16F1H5	B16F1D2
MEDIUM	6.00±0.63a	11.61±0.46a
TRYPSIN	1.99±0.03b	2.71±0.12b
CELL	1.92±0.04c	5.70±0.51c

Significance values (Student's t-test):

(a)(b)(c): 0.01>P

Table.5b: Total GAG synthesized in each cell culture fraction as measured by ^{3}H-GlcN incorporation (mean ± SEM; n=3)



The CS-GAG present in the medium and trypsin fractions from B16F1H5 and B16F1D2 cells possessed similar sulphation characteristics (fig.5c). The levels of incorporation of ^{35}S-isotope into the medium fraction (as assessed via the $^{3}H/^{35}S$ ratio; Table.5d) indicated that both the medium fraction of both B16 melanoma clones contained CS-GAG with very similar levels of sulphation, and the degree of CS-GAG sulphation as measured by Mono-Q-Sepharose IEC (fig.63) was in close agreement with this observation even though B16F1H5 CS-GAG eluted over a slightly wider range of salt concentrations indicating a wider range of sulphation levels. There was no significant difference in the $^{3}H/^{35}S$ ratio for the trypsin fraction CS-GAG from the melanoma clones (Table.5d). This corresponded with the Mono-Q-Sepharose IEC elution profile (fig.65) where the CS-GAG from B16F1D2 and B16F1H5 cells eluted over the same range of salt concentrations.

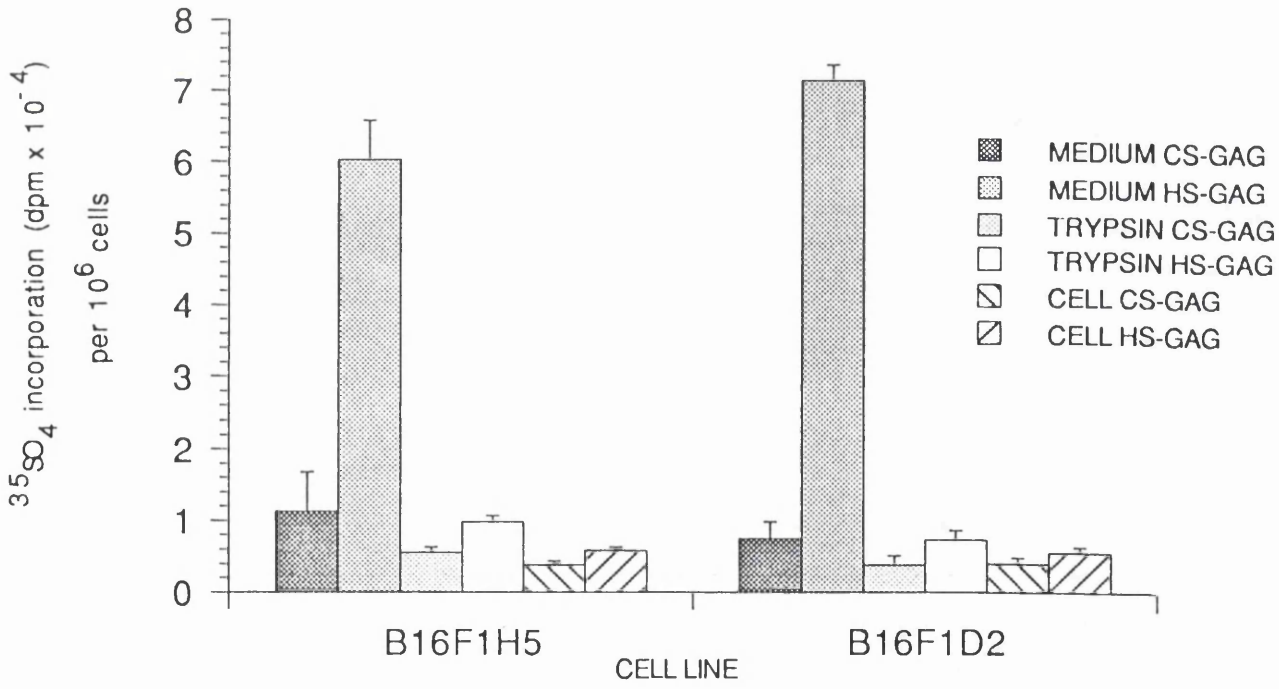
Table.5c $^{35}SO_4$ Incorporation into Glycosaminoglycan

$^{35}SO_4$ incorporation (dpm $x10^{-4}$) per 10^6 cells

(mean±SEM; n=3)

	B16F1H5		B16F1D2	
	CS-GAG	HS-GAG	CS-GAG	HS-GAG
MEDIUM	1.13±0.55	6.03±0.55	0.75±0.23	7.13±0.23
TRYPSIN	0.55±0.08	0.98±0.08	0.38±0.13	0.73±0.13
CELL	0.38±0.05	0.58±0.05	0.40±0.08	0.55±0.08

Table.5c: ^{35}Sulphate incorporation into GAG
(mean ± SEM; n=3)



The ratio of ^{3}H- to ^{35}S-incorporation ($^{3}H/^{35}S$) for the medium fraction HS-GAG also corresponded with the results obtained from Mono-Q Sepharose IEC. The HS-GAG from B16F1H5 eluted later than that of B16F1D2 cells (fig.66) and had a smaller $^{3}H/^{35}S$ ratio (Table.5d). These factors indicated that B16F1H5 cells incorporated more ^{35}S-radiolabel into their medium fraction HS-GAG than did B16F1D2 cells. However the $^{3}H/^{35}S$ ratio for the trypsin-releasable HS-GAG fraction did not correspond to the Mono-Q-Sepharose elution profile. The ratio of $^{3}H/^{35}S$ suggested that B16F1H5 synthesized HS-GAG with a significantly higher degree of ^{35}S-incorporation (0.05>P) but Mono-Q-Sepharose IEC failed to support this observation (fig.64).

Table.5d $^{3}H/^{35}S$ Ratio (mean±SEM; n=3)

	B16F1H5		B16F1D2	
	CS-GAG	HS-GAG	CS-GAG	HS-GAG
MEDIUM	4.92±0.19	2.03±0.18a	5.64±0.57	3.73±0.16a
TRYPSIN	5.95±0.51	3.23±0.25b	8.51±0.91	5.11±0.17b
CELL	8.31±1.17	4.91±0.18	11.60±3.94	9.17±2.57

Significance values (Student's t-test):
(a)(b): 0.05>P

Sephadex G50 GPC of HS-GAG deaminated with nitrous acid at low pH was used to determine the degree of N-sulphation present in these GAG chains. IEC (fig.62 & fig.66) and the $^{3}H/^{35}S$ ratio (Table.5d) suggested that the HS-GAG from the medium fraction of B1F1H5 cells was more sulphated than that of B16F1D2 cells. The ratio of N-:O-sulphate agreed with those observations (Table.5e) even though the difference was not statistically significant and may reflect the wider elution profile observed for B16F1D2 HS-GAG on Mono-Q-Sepharose.

Table.5e HS-GAG N-sulphate : O-sulphate ratio

(mean±SEM; n=3)

	B16F1D2	B16F1H5
MEDIUM	0.91±0.13	1.21±0.16
TRYPSIN	0.91±0.04	0.83±0.05

The trypsin-releasable fraction HS-GAG from B16F1D2 and B16F1H5 cells, which eluted at the same point on DEAE-Cellulose or Mono-Q-Sepharose (fig.61 & fig.64), had very similar levels of N-:O-sulphation. In most instances the levels of $^{35}SO_4$-incorporation into GAG (measured as the $^{3}H/^{35}S$ ratio) appeared to correspond with the elution profile obtained with Mono-Q-Sepharose IEC at low pH. Since discrepancies do arise, it may be more advisable to employ both methods to determine the degree of GAG sulphation.

c) B16F1D2 ± 0.5mM ß-D-xyloside

ß-D-xyloside induced significant alterations in the HS : CS ratios ($0.01>P$) of both the medium and cell-associated fractions (Table.6). ß-D-xyloside treatment had no significant effect upon the ratio of cell surface HS-GAG to CS-GAG but stimulated CS-GAG levels in the medium fraction, resulting in a near reversal of the HS : CS ratio. In addition, ß-D-xyloside stimulated the level of cell-associated HS-GAG radioactivity.

Table.6 % Glycosaminoglycan composition (mean±SEM; n=4)

	B16F1D2 Control		B16F1D2+ß-D-xyloside	
	CS-GAG	HS-GAG	CS-GAG	HS-GAG
MEDIUM	34.5±7.4a	65.5±7.4b	72.3±1.6a	27.7±1.6b
TRYPSIN	59.5±7.4	40.5±7.4	43.6±5.6	56.4±5.6
CELL	69.2±2.6c	30.8±2.6d	51.6±3.8c	48.4±3.8d

Significance values (Student's t-test):

(a)(b)(c)(d): 0.01>P

ß-D-xyloside treatment resulted in a significant decrease in the total amount of GAG present on the cell surface and a significant increase of that present in the culture medium (Table.7a and Table.7b). B16F1D2 cells treated with ß-D-xyloside showed a 267% increase in total medium GAG synthesized, and a 40% reduction in cell surface GAG, with no significant alteration in the level of cell-associated GAG.

These observations are in agreement with the proposed mechanism of action of ß-D-xylosides in that ß-D-xyloside stimulates GAG biosynthesis by inhibiting the polymerization of GAG chains onto core protein. The cell surface of ß-D-xyloside-treated cells did not appear to contain a full complement of GAG when compared with control cells, suggesting that either the normal cell surface complement of proteoglycan, or the number or size of GAG chains polymerized onto proteoglycan core proteins is reduced. It is also conceivable that the cell surface contains naked core protein bare of GAG chains.

ß-D-xyloside was observed to reduce the charge density of the cell-lysate CS-GAG (fig.71) and medium HS-GAG (fig.72) of B16F1D2 cells, and have negligible effects on the charge density of the medium fraction CS-GAG

(fig.73) and cell-lysate HS-GAG (fig.74). These observations conflict with the $^{3}H/^{35}S$ ratios which suggested that ß-D-xyloside treated cells incorporate more ^{35}S-isotope into their GAG than control cells (Table.7d). It is possible that changes in GAG charge density may reflect alterations in the size of the GAG chains induced by ß-D-xyloside and which were not detectable on Sepharose CL4B. Therefore the level of sulphation may not be the only factor involved in the charge density properties of the GAG induced by ß-D-xyloside. Other investigators report that ß-D-xyloside stimulates ^{35}S-incorporation into GAG, including increasing the degree of GAG sulphation (Murray *et al.*, 1983). This therefore requires confirmation using Mono-Q-Sepharose IEC.

Table.6: % Composition of GAG in each culture fraction isolated (mean ± SEM; n=4)

Significance values (Student's t-test):
(*)(•)(†)(◊) P<0.01

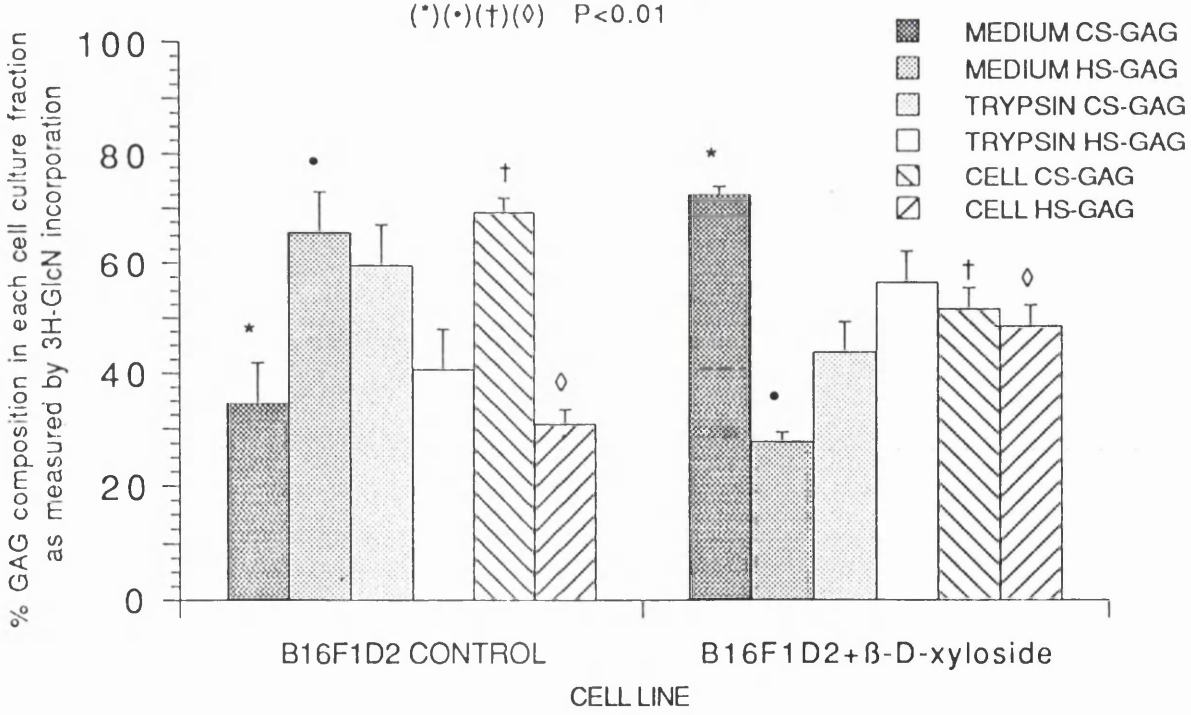


Table.7a Quantity of Glycosaminoglycan as Measured by ^{3}H-GlcN Incorporation

^{3}H-GlcN incorporation (dpm x 10^{-5}) per 10^{5}cells

(mean±SEM; n=4)

	B16F1D2 control		B16F1D2+ß-D-xyloside	
	CS-GAG	HS-GAG	CS-GAG	HS-GAG
MEDIUM	2.79±0.60a	5.30±0.60b	21.48±0.48a	8.23±0.48b
TRYPSIN	1.40±0.17c	0.95±0.17	0.61±0.08c	0.79±0.08
CELL	1.23±0.05d	0.55±0.05e	0.98±0.07d	0.92±0.07e

Significance values (Student's t-test):

(a): 0.001>P

(b)(c)(d)(e): 0.05>P

Table.7a: Quantity of GAG present in cell culture fraction (^{3}H-GlcN incorporation) (mean ± SEM; n=4)

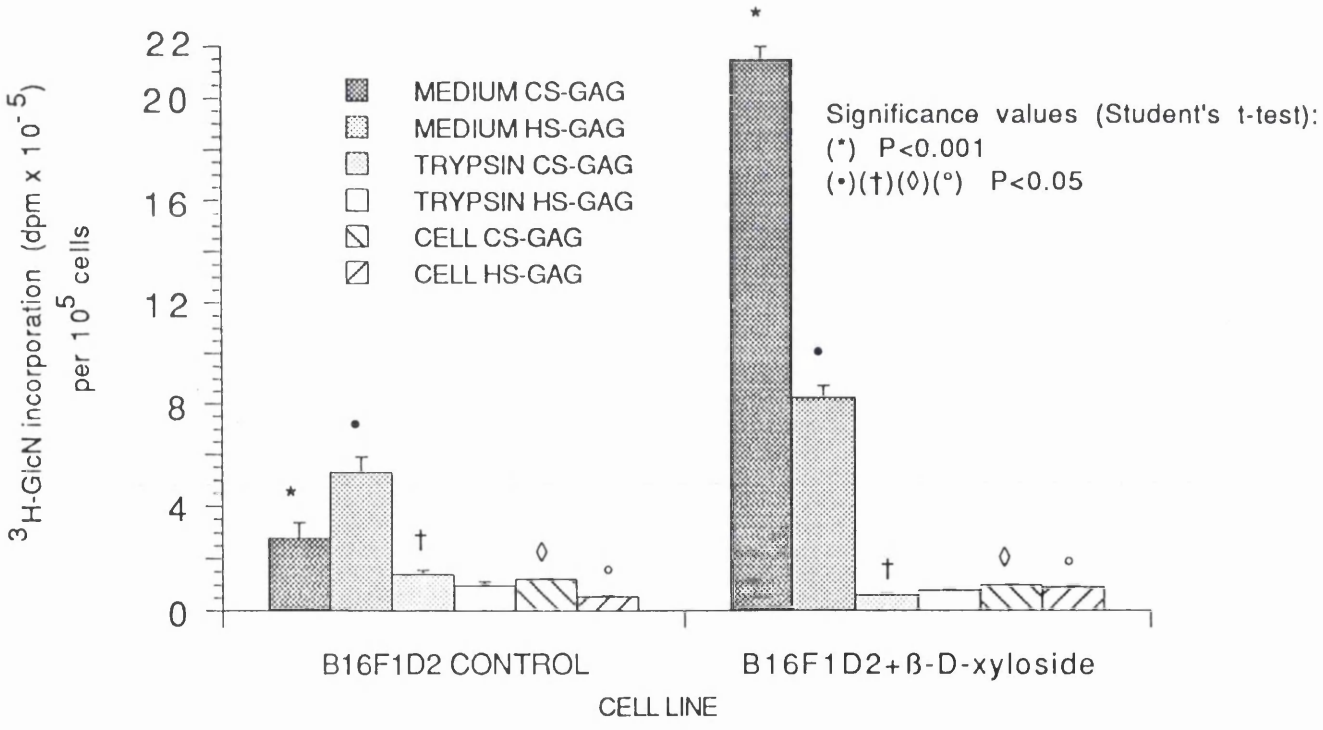


Table.7b Total Glycosaminoglycan as Measured by ^{3}H-GlcN Incorporation

^{3}H-GlcN incorporation (dpm x 10^{-5}) per 10^{5} cells

(mean±SEM; n=4)

	B16F1D2 control	B16F1D2+ß-D-xyloside
MEDIUM	8.09±0.83a	29.71±5.07a
TRYPSIN	2.35±0.04b	1.40±0.003b
CELL	1.78±0.12	1.90±0.52

Significance values (Student's t-test):

(a): 0.01>P

(b): 0.001>P

Table.7b: Total GAG synthesized in each cell culture fraction as measured by ^{3}H-GlcN incorporation (mean ± SEM; n=4)

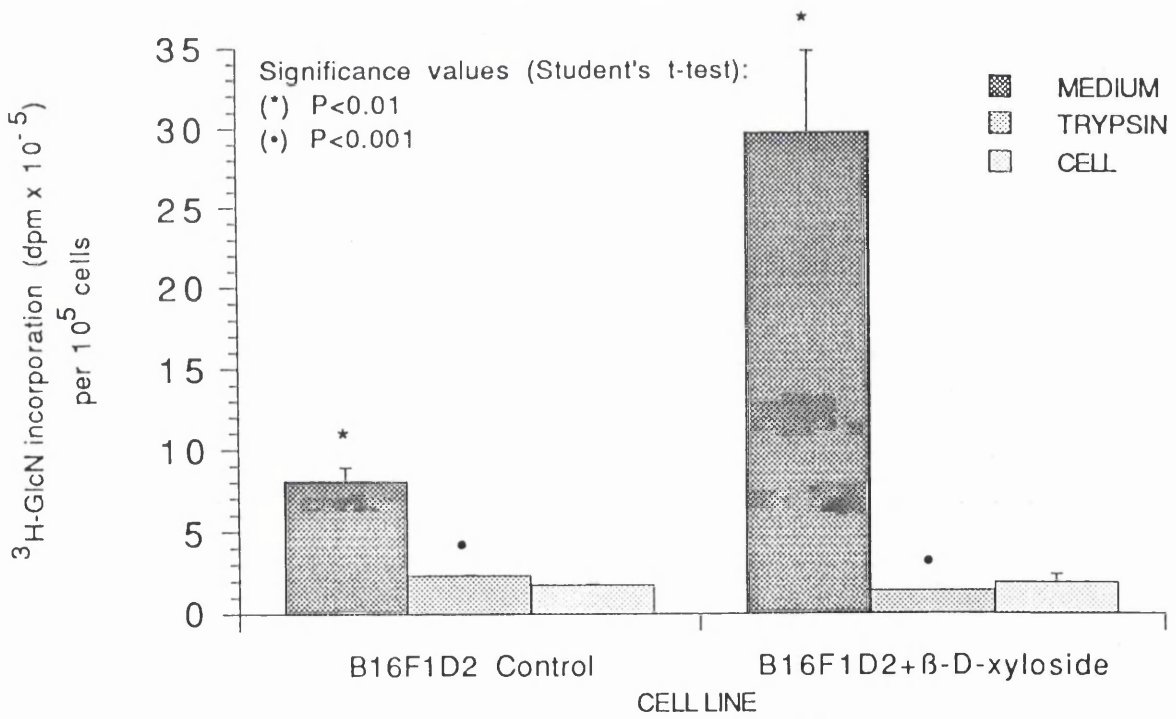


Table.7c $^{35}SO_4$ Incorporation into Glycosaminoglycan

$^{35}SO_4$ incorporation (dpm x 10^{-4}) per 10^6 cells

(mean±SEM; n=2)

	B16F1D2 control		B16F1D2+ß-D-xyloside	
	CS-GAG	HS-GAG	CS-GAG	HS-GAG
MEDIUM	2.84±0.50a	6.21±0.50b	34.07±2.97a	24.48±2.97b
TRYPSIN	0.86±0.09	0.90±0.09c	0.54±0.09	1.49±0.09c
CELL	0.99±0.03d	0.36±0.03e	0.63±0.01d	0.90±0.01e

Significance values (Student's t-test):

(a)(d)(e): 0.01>P

(b)(c): 0.05>P

Table.7c: ^{35}Sulphate incorporation into GAG
(mean ± SEM; n=2)

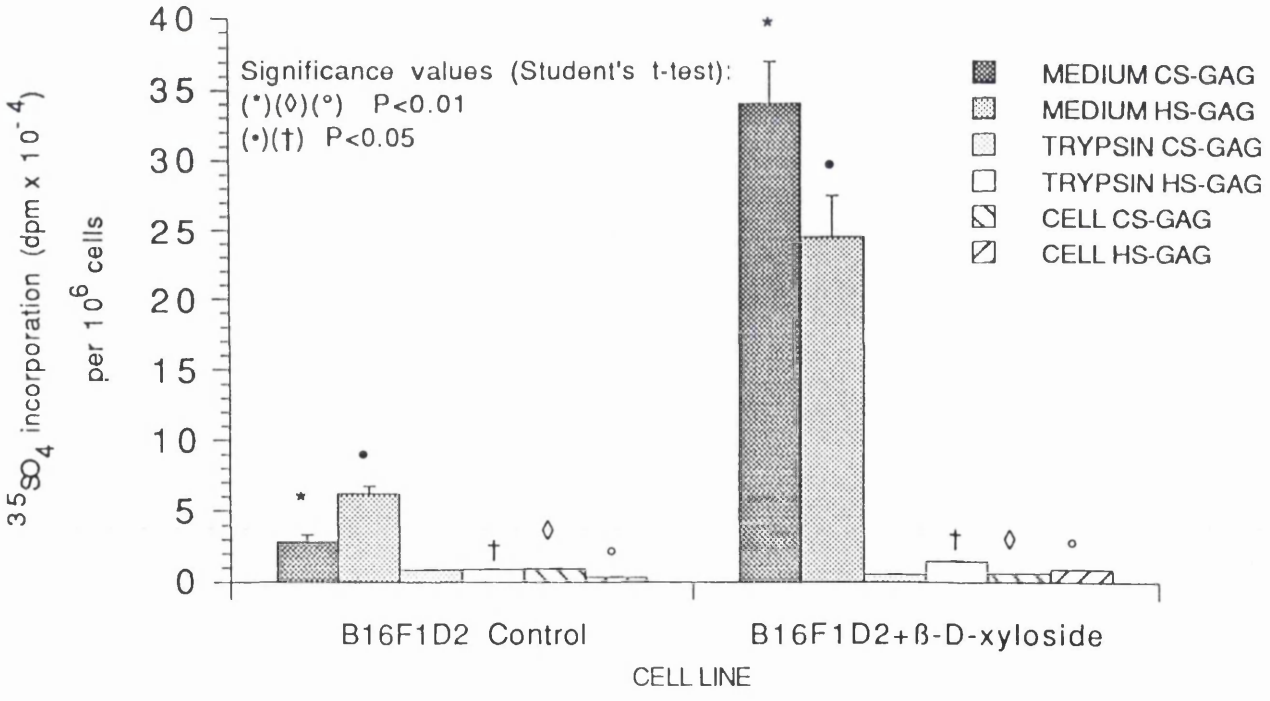


Table.7d $^{3}H/^{35}S$ Ratio (mean±SEM; n=2)

	B16F1D2 Control		B16F1D2+ß-D-xyloside	
	CS-GAG	HS-GAG	CS-GAG	HS-GAG
MEDIUM	6.16±0.59a	3.65±0.56b	3.38±0.26a	1.81±0.26b
TRYPSIN	8.06±1.56	4.04±0.74	5.61±0.26	2.65±0.25
CELL	6.81±0.42	6.53±1.18	7.93±0.22	4.78±1.15

Significance values (Student's t-test):
(a)(b): 0.05>P

The N-:O-sulphate ratios (Table.7e) indicate a lower level of N-sulphation of HS-GAG in the medium fraction from B16F1D2 control cells compared with ß-D-xyloside treated cells. This would contribute to the reduced charge density observed for the HS-GAG from ß-D-xyloside-treated as observed by DEAE-Cellulose IEC (fig.72). The finding that there was no significant difference in the N-:O-sulphate ratio for the cell-lysate HS-GAG fraction was in agreement with the observed IEC observation of no charge density difference for the cell-lysate HS-GAG from these cells (fig.74).

Table.7e HS-GAG N-sulphate : O-sulphate Ratio (x±SEM; n=2)

	B16F1D2 control	B16F1D2+ß-D-xyloside
MEDIUM	1.62±0.03a	8.59±1.02a
TRYPSIN	2.70±0.37	3.21±0.82

Significance values (Student's t-test):
(a): 0.05>P

Once again, one consistently identifiable trait correlated with metastatic potential; the level of cell surface GAG measured as radioactivity. B16

melanoma metastatic capacity appears to coincide with an increase in the total level of cell surface radioactivity: moderately metastatic B16F1M3 cells possessed approximately 20% more cell surface GAG than poorly metastatic B16F1 cells; highly metastatic B16F1D2 cells approximately 30% more than poorly metastatic B16F1H5 cells; and ß-D-xyloside, which inhibited lung colonization, reduced the level of cell surface radioactivity associated with B16F1D2 cells by 40%.

SECTION 7. DISCUSSION

Page
131: 5.1.1. Discussion
132: 5.1.2. Cell adhesion
138: 5.1.3. Differentiation
143: 5.1.4. Cell growth
145: 5.1.5. Matrix dissolution
147: 5.1.6. Proteoglycans
159: 5.1.7. Further work

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5.1.1. Discussion

The B16 murine melanoma system was developed initially as a tumour model for screening therapeutic agents, and has also proved to be well suited for studies concerning the biochemistry of tumour cell arrest and organ colonization (Fidler, 1973a, 1973; 1978). It is estimated that between 10^5-10^7 cells are shed daily from a given primary tumour (Butler & Gullino, 1975; Liotta *et al.*, 1976). Consequently, the intravenous bolus administration of tumour cell inoculi within this range is quantitatively comparable to the clinical situation, even though it fails to mimic the true clinical situation of a slower rate of cell release, often as cell aggregates. The system, although not truly representative of tumour cell metastasis since it bypasses the initial phases of the metastatic cascade, permits the rapid screening of putatative anti-metastatic therapies.

Metastatic potential can be modulated through the transference of plasma membrane from highly to poorly metastatic cells (Poste & Fidler, 1980). Therefore constituents of the tumour cell surface are responsible for the direct mediation of the metastatic phenotype. Proteoglycans and GAG play a variety of roles in a number of biological processes and phenomena; cell-cell and cell-matrix interactions (Izzard *et al.*, 1986; Rapraeger *et al.*, 1986, 1987; Ruoslahti, 1988a), matrix stabilization (Robinson *et al.*, 1984; Brauer *et al.*, 1988; Keller *et al.*, 1988), growth (Castellot *et al.*, 1982; Fedarko & Conrad, 1986; Benitz *et al.*, 1990), migration, (Funderburg & Markwald, 1986; Robertson *et al.*, 1989), morphology and differentiation (Thompson & Spooner, 1983; Carey *et al.*, 1987; Katoh-Semba *et al.*, 1989; Luikart *et al.*, 1990), and cell invasion and metastasis (Gunthert *et al.*, 1991; Faassen *et al.*, 1992). Since the functions of these molecules are becoming more widely understood, their role in, and the nature and relevence of their chemical properties in B16 melanoma metastasis was

evaluated.

5.1.2. Cell adhesion

Many biological processes are initiated by and are dependent upon cell adhesion, and evidence relates changes in cell adhesion with alterations in metastatic potential. Increases in cell-matrix interactions have been associated with increased metastatic potential (Terranova *et al.*, 1984; Chung *et al.*, 1988; Humphries *et al.*, 1989; Hunt, 1989; Hutchinson *et al.*, 1989; Tullberg *et al.*, 1989; Fridman *et al.*, 1990), but not all changes are concomitant with positive changes in metastatic potential (Nabi & Raz, 1988; Zoller & Matzku, 1989).

The cell adhesive characteristics of the B16 melanoma cell lines used in this study to defined ECM proteins had no apparent correlation with metastatic potential, except possibly collagen IV. Several investigators have demonstrated a correlation between cell attachment to collagen IV and metastatic potential (Terranova *et al.*, 1982, 1984; Liotta, 1986; Hunt & Sherbet, 1989), an interaction which can be mediated via laminin (Fligiel *et al.*, 1988). Some metastatic cell lines exhibited an increase in their rate of attachment to collagen IV when compared with poorly metastatic cells. This relationship was not universal for all cell lines tested; poorly metastatic B16F1H5 cells attached more readily, and with higher numbers, to collagen IV than highly metastatic B16F1D2 cells. The nature of these cells may reflect their originally clonal nature, and they may therefore constitute minor unrepresentative populations of the original B16F1M3 cell line from which they were derived.

However the observation questions the relationship between increased levels of matrix protein-specific cell attachment and metastatic

potential. Since increased rates and levels of attachment to collagen IV were only detected in metastatic B16 cell lines comprised of heterogeneous populations, and since it is possible to isolate and identify metastatic cell populations via their rapid attachment to ECM proteins, it would have to be determined whether the B16 cells that rapidly attached to collagen IV actually represented competent metastatic subpopulations. In addition, the adhesion assay reflects only those cells capable of adhering during the time frame of the experiment. In vivo, the attachment and extravasation of metastasizing cells is a rapid process, and multiple mechanisms may be employed simultaneously to enact these processes.

Intravenous inoculation can generate metastatic lesions of a monoclonal origin (Poste *et al.*, 1982b; Fidler & Talmadge, 1986). This observation and the fact that individual ECM proteins are encountered in vivo as complexes comprised of several different ECM proteins would lessens the importance of determining the adhesion profiles of heterogeneous cell populations to individual matrix proteins. If one assumes that metastatic tumour cells constitute minor subpopulations within a highly heterogeneous primary tumour cell population (a theory which is now under critical review) then the selection of metastatic cell populations via their ability to attach rapidly to defined ECM proteins points to an important function for these events in the metastatic phenotype. Therefore increased cell attachment to individual ECM components may contribute to a metastatic phenotype or be advantageous in certain phases of the process, but it is not appear to be indicative of metastatic potential for these tumour cell lines.

Although cell adhesion is central to the underlying processes of tumour cell metastasis, and anti-adhesive therapies are capable of interfering

with the process (McCarthy *et al.*, 1988; Saiki *et al.*, 1990a), it is difficult to fully assess the significance of changes in cell adhesion as they relate to the tumour cells ability to complete the metastatic process.

Since metastasizing tumour cells do not encounter ECM proteins as individual entities but rather as heterogeneous ECM complexes in tissues, the results of cell attachment to barriers which are encountered during metastasis are in all probability more relevant. Anti-adhesive therapies that inhibit cell attachment to ECM proteins and the invasion of BM structures (Gehlsen *et al.*, 1988) indicate that individual ECM proteins play an important role, but since cells simultaneously express multiple integrin and matrix-binding receptor species, the importance of any one specific receptor type in mediating attachment to a complex ECM is diminished. The expression and activity of multiple matrix-receptor systems may be additive in respect to their effect on cell attachment to complex ECMs, and may therefore negate any qualitative or quantitative differences detected in adhesion to individual matrix proteins

.

Metastasis requires that tumour cells lodge in an appropriate target organ, capable of supporting their growth, and penetrate the capillary endothelium and subendothelial matrix. Several studies have failed to detect quantitative differences in cell lodgement within target organs by metastatic and non- or poorly metastatic cells. Further studies revealed that the retention of cells within the organ is a critical factor, with metastatic cells being retained for longer. When the levels and rates of B16 cell attachment to CPAE endothelial monolayers and their underlying subendothelial matrix was measured, it was observed that there were no significant differences between cells of differing metastatic potential.

This may be that this is a consequence of at least two factors; the histological source of the endothelial cells (arteriole as opposed to capillary), and the animal source (bovine as opposed to murine). Many cell lines have been observed to display selective and preferential adhesion to capillary endothelial cells and matrices from their preferred target organ (Nicolson & Winkelhake, 1975; Nicolson, 1982a, 1988a; Lichtner *et al.*, 1989; Bevacqua *et al.*, 1990). In addition, the attachment of B16 cells to CPAE monolayers may actually be mediated via fibronectin (from culture serum) absorbed onto the endothelial cell surface. Furthermore, since melanoma cells originate within the basal epithelia, one may expect them to exhibit rapid and high levels of attachment to BM-like structures. The failure to detect quantitative differences in cell attachment to complex matrices supports the arguement that these cells may utilize multiple adhesion mechanisms in a simultaneous fashion.

Events subsequent to cell adhesion may be more important to, and more reflective of, metastatic potential. This would include the production or activation of matrix-degrading proteinases and the stimulation of cell migration. Other factors must also be considered such as the capacity of the host organ stroma to support further tumour cell growth. Many tumour cell lines show preferential organ colonization, and the presence of particular factors (Cauvanaugh & Nicolson, 1989) and conditions within the organ stroma are critical. In addition to cell adhesion being a major determinant of organ colonization the organ microenvironment is also intrinsically involved.

Though quantitative and qualitative differences in cell attachment to individual matrix proteins could be demonstrated, their significance in

lung colonization is unclear since they had no bearing upon the levels and rate of attachment to complex ECMs or cell monolayers. Although cells may initially attach to only one, undetermined ECM protein, other systems may come rapidly into play. An enhanced level of attachment to an individual matrix protein may result in a more rapid inducement of those cellular processes required to complete the step of extravasation.

It is interesting to observe that heterogeneity in adhesive responses to ECM substrates occurred within the cell clones B16F1H5 and B16F1D2 (figs.15, 19, 24, & 28). Within these clonal cell populations, all cells were not capable of attaching to the ECM substrates within the time period of the experiment suggesting that biological diversity in cellular responses had arisen. It is possible that different subpopulations, possibly with different metastatic potentials, had arisen during the period of cell expansion and culture.

Pretreatment of B16F1D2 melanoma cells with ß-D-xyloside had a significant suppressive effect on the level of cell attachment to collagen IV. It is possible that the other effects attributed to ß-D-xyloside (i.e. the reduction in total cell surface GAG and inhibition of lung-colonization) may be related to the inhibitory effect of ß-D-xyloside on cell adhesion to collagen IV. Furthermore, afunction of proteoglycans may therefore be to mediate cell attachment to collagen IV, a process that is apparently critical for metastasis. Alternatively, the loss of cell surface laminin may be the causative means of reduced attachment to collagen IV. Since laminin possesses a well characterized GAG-binding domain, and cell surface laminin correlates with metastatic potential, the reduction in cell surface GAG induced by ß-D-xyloside may result in a reduction in cell

surface-bound laminin.

Tumour cell aggregation is particularly relevant to metastasis. The production of tumour cell aggregates in vitro failed to demonstrate any significant difference between B16F1, B16F1M3 and DMF-treated cells although visual observations suggested that DMF stimulated cell-cell aggregation. The failure of the assay to support the visual observations may be primarily due to the assay's failure to accommodate for the cell collisions that occur between cells during the haematogeneous phase of tumour cell dissemination. Evidence for the visual observations was obtained from the cell adhesion assays to homotypic monolayers; cells treated with DMF adhered more rapidly and in higher numbers (up to 20% more) than either B16F1M3 or B16F1 cells (fig.33). This increase in the aggregative potential may contribute to the increase in lung colony formation, by both increasing the probability of cell lodgement and affording the cells physical protection from mechanical damage.

A correlation between cell spreading on culture plastic in vitro and metastatic potential was demonstrated: poorly metastatic B16F1 cells were significantly less well spread ($P<0.01$) than B16F1M3 cells which were also significantly less well spread ($P<0.01$) than their DMF-treated counterparts (fig.9). This could either reflect an increase in tumour cell size, which in turn may contribute to an increased probability of physical entrapment of more metastatic cells within the pulmonary capillaries. Secondly, the increased spread area may indicate that metastatic tumour cells are more able to reorganize their cytoskeletons, a process which may be related to alterations in cell motility and gene expression which may be beneficial to or necessary for the metastatic phenotype. The

increase in cell spread area in vitro may be related to the findings of Baniyash *et al.* (1981) who demonstrated that that an increase in the metastatic potential of B16 melanoma cells was related to a decrease in cell density (as a measure of mass and volume). The findings for the cell clones, B16F1H5 and B16F1D2, do not support this hypothesis since these cell spread area of these cell lines in vitro did not differ significantly. It is possible though that the clones are not truly representative of the metastatic and non-metastatic subpopulations within the primary tumour from which they are derived. Therefore the role of cell spreading and its relationship to cell phenotype merit further evaluation.

5.1.3. Differentiation

Tumour cell metastasis has been described as a cloning process since tumours can arise from single progenitor cells (Poste *et al.*, 1982a; Fidler & Talmadge, 1986; Hu *et al.*, 1987). Tumorigenesis and malignancy may arise through cellular dedifferentiation and the induction of a more differentiated cell phenotype may be concomitant with reduced malignant potential and improved host survival times. Haematological neoplasms are susceptible to polar solvents which induce both morphological and functional differentiation (Collins *et al.*, 1978; Breitman & He, 1990). B16F1M3 melanoma cells were cultured in the presence of DMF in an attempt to induce a more differentiated phenotype and to observe whether the polar solvent influenced their metastatic potential. In some respects differentiation was achieved. DMF induced a dose-dependent increase in intracellular melanin and tyrosinase activity, commonly used markers for the differentiation of pigmented cells. The increases recorded for the levels of these markers induced by 0.5% DMF in B16F1M3 cells closely reflected those levels present in poorly metastatic B16F1 cells.

However, morphological and functional differentiation was not achieved: DMF-treated cells became highly flattened and epithelioid and their lung colonizing potential was augmented (Table.1). The effects of DMF were also readily reversed upon removal of the agent. The results suggest that DMF is not a differentiation-inducing agent for B16 melanoma cells and that melanin and tyrosinase, in conjunction with DMF, appear to be inadequate markers of B16 melanoma cell differentiation. It is possible that DMF, while inducing characteristics associated with differentiated cells, also induces characteristics associated with dedifferentiation which might negate any positive effect that the solvent had on cell differentiation. Another closely related polar solvent, N-methylformamide (N-MF), also fails to induce the cellular differentiation of B16 melanoma cells (Spremulli & Dexter, 1984), and in humans DMF is metabolized to N-MF. It is possible therefore that the ineffectiveness of DMF is a consequence of its metabolism to N-MF.

DMF had little effect upon the interactions between cells and matrix proteins except to suppress cell attachment to laminin-nidogen. This suppressed level was comparable to that observed for B16F1 cells, and may suggest that, in this instance, lung colonization is not closely related to the ability of these B16 melanoma cells to attach to laminin.

DMF induced B16F1M3 cells to synthesize a cell surface HS-GAG:CS-GAG composition similar to that observed for B16F1 cells (Table.2). Cell proteoglycans have been demonstrated to interfere with cell adhesion to ECM components (Lewandoska *et al.*, 1987). Since DMF-treatment reduced the level of B16F1M3 cell attachment to laminin/nidogen to that observed for B16F1 cells, the increase in cell surface CS-GAG induced by DMF may

contribute to this reduction, possibly by interfering with the interaction between HS-GAG and the GAG-binding domain of laminin.

The increased lung colonization potential of DMF-treated cells was not due to the selection of highly metastatic tumour cell subpopulations from the B16 cell population. DMF stimulated the lung-colonization potential of the tumour cell population as a whole since removal of the agent resulted in the re-acquisition of the original level of lung-colonizing ability even with long-term cultures.

The mode of action of differentiation-inducing agents are multifold, causing changes in enzyme activities, chromatin structure, antigenicity, surface membrane composition, fluidity, and growth kinetics (Spremulli & Dexter, 1984). This study did not determine whether the increase in tyrosinase activity induced by DMF was quantitative or qualitative. Tyrosinase activity peaked with 0.5% DMF (fig.8a) and was similar to the level exhibited by the more melanotic B16F1 cells and their DMF-treated counterparts, suggesting that the activity was maximal. By virtue of its small molecular weight and polar nature, DMF partitions into cellular membranes and may modify tyrosinase activity either by directly influencing gene expression or membrane fluidity (Spremulli & Dexter, 1984). The increase in tyrosinase activity is reflected in the accumulation of intracellular melanin (and extracellular levels). Similar increases in the levels of melanin and tyrosinase induced by DMF and N-MF in B16 melanomas have also been detected by other investigators (Spremulli & Dexter, 1986). Since melanin levels and tyrosinase activity showed a poor correlation with the cellular differentiation of the B16 melanoma cells used in this study, their role as markers of cellular differentiation may

only apply to the differentiation of normal, non-transformed, pigmented cells.

In conclusion, although DMF induced some aspects of differentiation over a range of concentrations, their functional potential in restoring a less metastatic phenotype appears to have been negated, possibly through the expression of other uncharacterized side-effects induced by DMF. It is also possible that the observed increase in metastatic potential may only reflect the increases in cell size, homotypic cell-cell interactions and alterations in GAG biosynthesis since DMF-treated cells failed to demonstrate any significant positive differences in their ability to degrade subendothelial matrix or attach to ECM proteins. Bennett *et al.* (1986) also observed that the induction of melanoma cell differentiation with α-MSH, using melanin and tyrosinase as markers, also induced an increase in metastatic potential. The differentiative effects accorded to polar solvents may be as a result of the direct effects of the agents on DNA and the factors that control its transcription, or their effects on cell-mediated cytotoxicity (Spremulli & Dexter, 1984).

Other reports of the influence of polar solvents, including DMSO and DMF, upon lung colonization have been recorded (Takenaga, 1984; Bentel *et al.*, 1990). Such agents stimulate the lung colonizing potential of poorly metastatic LLC cells and elevate both the levels of cathepsin B and plasminogen activator and the degradation of subendothelial matrix (Takenaga, 1984). Cells pretreated with polar solvents are also more resistant to detachment from culture plastic by trypsin, and DMSO has been demonstrated to induce a more organized cytoskeleton in B16 melanoma cells (Lampugnani *et al.*, 1987). Cytoskeletal reorganization

affects various cellular processes including growth and gene expression. The presence of proteoglycans in focal contacts and their involvement in the linkage of the cytoskeleton to the ECM suggests a role for cellular proteoglycans in the development of cell morphology and phenotype. Since DMF was observed to induce a more flattened cell morphology and to stimulate GAG biosynthesis, it could be suggested that these two events are linked; increased biosynthesis of cell associated GAG may indicate an increase in proteoglycan biosynthesis. This may be concomitant with in an increase in their accumulation within focal contacts resulting in an increase in cell-matrix stabilization and altered cell morphology. This hypothesis also applies to the untreated B16F1M3 cells which also display increases in GAG biosynthesis and more flattened cell morphology when compared with B16F1 cells. The altered cell morphology, if a consequence of increased proteoglycan biosynthesis, may induce the changes in cell phenotype that differentiate between these cell lines, thereby possibly implicating a role for proteoglycans in the regulation of gene expression.

DMF may mediate its initial growth inhibitory effects either through direct genetic effects or by the synthesis of growth-inhibitory molecules. The growth of human colon carcinoma cells is reversibly supressed by N-MF through its inhibition of c-myc expression (Chatterjee *et al.*, 1989), and the induction of a non-transformed cell phenotype with reduced growth rate in AKR-MCA cells by DMF may be a consequence of its stimulation of the synthesis of a TGF-ß-like protein (Levine *et al.*, 1987).

An increase in, or acquisition of, metastatic potential may represent a cellular response to the development of stress conditions (e.g. hypoxia, nutrient deficiencies and necrosis) within cells and tumours (Alterman *et*

al., 1989; Young *et al.*, 1988; Hill, 1990; Young & Hill, 1990), and once the conditions of stress are eleviated the cells may reacquire a more normal phenotype. The DMF-induced increase in lung-colonizing potential may therefore be a consequence of, or response to, environmental or cellular stress(es). Whether DMF itself is a stress agent, or whether it induces the synthesis or accumulation of stress factors is undetermined. DMF was observed however to rapidly induce the synthesis of cytotoxic levels of extracellular melanin. The accumulation of this substance may represent a stress factor which induces an enhancement in metastatic potential. Under these conditions the metastatic phenotype may represent an "escape response" elicited in order to improve the cells probability of survival.

5.1.4. Cell Growth

Although DMF had an initial growth inhibitory effect on B16F1M3 cell growth, the effect did not endure and DMF-treated cells appeared to regain growth rates similar to their original rates of growth. The reacquisition of a normal growth rate may reflect an adaptation response by the cells to DMF by which cells become less sensitive to its effectsF. It is also possible that DMF activates, in a temporal manner, the expression or accumulation of other factor which account for the normalizing of cell growth rate. This may be effected through the induction of enzymes capable of metabolizing DMF to a less active form or the synthesis of molecules which are capable of either transporting DMF out of the cell or inactivate it through complexing it into an inert form.

The initial DMF-induced inhibition of cell growth may possibly be related to the positive effects of DMF on HS-GAG sulphation. Heparin-like HS-GAG have been identified as regulators of cell growth (Kawakami & Terayama,

1981; Castellot *et al.*, 1982; Gordon *et al.*, 1988, 1989). Therefore the anticancer and growth inhibitory effects of polar solvents on in situ tumours (Spremulli & Dexter, 1984) may be related to the induction of similar GAG alterations. Unfortunately, the influence of cell surface or medium fraction HS-GAG in the regulation of B16 melanoma cell growth was not determined. However, if B16 melanoma-derived HS-GAG is growth inhibitory for other cell populations within a tumour it may provide a selective growth advantage by permitting the expansion of those tumour cell subpopulations which are resistant and which may be metastatic.

N-MF, and therefore possibly DMF, suppresses the expression of the c-myc proto-oncogene, by reversibly inhibiting its transcription (Chatterjee *et al.*, 1989). Suppression of c-myc may be associated with the ability of N-MF to increase cell doubling times. Heparin and endothelial cell HS-GAG inhibits smooth muscle cell growth and also inhibits c-fos and c-myc expression (Pukac *et al.*, 1990), possibly through the selective inhibition of a Protein Kinase C-dependent pathway for proto-oncogene induction. Although the link between the synthesis of highly sulphated HS-GAG and B16 melanoma cell growth is at present circumstantial, it is nonetheless seductive. B16F1 cells were observed to synthesize medium HS-GAG with a higher degree of sulphation than B16F1M3 cells and subsequently possessed a slower growth rate. DMF which induced B16F1M3 cells to synthesize oversulphated medium HS-GAG and was accompanied by an initial reduction in their growth rate. Consequently, the inhibitory effect of DMF on cell growth may be twofold; DMF elicits its growth inhibition through increasing HS-GAG sulphation and inhibiting c-myc expression.

Unfortunately this potential relationship does not hold for the B16

melanoma cell clones; B16F1H5 cells synthesize, on average, a more sulphated HSPG than B16F1D2 cells, but possess a faster growth rate. However, it is also possible that B16F1H5 cells represent a subpopulation of cells resistant to any growth inhibitory properties associated with HS-GAG. Heparin was however observed to elicit no effect on the growth rate of B16F1M3 cells. This may either indicate that heparin is a selective growth inhibitor for some cell types, or that any growth inhibitory property associated with B16 melanoma HS-GAG is specific for these B16 melanoma cells. Alternatively, the growth inhibitory properties of DMF may be only related to an inhibition of proto-oncogene expression.

5.1.5. Matrix dissolution

The degradation of BM collagen IV disrupts the structural integrity of this matrix. Whereas differences in the abilities of cells to adhere to individual ECM components may not reflect their capacity to attach to complex matrices, it may be critical and reflective of their ability to degrade ECM. The interaction between tumour cells and laminin is known to induce in some cell types the synthesis of type IV collagenase (Turpeenniemi-Hujanen *et al.*, 1986; Emonard *et al.*, 1990) and to correlate with metastatic potential (Turpeenniemi-Hujanen *et al.*, 1985; Reich *et al.*, 1988b; Hoyhtya *et al.*, 1990). In this study however, the attachment of B16 melanoma cells to laminin-nidogen in vitro and their abilities to liberate ^{3}H-proline from metabolically labelled subendothelial BM was inconsistent with metastatic potential, indicating that the B16 cell lines did not differ significantly in collagenase activity. Additionally, the efficiency of tumour cell attachment to laminin did not reflect collagenolytic activity, and might therefore suggest that laminin does not induce collagenase synthesis in these B16 melanoma cell lines. To what

extent melanocytes are involved in the normal maintenance and turnover of the BM and its constituent proteins is not known. However since these results indicate an intrinsic level of collagenase activity in B16 cells this may reflect a normal function of melanoma cell precursors in the maintenance and turnover of BM collagen IV. Although the results indicate that collagen IV degaradation was insufficient in itself to reflect metastatic potential, it may be reflective of invasive potential which was not investigated in this study. Metastatic ability probably involves other co-ordinated and possibly related activities. Collagenase activity stimulates cell migration and metastatic cell populations may be more responsive to the migratory signals and factors elicited through the action of these enzymes.

BM HSPG is believed to assist in the maintenance of the structural integrity of the BM by interacting with different matrix proteins through their GAG-binding domains. Degradation of the GAG chains could result in the loss of this integrity and loosen the matrix structure, facilitating matrix invasion. Degradation of matrix HSPG may also liberate GAG-bound growth factors and enhance the activity of matrix-associated proteinases (Falcone, 1989; Watahiki *et al.*, 1989). The actions of heparanases have the potential to elicit a wide range of biological phenomena that would be advantageous for invading cells. Consequently, heparanase activity has been demonstrated to correlate positively with the metasatic potential of several cell lines (Nakajima *et al.*, 1983; Savion *et al.*, 1987; Vlodavsky *et al.*, 1988). In this study ^{35}S-releasing activity, suggestive of heparanase activity, also correlated positively with lung colonizing potential: more metastatic B16 melanoma cell lines and clones possessed higher levels of this activity than their poorly metastatic counterparts although DMF did

not influence the level of this activity in B16F1M3 cells. Polar solvents modulate protease activities in a range of different cell lines (Spremulli & Dexter, 1984; Takenaga, 1984), but the increase in lung colonization attributed to DMF in this study does not appear to be a consequence of its modulation of heparanase activity. There may be a subsequent relationship between ^{3}H-proline- and ^{35}S-releasing activities and metastatic potential. It is probable that the joint actions of collagenases and heparanases have deleterious effects upon the integrity of the BM matrix. Cells with enhanced heparanase activity may disrupt the BM more severely and more rapidly, thereby improving their prospects of escaping from the constraints imposed by the matrix.

5.1.6. Proteoglycans

Glycosaminoglycans exert strong influences on several biological processes intrinsic to the metastatic process. Matrix invasion is enhanced by both heparin and HS-GAG (Robertson *et al.*, 1989), and can be blocked specifically by pretreatment of cells with heparins, HS-GAG or highly sulphated chitins (Saiki *et al.*, 1990b); the inhibitory effects of these molecules appears to be related to their degree of polymer sulphation. Heparin is also capable of blocking the attachment and migration of B16 cells to laminin-coated substrates (Saiki *et al.*, 1990b), and heparin and chitin analogues inhibit heparanase and type IV collagenolytic activities. Cathepsin B and plasminogen activator (PA) activities can correlate with B16 melanoma lung-colonizing ability (Wang *et al.*, 1980; Sloane *et al.*, 1981, 1982) and can also be modulated by GAG. Consequently, since cell-derived GAG possesses the potential to modulate both cellular behaviour and the actions of environmental factors, the influence of local concentration, distribution, composition, and intrinsic properties of GAG

merits evaluation.

From data derived from qualitative GAG studies there appeared to be no consistent correlation relating charge density and the degree of GAG sulphation to the lung colonizing potential of B16 melanoma variants.

DMF had a positive effect upon both the charge density and the degree of sulphation of HS-GAG synthesized by B16F1M3 cells (which was countered by a concomitant negative effect upon both CS-GAG charge density and sulphation). Similarly, cells cultured in the presence of heparin synthesized oversulphated medium HS-GAG, yet heparin did not influence lung colonization; poorly metastatic B16F1H5 cells also synthesized a medium HS-GAG fraction with a higher charge density and degree of sulphation than highly metastatic B16F1D2 cells. Although increases in the degree of sulphation and therefore the charge density of HS-GAG may be interpreted as being potentially beneficial since many GAG-binding activities are charge-dependent, the level of HS-GAG sulphation, whether increased or decreased, had no direct effect on metastatic potential. The effect of DMF on both HS-GAG and CS-GAG sulphation were similar to those observed for low cell-density, low lung-colonizing potential B16 melanoma cells and for cells treated with retinoic acid-treated which inhibits lung-colonization (Edward & Mackie, 1989). Such observations suggest that even gross alterations in the properties of B16 melanoma GAG have little effect in influencing lung colonization. The mechanisms by which DMF and retinoic acid influence metastatic potential are not fully elucidated. Subsequently, their effects upon GAG biosynthesis may be a minor consequence of their potential to influence both gene expression and enzyme activity, and may represent no more than the modulation of those

enzymes which influence, or are integral components of the GAG polymer modification pathway.

It is difficult to assess the role of the medium fraction proteoglycans and GAG in tumour cell metastasis using the artificial metastasis system. In washed cell suspensions their presence would be minimal, but their levels and properties may influence the environment, growth, and phenotype of the cell populations within the primary tumour and metastases by altering the physical and chemical properties of the pericellular matrix and by modulating cell interactions with the matrix in situ.

As indicated previously, HSPG are implicated in cell adhesion and growth. Modulation of HS-GAG charge properties by chlorate or heparin had no significant effect on the lung colonizing potential or growth rates of moderately metastatic B16F1M3 cells. Heparin had a positive effect upon the sulphation pattern of medium fraction HS-GAG, whereas chlorate had the opposite effect, reducing the degree of HS-GAG sulphation in both the medium and trypsin fractions. It is possible that the failure of these agents to affect lung colonization is a reflection of the moderate lung colonizing capacity of the B16F1M3 cells. Any detectable significant effect that these agents may have had upon the metastatic potential of highly (or poorly) metastatic cell populations may be diluted out as a consequence of the heterogeneity of the cell line. Consequently, the actions of these agents on the metastatic potential of highly metastatic cells merits evaluation. Highly metastatic, less heterogeneous cell populations, such as B16F1D2, may have proved to be more informative since only one population of highly metastatic tumour cells would be evaluated, and any modification in metastatic behaviour caused by the

modulation of cell GAG would not be masked by similar alterations in non- and poorly metastatic cell populations.

ß-D-xyloside also affected the level of GAG sulphation by reducing the amount of ^{35}S-incorporation into medium and trypsin HS-GAG and CS-GAG, although this only translated into reduced sulphation in cell surface CS-GAG and medium HS-GAG as measured by Mono-Q-Sepharose. These results confirmed that the degree of GAG sulphation did not correspond with metastatic potential. ß-D-xyloside inhibits the activities of the polymer modifying enzymes, uronosyl C-5 epimerase and 4-sulphotransferase (Coster *et al.*, 1991), without greatly altering the sulphation pattern, and the activities of these enzymes are believed to be regulated by proteoglycan core protein. This finding would explain the reduction in CS-GAG sulphation observed in the culture medium. The large and near-exclusive stimulation of CS-GAG synthesis also indicate that specific signals within core proteins determine the proteoglycan species, and that the galactosyltransferases involved in CS-GAG biosynthesis are less specific than those involved in HS-GAG synthesis (Stevens & Austen, 1982).

Although the charge density properties of the GAG synthesized by the B16 melanomas used in this study did not appear to be related to the lung colonizing potential, there appeared to be an important correlation with the level of proteoglycan expression and/or its degree of glycosylation, measured either as an increase in GAG chain number or size. B16F1D2 cells synthesized cell surface GAG that was predominantly expressed as proteoglycan; B16F1D2 cells synthesized cell-lysate HSPG and CSPG and medium CSPG which eluted prior to that of B16F1H5 cells on Sepharose

CL4B indicating a larger molecular mass. The large CS-GAG species present in the culture medium after pronase digestion was also larger for B16F1D2 cells, and B16F1D2 cells synthesized significantly more medium and trypsin fraction GAG than B16F1H5 cells. Consequently, it is possible that differences in the molecular size of the proteoglycans synthesized by the cell clones can be attributed to differences in their levels of glycosylation, e.g. B16F1D2 cells may synthesize proteoglycans with an increased degree of glycosylation. Although this was not established *per se* for B16F1, B16F1M3 cells and their DMF-treated counterparts, the increase in the levels of cell surface GAG that correlate with increased lung-colonizing potential may also be related to an increase in proteoglycan glycosylation. There is some evidence to support this theory. Timar & Lapis (1990) reported that a decrease in HSPG epitopes occurred in highly metastatic carcinoma and B16F10 cells, and was accompanied by an increase in total HS-GAG in the carcinoma cells. The reduction in HSPG epitopes was attributed to either the loss of epitopes or their masking through increased glycosylation. Similarly, Yeo *et al.* (1991) reported that increased CSPG glycosylation was a feature common to tumour stroma and healing wounds, both of which share common pathological entities.

Protein glycosylation is believed to be protective in certain respects by masking specific immunologically-sensitive epitopes or by protecting core proteins from proteolysis. The role of increased proteoglycan glycosylation may be to protect functionally important cell surface proteoglycans from proteolysis or increase their likelihood of successful participation in charge-dependent interactions such as the sequestration of GAG-binding growth factors (Roberts *et al.*, 1988; Gospodarowicz *et al.*, 1987; Gordon *et al.*, 1988; Luikart *et al.*, 1990). Increased glycosylation of

cell surface proteoglycans or increases in GAG chain length would have the to potential influence the hydration properties of both the cell surface and the pericellular matrix which may contribute to successful metastasis. On an immunological basis, the masking of epitopes through glycosylation may be critical. The HSPG transferrin receptor (Fransson *et al.*, 1984), has been postulated as a target for human NK cells (Perl *et al.*, 1986) and the loss of HS-GAG chains may expose epitopes recognized by NK cells. Conversely, reduced B16 melanoma cell susceptibility to NK cells has also been attributed to a reduction in the level of cell surface HS-GAG, and may suggest that GAG chains themselves represent epitopes recognized by the immune effector system. Therefore alterations or perturbations in the enzymes that facilitate GAG polymer modifications may result in the expression of sensitive epitopes. This however remains to be resolved.

The failure in this study to associate distinct qualitative differences in GAG biosynthesis does not necessarily suggest that such changes are not relevant in tumour cell metastasis. Subtle alterations in the degree or types of O-sulphation or the degree of epimerization or N-sulphation may be significantly important. The undersulphation of HS-GAG that can accompany viral or spontaneous transformation is usually the consequence of reduced O-sulphation (Underhill & Keller, 1977; Winterbourne & Mora, 1981; Smith *et al.*, 1987). Specific oligosaccharide sequences may confer specialized functions. Kure *et al.* (1987) demonstrated that reduced cell surface HS-GAG correlated with increased B16 melanoma metastatic potential, and that cells selected for reduced cell surface HS-GAG levels possessed an increased potential. The criterion for selection was a HS-GAG-specific monoclonal antibody suggesting that the loss of a

specific HS-GAG epitope correlated with increased metastatic potential.

As expected, sodium chlorate had an inhibitory effect on the charge density of the HS-GAG synthesized by B16F1M3 cells. It is reported that sodium chlorate inhibits both O-sulphation, GlcUA C5-epimerization and the subsequent N-sulphation of deacetylated GlcN (Keller *et al.*, 1989). In agreement with other reports (Greve *et al.*, 1988; Humphries & Silbert, 1988; Walters *et al.*, 1988), sodium chlorate also reduced the charge density of the CS-GAG synthesized, supporting the view that the sulphotransferases involved in CS-GAG synthesis are more sensitive to the action of sodium chlorate. In contrast, heparin induced an increase in the degree of sulphation of the medium fraction HS-GAG. Heparin has been shown to specifically stimulate the degree of sulphation of the HS-GAG synthesized by rabbit endothelial cells with an enrichment of uronic acid sulphation (Nader *et al.*, 1989). The findings that it was possible to chemically induce gross alterations in the charge density properties of cell-derived GAG without significantly affecting colonization suggests that the extent and nature of these alterations plays a minor role in the latter stages of lung colonization. The GAG modifications associated with neoplastic transformation and/or acquisition of a metastatic phenotype may be beneficial to that phenotype, but would appear to be an effect of the altered cell phenotype rather than a cause.

Sepharose CL4B GPC indicated that both B16F1D2 cells and their ß-D-xyloside-treated counterparts synthesized proteoglycans and GAGs with similar elution profiles. Size exclusion analysis of these structures suggested that ß-D-xyloside did not appear to significantly alter the size of the proteoglycans synthesized but reduced their levels of expression on

the cell surface. Consequently, the reduction in lung colonization attributed to ß-D-xyloside-treatment suggests that the underexpression of proteoglycans is the basis of this reduction in metastatic potential. A change in GAG chain length cannot be ruled out since Yeo *et al.* (1991), reported that an increase in CS-GAG glycosylation in tumour stroma and healing wounds could be attributed to an increase in CS-GAG chain size. The effect of alterations in GAG chain length on proteoglycan functioning and properties and cell behaviour is poorly understood. Increased chain length may result in the local modification of tumour microenvironments ranging from increased ligand binding, to modulation of degradative enzyme-inhibitor balances to variance in the biophysical and biochemical properties of the tumour extracellular matrix.

Comparison of the cell surface proteoglycan profiles of the two lots of B16F1D2 cells used in the studies with ß-D-xyloside-treated cells and B16F1H5 cells revealed a reduction in the relative proportion of proteoglycan to free GAG chains. This may reflect the effect of the polar solvent, DMSO, which was used to dissolve the ß-D-xyloside, and was present in the medium of control B16F1D2 cell cultures at a concentration of 0.17%.

The Sepharose CL4B GPC results also suggest that ß-D-xyloside does not affect the level of proteoglycan glycosylation: there was no distinct peak to indicate the presence of partially-glycosylated proteoglycan forms. ß-D-xyloside only appears to suppress the numbers of fully glycosylated proteoglycans although non-glycosylated proteoglycan core proteins may have been synthesized and expressed. If naked core proteins are expressed in the plasma membrane, they may represent epitopes recognized by NK

cells, and thereby prove to be a potential mechanism for the reduced lung-colonizing potential observed for ß-D-xyloside-treated cells. The failure to detect partially-glycosylated proteoglycans might indicate that the proteoglycan biosynthetic apparatus is capable of synthesizing and expressing only fully glycosylated proteoglycan (or naked core protein). The presence of apparently mature proteoglycans in the plasma membrane of ß-D-xyloside-treated cells emphasizes the importance of these structures for the normal biological functioning of cells.

GAG-deficient BHK cells have been observed to exhibit altered adhesion responses and fail to form stress fibres and focal contacts (Couchman *et al.*, 1988; LeBaron *et al.*, 1988). HSPG are involved in cell-substrate attachment (Gill *et al.*, 1986). Proteoglycans, in particular HSPG, are implicated in tumour cell metastasis since the process can be perturbed by the heparin-binding domain of fibronectin (McCarthy *et al.*, 1988). The inhibitory effect of ß-D-xyloside on cell attachment to collagen IV suggests that cell attachment to this protein may be mediated, in part, by proteoglycans. Both B16F1M3 cells and their DMF-treated counterparts possess more cell surface GAG than B16F1 cells and show an increased rate of cell attachment to collagen IV. Cell surface proteoglycans may therefore be involved in cell attachment to collagen IV as appears to be the case for attachment to laminin and fibronectin, at least in the initial phases of the process. The level of cell surface GAG however, does not reflect the final level of cell attachment to collagen IV since B16F1, B16F1M3 and DMF-treated cells possessed similar levels of attachment after 90 mins. GAG may possess multiple roles in cell attachment. Since the GAG side-chains are highly flexible molecules and possess a large volume to size ratio, they may represent primary, non-integrin adhesive

mechanisms: GAG may represent the first molecules to interact with matrix proteins through their GAG-binding domains, and the resultant receptor-ligand interaction may prove stable enough temporalily, to permit the later interactions between integrins and matrix proteins. Further, the GAG side-chains may stabilize this receptor-ligand complex and assist in the formation of adhesion plaques and cytoskeletal reorganization and stabilization.

Although ß-D-xyloside suppresses lung colonization and the expression of cell surface proteoglycans, which may be related factors, and that cell surface proteoglycans may be a potentially important factor in this phase of the metastatic process, it is possible that the inhibitory effect on lung colonization is mediated through the effect of ß-D-xyloside on the biosynthesis of other important glycoconjugates.

It was consistently observed among all B16 cells whose proteoglycans were analyzed via Sepharose CL4B GPC that although free CS-GAG and HS-GAG chains appeared to be released into the culture medium, only CS-GAG was polymerized as proteoglycan. There also appeared in the culture medium a large chondroitin sulphate-species which eluted from the GPC column in the same region as proteoglycans but which was pronase-resistant and chondroitinase ABC sensitive, indicative of either a larger molecular weight CS-GAG or a CSPG protected from proteolysis due to its level of glycosylation. The nature and function of this chondroitin sulphate was not determined and may be important. Whether a similar species is detectable on B16F1M3 cells and B16F1 cells and whether DMF influences its physical characteristics needs to be determined. Attention has now focussed on the CD44 CSPG, the expression of which correlates

with metastatic potential (Gunthert *et al.*, 1991; Faassen *et al.*, 1992). Whether the metastatic B16 cells express this molecule and if so whether its physical properties are altered is worth evaluation. The ability of this molecule to confer metastatic potential is apparently related to the generation of different isoforms through alternative splicing. This is particularly intruiging since B16F1D2 cells synthesize a larger cell surface CSPG species than B16F1H5 cells.

Changes in GAG charge density appears to be relatively unimportant in the later stages of B16 melanoma metastasis. Increases in charge density and in particular to the degree of sulphation have been equated with improvements in binding activities for various macromolecules and growth factors. The observed differences in charge density and degree of chain sulphation, whether naturally occurring or chemically induced, are inconsistent with the observed metastatic potentials and adhesion characteristics to defined ECM components. Many binding properties of GAG can be substituted for by increases in GAG chain numbers since charge is often the major determinant of GAG binding activity. The observation that all metastatic cell lines express elevated levels of total cell GAG when compared with the poorly metastatic cell lines corresponds with this, and it may be that although increases in the charge density of specific GAG may provide a selective advantage, these may be countered by increases in the total cell surface GAG charge density by the increase in GAG chain numbers. Increases in cell surface GAG may result in an increase in the total cell surface negative charge. Whether this has important consequences on cell phenotype and properties also merits further evaluation.

An Increase in GAG biosynthesis has the potential to alter the physicochemical properties of the ECM surrounding cells. Increased GAG levels would affect the hydration properties of the pericellular matrix and ECM, and may also increase the availability of GAG-binding growth factors and strengthen, or interfere with, the cellular interactions with ECM components. The effect of B16 cell-derived GAG on other cell types has not been evaluated. Tumour cells, including B16 melanomas, can induce other cell types to remodel and alter the physicochemical properties of the ECM, either by producing factors which induce collagenolysis (Biswas & Nugent, 1987) or stimulate alterations in GAG biosynthesis (Edward *et al.*, in press). Therefore the effect of melanoma-derived ECM and proteoglycans upon the behaviour of normal cells also merits further study.

The reduction in total GAG present in the cell surface, whether as proteoglycan or free GAG chains would appear to correspond with the reduction in lung colonization ability. It may be that a reduction in GAG results in the loss of some cell surface factor, or a reduction or loss of some GAG-dependent activity important for lung colonization, or the subsequent early events that occur after lodgement.

In conclusion, the effects of the chemical properties of proteoglycans and GAG properties on lung colonization appear to be minimal. The levels of cell surface GAG and ^{35}S-releasing activity though did correlate consistently with metastatic potential, underling the importance of both cell surface and matrix proteoglycans in both maintaining tissue integrity and regulating cell behaviour. However the importance of cellular preferences for ECM proteins in cell attachment is unclear, since it

appears that different tumour cell subpopulations display marked differences in attachment to defined ECM substates irrespective of their metastatic potential.

5.1.7. Further work.

It shouldbe noted that the GAG properties and profiles observed in vitro may be only a consequence of cell culture. In situ, one might expect that the cell enviroment, a readily modulated factor, would influence the texpression and properties of cell-derived GAG and proteoglycan. Subsequently the properties of the GAG synthesized by tumour cells in situ need to be addressed. The expression of larger cell surface proteoglycans by metastatic B16F1D2 cells and the increased level of radioactivity associated with metastatic B16F1M3 cells and DMF-treated cells suggests that the proteoglycan size may be an important factor, at least with the cell lines used in this study. Therefore it may be worthwhile to further characterize the proteoglycans synthesized by both B16F1M3 and B16F1 melanomas and determine whether increased proteoglycan size is consistent with metastatic potential and whether it is a function of increased glycosylation, or increased GAG chain or core protein size. In addition, it should be determined whether these B16 melanomas express CD44, and if so, whether there are differences in its expression and properties between metastatic and non-metastatic cells. It would also be advisable to characterize several B16F1M3-derived cell clones in order to generate sufficient data to relate GAG differences to differences in cell adhesion and metastasis.

The observation that ^{35}S-releasing activity but not ^{3}H-Proline releasing activity correlated with metastatic potential requires that the effect of these activities on cell migration also be investigated. It may transpire

that the metastatic B16 populations are more responsive to matrix degradation products than their non-metastatic counterparts, and that the levels of collagenase activity recorded are sufficient to stimulate the (directed) motility of metastatic cells.

Very little could be said concerning the medium fraction GAG since their role in the latter phases of metastasis could not be evaluated. However, these molecules may be influencial in situ. It is observed that tumour cells can directly modulate the behaviour and activities, including the biosynthesis of macromolecules by neighbouring cells. The modulation of the activities of other neighbouring cells by specific subpopulations of tumour cells may confer some selective advantage(s) upon those cells in situ. Therefore the effect of tumour cell-derived GAG and proteoglycans on the activities of fibroblasts, endothelial, epithelial cell and melanoma cell cultures also merits close examination.

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