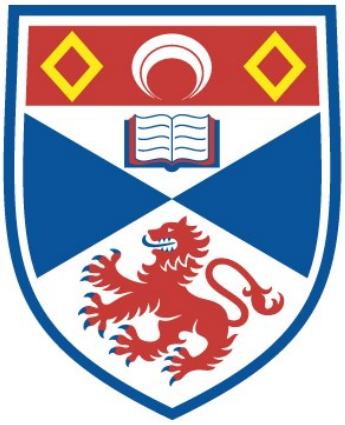
ADHESION OF B16 MALIGNANT MELANOMA CELLS TO THE ENDOTHELIUM AND TO SUBENDOTHELIA MATRIX COMPONENTS

Corrado D'Arrigo

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Adhesion of B16 Malignant Melanoma Cells to the Endothelium and to Subendothelial Matrix Components.

A thesis submitted to the University of St. Andrews
for the degree of Doctor of Philosophy by Corrado D'Arrigo

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Preface

I, Corrado D'Arrigo, hereby certify that this thesis has been composed by myself, that it is a record of my own work and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General no 12 on October 1984 and as a candidate for the degree of Ph.D. on November 1985.

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the degree of Ph.D.

(Dr. J. F. Aiton)

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Acknowledgements

Many people have made this work possible. I would like to thank Clive Evans for the excellent teaching that he has given me in these years. For all the discussions, and for all the science he has poured into me, not to mention few other things like the hall wall or the stairs carpet, thank you Clive. Next in line is David Odgen who has put up with all my impossible devices with stoic patience while trying to point me discretely towards more feasible solutions. Many thanks also to Jim Aiton who inherited the hardest job of all, the one to teach me how to actually put my work into words of a language which is not mine! Much apreciated Jim.

My thanks also to all the people that have been working in the laboratory during this time, Chris, Sue, Fiona, Richard, Allan, Katriona, Ruth, Nicki, to all members of the department and to Graham Kemp for his separation expertise. I would also like to thank Prof. J. S. Beck and Dr. Paul Elvin for their help in the final review of this script.

I would like to remember my father who would probably be very interested in this research and thank my family and my relatives for being so understanding and not locking me up when I told them I was abandoning ship for a precarious career in British academia.

Oh, yes, almost forgot, how could it slip my mind? My very special thanks to Wendy, for all her support, patience, understanding, encouragement, guidance, for all the discussions, for the good food and the chicken pies (!), for the keen interest in matters of mechanics, wiring, plumbing, plastering and stone working, for keeping me clean and well dressed and for her excellent taste in hi-fi matters! Without you I wouldn't even be here, thank you.

Abstract

During the haematogenous spread of tumours, the metastasizing cells must arrest within the blood vessels of the organs they colonize. There is still much debate upon the mechanism of such arrest, whether it is due to mechanical trapping or, more specifically, to adhesion of the tumour cells to the blood vessel wall. This work demonstrates that tumour cells are capable of adhering to blood vessel wall components.

According to the hypothesis of specific adhesion, it is thought that metastasizing tumour cells would only come into contact with the vessel wall for a very short time and therefore their adhesion to the vessel wall must be extremely rapid. It has been shown in the past that tumour cells can adhere rapidly to components of the blood vessel wall such as exposed sub-endothelial matrix. Adhesion to endothelial cells was believed to occur at a much slower rate and therefore the involvement of the endothelium during the arrest phase of the metastatic process was thought to be marginal.

The experiments carried out during this study show that, in vitro, tumour cells do adhere to the endothelium at a rate comparable to that for isolated components of the sub-endothelial matrix. Furthermore, this work provides some evidence that the molecular basis for such rapid adhesion to the endothelium may be different from the ones involved in the adhesion to known components of the sub-endothelial matrix.

Table of abbreviations used

A	Angstrom
BAE	bovine aortic endothelial cells
BAT	HLA-B associated transcript
b.s.	binding site
BSA	bovine serum albumin
BSP	bone sialoprotein
CIG	cold insoluble globulin
CLGP	collagen-like glycoprotein (collagen VI)
Co	collagen
CR3	complement receptor type-3
CR4	complement receptor type-4
$C1_q$	fragment q of complement component 1
C3	complement component 3
$C3_b$	fragment b of complement component 3
$C3_{bi}$	fragment bi of complement component 3
$C5_b$-7	complex of complement components 5_b and 7
$C5_b$-7..9	complex of complement components 5_b, 7, 8 and 9
D	Dalton
D	calcium- and magnesium-free PBS
EDTA	Ethylenediaminetetra-acetic acid
Fb	fibrinogen (fibrin)
FCS	fetal calf serum
Fn	fibronectin
FnR	fibronectin receptor
GAG	glycosaminoglycan
Gap B3	galactoprotein B3
Ge	gelatin
gpIa/IIa	complex of platelet glycoproteins Ia and IIa
gpIc/IIa	complex of platelet glycoproteins Ic and IIa
gpIc'/IIa	complex of platelet glycoproteins Ic' and IIa
gpIb	platelet glycoprotein Ib
gpIIb/IIIa	complex of platelet glycoproteins IIb/IIIa
GRGDS	glycyl-arginyl-glycyl-aspartyl-serine
HLA	human lymphocyte antigens
HRGP	hystidine-rich glycoprotein
i.d.	internal diameter
IU	international unit
Iv	invasin

kD	kilodalton
K_d	constant of dissociation
K_i	constant of inhibition
ICAM	intercellular adhesion molecule
LDV	leucyl-aspartyl-valine
LFA-1	lymphocyte function-associated antigen-1
Lm	laminin
LPAM	lymphocyte-Payer's patch adhesion molecule
LRI	leukocyte response integrin
Mac-1	macrophage antigen-1
MEM	minimum essential medium
MFP-1	microfibrillar protein-1 (collagen VI)
M_r	relative molecular mass (molecular weight)
nRNA	nuclear or transcriptional RNA
Op	osteopontin
PBS	phosphate buffered saline
PgD_2	prostaglandin D_2
PgE_2	prostaglandin E_2
PgF_{2a}	prostaglandin F_{2a}
PgI_2	prostaglandin I_2
pI	isoelectric point
PP-PCVE	Payer's patches post capillary venule endothelium
PS	position specific (antigens) of Drosophila
REDV	arginyl-glutamyl-aspartyl-valine
RGD	arginyl-glycyl-aspartic acid
RGDS	arginyl-glycyl-aspartyl-serine
S	Svedberg units
SDS-PAGE	polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate
T	calcium- and magnesium-free TBS
TBS	tris-buffered saline
TSP	thrombospondin
um	micrometre
VCAM	vascular cell adhesion molecule
VLA	very late-activation antigen
Vn	vitronectin
VnR	vitronectin receptor
vWf	von Willebrand factor
YIGSR	tyrosyl-isoleucyl-glycyl-seryl-arginine
~	circa

List of tables and figures

Introduction

Aims of the project

One of the crucial phases of the metastatic process is that of arrest of the metastasizing cells within the microvasculature of the target organ. Tumour cells, detached from the primary mass, are carried by the blood stream and are known to exit the circulation and establish foci of growth in distant organs. While in the blood stream, tumour cells of non-lymphoid or myeloid origin are thought to be most vulnerable because of the turbulence of flow in the arterial side and because of the difficulty to negotiate the narrow passages of the reticulo-endothelial system. It is postulated that tumour cells that enter the circulation either successfully arrest within the first few artero-venous passages or they die (Weiss, 1985). If this hypothesis proves to be true, the prevention of haematic spread of tumours could be achieved by interfering with the mechanisms these cells use in the arrest phase.

The project described in this thesis had two aims:

1. To demonstrate an active role of the endothelium during the arrest phase of circulating metastasizing tumour cells.
2. To extract and characterize the endothelial molecule or molecules responsible for promoting the adhesion of tumour cells.

For this purpose it was necessary to devise an in vitro assay to study the rapid adhesion of a suspension of tumour cells to either an endothelial monolayer or a molecular substratum. This assay allowed the study and comparison of the adhesion-promoting activity of various substrata.

A second assay, called spreading assay, was also devised and used to investigate the effects of various molecular substrata on the morphology of the adherent tumour cells. While the first (adhesion) assay was used to measure very rapid (1-5 minutes) events, the events measured by the second (spreading) assay were of much slower onset (30-60 minutes).

Arrest and extravasation of metastatic cells

A metastasis is a focus of tumour growth not contiguous with and not necessarily similar to the one of origin. The ability to metastasize is a characteristic of malignant neoplasms and is often what makes the therapy of these tumours unsuccessful.

Although it is thought that tumour cells may reach the distant metastatic site in a variety of ways (spread by direct contact, intra-coelomic spread, spread through the cerebrospinal fluid), the most predominant routes of dissemination is via the two established systems of transport in the body: the lymphatic and the haematic systems.

When tumour cells metastasize via the blood, as is generally the case for sarcomas, they may gain access to the blood stream in different ways. In some cases the tumour destroys the wall of a blood vessel and invades into the lumen where cells can be shed into the circulation as single cells or as clumps. The invasion of blood vessels is usually restricted to the venous side of the circulation, arterial vessels rarely being involved. Within the venous tree, vessels of all calibers may be affected, even the larger ones as is the case of the jugular vein in tumours of the pharynx and larynx (Willis, 1973). Another way in which tumour cells can enter the blood stream is via the lymphatic-venous shunts at all levels of the lymphatic tree. Cells could invade the lymphatics or could be washed into them by interstitial fluid currents through the clefts of the walls of the lymphatic capillaries and then either pass through the lymphatic tissue or be retained by it to be released at later stages (Willis, 1973; Scanlon, 1985). A more specific way to gain access to the blood circulation is by

migration through the walls of small calibre veins in the same way that lymphocytes are thought to migrate through the walls of the high endothelium venules (Harlan, 1985).

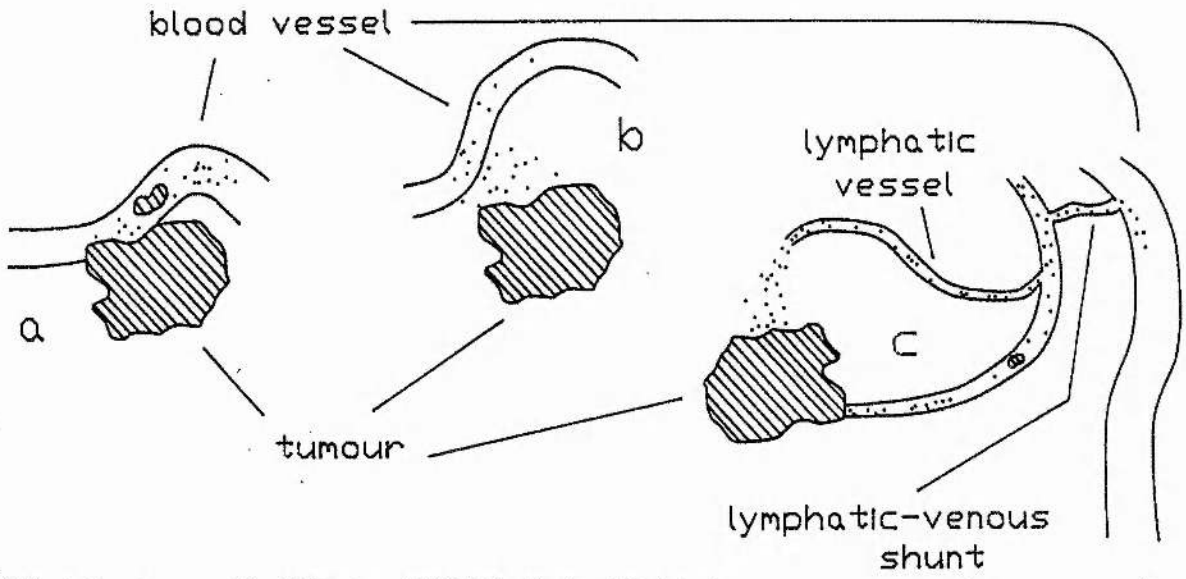


Figure 1: Blood-borne metastasis: mechanisms of access to the circulation.

a: destruction of the vessel wall and invasion into the lumen followed by shedding of single cells or tumour emboli.
b: migration of single cells through the vessel wall.
c: tumour cells may gain entry into the lymphatics either by direct invasion (bottom) or by means of interstitial fluid currents (top), and later arrive into the blood stream via a lymphatic-venous shunt.

Once tumour cells are in the blood circulation, they have to arrest at a distant site in order to form a metastatic growth. Again this event may happen in a variety of ways. Tumour cells can be physically trapped in the vascular compartment at the metastatic site. This trapping may happen both to clumps of tumour cells, usually seen caught at bifurcations on the arterial side of the circulation, or to single cells, that can be trapped while progressing through the capillary bed (Willis, 1973). Platelets have been implicated in this phenomenon and are said to form

aggregates with tumour cells that facilitate the lodging of the metastatic cells entrapped in such thrombi (Weiss, 1985).

Another way circulating tumour cells can extravasate is by adhering to the endothelium. A link has been established between the in vivo colonization pattern of some experimental tumours with their in vitro preference for adhesion to endothelium derived from the parenchyma of the organs colonized (Nicolson, 1982).

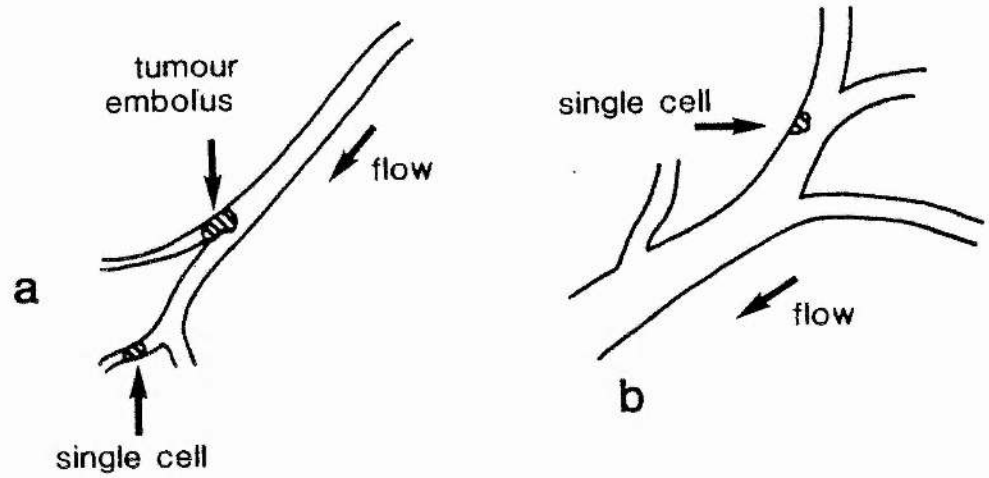


Figure 2: Blood-borne metastasis: arrest at metastatic site.

Cells can be mechanically trapped in the arterial side of the circulation (a) or they can arrest specifically in postcapillary venules (b).

Once tumour cells are arrested, they may migrate through the endothelium, or they can begin to grow within the lumen of the vessel and may eventually erode the wall and infiltrate the tissue (Nicolson, 1982). Metastatic tumour cells that extravasate in this former way can begin to grow between the endothelium and the basement membrane or directly invade the tissue and then establish a focus of growth (Nicolson, 1982). Because neoplastic growth is characterized by marked heterogeneity (Heppner, 1984), both within a neoplasm and between neoplasms, any of the different ways of gaining access to the blood stream could be used by

any type of tumour. This is also true for the different ways these cells can extravasate and thus tumours can theoretically use any of these mechanisms, or even a combination of them, to establish a metastasis.

In the case of extravasation by adhesion to the endothelium, tumour cells would need to possess a molecular mechanism to allow them to establish an adhesion to components of the endothelium. It has been argued that tumour cells do not adhere to the endothelium but to exposed sub-endothelial matrix (Kramer et al., 1980). This matrix is rich in adhesive molecules such as laminin, fibronectin, collagen type IV, vitronectin and thrombospondin (see pages 25-66) and would be an ideal target for the adhesion of circulating tumour cells.

Although neoplastic cells have been shown in vitro to be able to adhere quite effectively to the sub-endothelial matrix or to one or more of its known components (reviewed in Roos, 1984), there are some doubts as to whether the arrest of tumour cells at the metastatic site is due to their adhesion to sub-endothelial matrix exposed through wounded endothelium. Such a supposition would in fact tend to underestimate the efficiency of two physiological phenomena: the ability of the endothelium to re-establish its continuity and the thrombogenic activity of platelets. The process of healing of a wound in an endothelial monolayer is very rapid and efficient, since the endothelium has the ability to stretch and fill the gaps left by dead endothelial cells (see pages 18-19). Circulating platelets are activated by exposed sub-endothelial matrix to which they rapidly adhere and spread to form the basis for further platelet-platelet aggregation and the development of a thrombus, if the severity of the lesion requires it (Weiss et al., 1986).

The ability of the endothelium to repair itself, combined, in the case of non-haematological tumours, with the overwhelming number of platelets in the blood when compared with that of circulating tumour cells, make it

unlikely, although not impossible, for the tumour cells ever to come into contact with exposed sub-endothelial matrix.

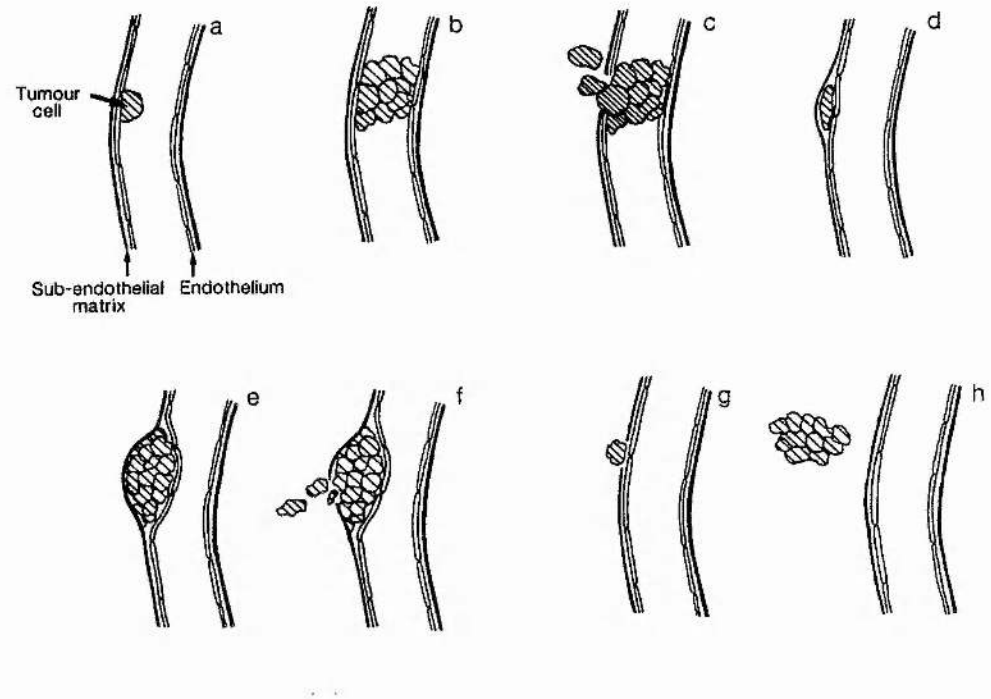


Figure 3: Blood-borne metastasis: different mechanisms that can lead to metastatic growth.

After the arrest phase (a), a tumour can grow within the lumen of the vessel (b) and later break through the vessel wall (c) or pass through the endothelium but not the subendothelial matrix (d) where it can grow (e) before invading the tissue (f). Alternatively tumour cells can extravasate and migrate into the tissue (g) before establishing a focus of growth (h).

Although there are no data available to rule out the hypothesis that tumour cells do occasionally arrest by adhering to exposed sub-endothelial matrix, in view of the above arguments it seems likely that a more common mechanism is dependent on molecular interactions between the endothelium and the tumour cell surface.

The endothelium

Until recently it was believed that the main functions of the endothelium were to provide a smooth and suitable surface to facilitate blood flow and to control transcapillary solute movements. The first observation of an active role played by the pulmonary endothelium was made two decades ago (Ryan et al., 1968) but it is only very recently that a new concept of the endothelium has emerged. It is now justifiable to look at the endothelium as an extensive and contiguous organ with very complex and important functions which are shared throughout the vascular tree, certain features being of more significance in particular organs or in vessels of particular dimensions.

These functions can be generally divided into 5 categories:

1. Maintenance of homeostasis.

The endothelium is capable of metabolizing a wide variety of molecules such as hormones, drugs, neurotransmitters, derivatives of arachidonic acid, biogenic amines and nucleotides. For instance, the endothelium clears noradrenalin and serotonin from the circulation, both effectively and selectively (adrenalin or histamine are not metabolized), prostaglandins of the series E and F are synthesized and degraded by the endothelial cell membrane, there is an endothelial cell surface carbonic anhydrase which enhances CO_2 removal from plasma bicarbonates and controls pH, and there are enzymes such as carboxypeptidase N for the metabolism of anaphylotoxin (Ryan and Ryan, 1977; Parnavelas et al., 1985; Ryan, 1986 a; Vanhoutte et al., 1986).

2. Repair and angiogenesis.

Endothelial cells are capable of rapidly and effectively restoring the continuity of a damaged endothelial monolayer through proliferation and directional migration (Ryan et al., 1982 b). They are also able to respond to appropriate stimuli by initiating endothelial sprouting and to continue the angiogenic process through the formation of anastomoses and the process of canalization to allow blood flow in the newly formed blood vessels (Folkman, 1986). The process of angiogenesis, which in the human embryo contributes to the rapid formation of the first functional system (the cardiovascular system) is not only an obvious necessity for the developing embryo but is also of prime importance for all repair processes. The ability of the endothelium to respond to angiogenic stimuli and the effectiveness of the angiogenic process is unfortunately well exploited by the growing neoplasm and can be considered an integral part of neoplastic growth (Folkman, 1986).

A number of diffusible angiogenic factors have been identified. They fall broadly into two categories: structurally related factors that bind heparin and which are found in tissues, and structurally unrelated factors that do not bind heparin and are mainly found in biological fluids. In adition there are a number of low M_r (200-15,000) factors, only partially characterized, that are mitogenic and/or chemotactic for endothelial cells. Among the heparin-binding factors there are the basic and the acidic fibroblast growth factors (basic-FGF and acidic-FGF), tumour angiogenic factor (TAF) and endothelial cell growth factor (ECGF). Among the non heparin-binding factors there are angiogenin, alpha and beta transforming growth factors (TGF-alpha and TGF-beta) and the endothelial cell stimulating angiogenic factor (ESAF) (Folkman and Klagsbrun, 1987).

All these factors can stimulate angiogenesis in vivo but have quite different effects on endothelial cells in vitro. Many are not specific for

these cells, some stimulate their proliferation or locomotion, some even cause inhibition rather than stimulation of these functions, and finally some factors do not seem to have the endothelial cells as targets. It seems therefore that angiogenesis can be evoked in a number of different ways which are likely to include both direct and indirect pathways (Folkman and Klagsbrun, 1987; Findlay, 1986).

3. Immuno-modulation.

The vast surface area of the smaller calibre vessels provides an interface between the tissues and circulating immunocompetent cells. This interface is very sensitive to local changes within the parenchyma that lies immediately behind it. Leukocytes continually scan the endothelium, principally in the small branches of the venous circulation, by adhering to and migrating across the luminal surface of the endothelium for many endothelial cell diameters before de-adhering to continue their circulation (Harlan, 1985). Subtle stimuli provided by tissue macrophages or mast cells, as well as more potent and decisive stimuli such as bacterial toxins, can cause changes in the endothelium which can be detected by the scanning leukocytes and lead to their migration through the endothelium and into the tissue (Faustmann and Dermietzel, 1985). Neutrophils are normally heavily involved in this activity but in certain immunological events lymphocytes are known to specifically adhere to and migrate through the endothelium (Harlan, 1985). Such a mechanism can be postulated to be used also by metastasizing tumour cells. Selective extravasation is a very precise and efficient process whose molecular mechanisms and, in particular, the contribution of the endothelial surface, are far from being understood. The luminal surface of the endothelial cell is capable of expressing receptors for the Fc tail of immunoglobulins G, receptors for two activated components of the complement cascade, $C3_b$

and $C1_q$, and can also be induced to express DR antigens and function as an antigen-presenting cell (McCarron et al., 1985; Ryan, 1986 a; Ryan, 1986 b). The contribution of these molecules to the selective extravasation of white blood cells has not been assessed but it is likely to be of importance.

4. Haemostasis.

The endothelium provides an active and very effective anti-thrombogenic surface, principally by means of the production of PgI_2, plasminogen activator and antithrombin III, as well as through the uptake and clearance of thrombin (Van Mourik et al., 1984; De Caterina et al., 1985; Ryan, 1986 a).

The anti-thrombogenic state can be very readily reversed and the endothelium can be transformed into a surface capable of supporting procoagulant activity. To achieve this, production of PgI_2 and antithrombin III are inhibited, thromboplastin activity appears, factor V is synthesized which, together with factor X_a activates prothrombin. Shifting from anti-thrombogenic to procoagulant activity is triggered by stimuli such as endotoxin, interleukins or decreased blood flow (Bevilacqua et al., 1985; Frangos et al., 1985; Hall et al., 1986). Such stimuli, although injurious, are far from being damaging and they certainly do not suggest the occurrence of a gross trauma to the endothelium as it would be in the case of the exposure of sub-endothelial matrix.

5. Phagocytosis.

It has been observed in vitro that the endothelial cell, traditionally considered non-phagocytic, is capable of ingesting large quantities of fixed red blood cells, heat killed Staphylococci, or inert particles (Ryan, 1986 a). In in vitro experiments many endothelial cells may ingest 10 or

more beads of 5-10 um in diameter. It has also been reported that, during wound repair experiments, phagocytosis activates endothelial cell division and migration as well as the expression of Fc receptors and DR antigens (Ryan, 1986 a). When confronted with larger particles the endothelial cells rapidly spread their cytoplasm around the foreign body (Ryan et al., 1982 a).

The phagocytic property of the endothelium has been successfully exploited as a way of obtaining arterial or venous endothelial cells explants from many organs of laboratory animals. The main advantage over previously used methods of isolation is the possibility to determine the size of the blood vessels from which the endothelial cells will be taken (Ryan et al., 1982 a): the organ of choice is canulated and perfused with a suspension of microcarrier beads and, by reversing the flow of the perfusing system, beads covered in endothelial cells are recovered.

The phagocytic ability of the endothelial cells is such that only a small proportion of beads of 5-10 um in diameter are returned from such experiments. This behaviour encourages speculation that the endothelium may serve as a very efficient filter to clear lodging emboli or thrombi: when these are small enough they are phagocytosed and the lumen of the vessel is rapidly recanalized to allow blood flow. In the case of a large particle the endothelial cells surround it and begin to permeate it with sprouts while encouraging migration of scavengers from the surrounding tissue, in a process similar to that occurring during the organization of thrombi.

Surface morphology.

Conventional thin section electron microscopy shows the endothelial cell as being extremely thin, with a rather smooth luminal surface, a prominent nucleus and scanty cytoplasm, with very few organelles and only a hint of

structural specialization. However, this picture of endothelial cells is far from accurate and some special electron microscopical techniques such as grazing sections or surface replication of freeze fractured cells have demonstrated the existence of a number of structures that once recognized can be readily identified in thin section profiles (Smith and Ryan, 1973; Ryan, 1986 b).

The endothelial cells have compressed within their flat dimensions all the major intracellular organelles: abundant elongated mitochondria and a large number of polyribosomes attached to the cisternae of a pronounced endoplasmic reticulum in close association with the numerous nuclear pores can all be seen (Haudenschild et al., 1975). Pictures showing the compact disposition of all these organelles and the way in which they are all placed so close to the luminal surface and to the nucleus, help to explain the speed and the efficiency with which these cells execute their metabolic tasks.

There are three structures present on the luminal surface of the endothelium that have significant functional importance: the endothelial cell is covered by projections of varying size, shape, distribution and density, it possesses large numbers of vesicles (caveolae) and it is covered by a supramembranous layer of amorphous material called the glycocalyx (Ryan, 1986 a).

Endothelial projections have the immediate effect of increasing the surface area of the cells and can be seen at times just above the surface of the glycocalyx. Their presence could be interpreted as being necessary to hold the glycocalyx in place and they may have a functional role when the glycocalyx is disorganized. It is nevertheless only through these projections that the plasma membrane is normally directly in contact with the cell free layer of plasma.

The glycocalyx appears in electron micrographs of surface replicas as a

deep pile carpet several hundred A thick covering the luminal surface. It may serve as a mass, shape or charge barrier that can facilitate or restrict access of solute molecules from the plasma of the boundary layer to the cell membrane. The composition of the glycocalyx can certainly be modified by the cell in response to certain stimuli (for instance, interleukins and interferon produced by activated leukocytes) or indeed it can be completely disarrayed, unmasking the underlying membrane (Ryan, 1986 a).

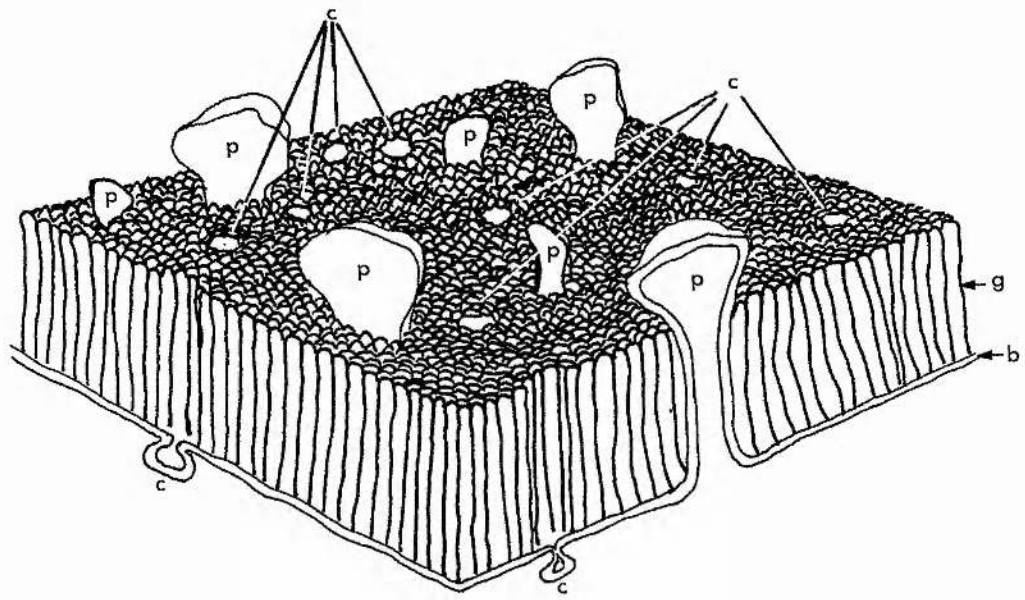


Figure 4: Morphology of the endothelial surface.

The lipid bilayer (b) is entirely covered by the glycocalyx (g); finger-like projections (p) emerge from the thick carpet formed by the glycocalyx; small indentations of the plasma membrane or caveolae (c) are also shown. This morphology can be rapidly changed if perturbing agents (such as antibodies against surface proteins) are used: the glycocalyx will be disarrayed and will show clumps and pits.

The caveolae are present at high density, usually remote from intercellular junctions. These vesicles are mostly round, with a diameter rarely bigger than 0.1 um, and they frequently possess a delicate

diaphragm at their entrance. At the caveolae the plasma membrane seems enriched in enzymes such as carboxypeptidase N, kininase II and 5' nucleotidase (Ryan, 1986 b). The presence of the caveolae and projections greatly increases the surface area of the endothelium (Smith and Ryan, 1973). The luminal surface of the endothelium is summarized in figure 4.

Sub-endothelial matrix

Within a tissue, cells are in contact with a dense latticework of collagen and elastin fibers which are embedded in a viscous medium composed of proteoglycans and glycoproteins. This structure is called the extracellular matrix. Its components are secreted and assembled by the parenchymal cells and although extracellular matrices throughout the body have the same overall structure, they differ in the relative quantity of their molecular components. This confers specificity to individual matrices and, as well as mediating cell adhesion, the extracellular matrix is thought to modulate differentiation, mitosis and migration and therefore determine the architecture of the tissue (Kleinman et al., 1984; von der Mark et al., 1984; Grinnell, 1982; McClay and Ettersohn, 1987; Ekblom et al., 1986).

Every organ is composed of interstitial stroma and a collection of various tissue compartments, all bordered by basement membrane. Basement membranes are very thin sheets of extracellular matrix rich in type IV collagen, which provides their structural backbone. Ultrastructural studies reveal that the basement membrane is composed of an electron lucid region, called the lamina lucida, immediately adjacent to the cell layer and a distal, electron dense region called the lamina densa (von der Mark and Kuhl, 1985; Kleinman et al., 1984).

As for any extracellular matrix, the composition and the supramolecular characteristics of the basement membrane vary in relation to its location and to the type of cells responsible for its assembly. The mode of deposition of basement membranes is not clear and currently there are three postulated models of assembly. According to the layered model,

layers of collagen and structural glycoproteins are alternatively and separately deposited in the matrix and the interactions between molecules of different layers contribute to produce a three dimensional structure. The matrisome model suggests that the matrix is deposited as assembled units; these units contain the major components of the basement membrane in stable complexes of well defined stochiometry and, only after deposition, the collagen IV molecules rearrange into a fibrillar network. The third model, or polymorphic polymerization model, postulates that the properties and structure of individual matrices are determined by variations in the synthesis and secretions of the individual components. The layered model (Schwartz and Veis, 1980) is based on observations that the main components of the matrix can form homopolymers and indeed in some basement membranes there is evidence of the presence of layers with distinct antigenic characteristics. The matrisome model (Martin et al., 1984) draws evidence from the fact that it is possible to extract from matrices soluble complexes containing major structural components. As for the polymorphic polymerization model (Furthmayr et al., 1985), there is some evidence to suggest that cells may be able to alter the composition of the matrix at discrete sites (Martin and Timpl, 1987). Matrix deposition, although a very fundamental phenomenon, has proven quite elusive to study and, to date, evidence to suggest that matrix is assembled following any of such hypothetical models is all indirect and there is much uncertainty on the likelihood of their validity.

The specialized basement membrane present on the basal aspect of the vascular endothelium, separating its cells from other structures, is called the sub-endothelial matrix. The constituents of the sub-endothelial matrix can be classified into four categories: elastin, the collagens, the glycosaminoglycans and the structural glycoproteins.

Elastin is a highly insoluble protein, rich in hydrophobic amino acids

and possessing very few polar groups. It is derived from a soluble precursor, a M_r 72,000 protein called tropoelastin. Elastin molecules are extensively crosslinked to each other to form a network of filaments which, together with the collagen fibres, constitute the structural framework of any basement membrane. While the collagen fibres confer resistance, elastin, due to its random coiled structure, gives the matrix a great degree of elasticity (Eyre et al., 1984).

Collagens

The collagens are large molecules composed of three polypeptide chains that have a helical tertiary structure. They can either be hetero or homotrimers and, while at present there are at least 11 recognized types of collagen, it is likely that other types will be identified in the future. Two criteria must be fulfilled in order to classify any molecule as collagen: firstly a sizable part of this molecule must be arranged in the typical triple helix and secondly it must form extracellular aggregates whose main function is support. The collagens can be separated into three different groups according to their size and tertiary structure. One group is composed of molecules having chains with M_r> 95,000 and possessing long (~300 nm) uninterrupted helical domains. Collagen type I, II, III, V and K (XI) belong to this group and they are the main collagens found in fibers and fibrils. The second group molecules, like those of the first group, have chains of M_r> 95,000 but have a number of helical domains separated by non helical (usually globular) regions. These molecules are collagen type IV, VI, VII and VIII. A third group is composed of molecules with chains of M_r< 95,000 and type IX and type X belong to this group. All collagens are synthesized and secreted as larger precursor molecules (procollagens) which possess additional domains of non-collagenous structure. These domains, which resemble those of the structural

glycoproteins (see page 32) with asparagine-linked carbohydrate side chains and an overall globular structure, are removed in the case of the type I, II and III collagens. The collagens found in the sub-endothelial matrix are predominantly of type IV and type VI (Sage et al., 1981).

Collagen type IV seems to be present exclusively in basement membrane, usually as aggregates which are not readily dissociated. Pepsin readily disperse these aggregates but also causes fragmentation of the molecule. It is composed of three chains: two alpha1(IV), each of M_r 185,000, and one alpha2(IV), of M_r 170,000. Differently from the collagens of the first group, the precursors of these chains, pro-alpha1(IV) and pro-alpha2(IV), undergo little or no post transcriptional processing.

The monomeric form of type IV collagen is a molecule approximately 400 nm long with a collagenous domain at the NH_2 end called 7S and a non collagenous globular domain at the COOH terminus called NC1 which are joint by a long non collagenous domain called NC2 (see figure 5). It is thought that dimer formation is achieved by interaction of the NC1 domains, while tetramers are formed by interactions of four monomers at the 7S domain. This type of polymerization gives rise to an open mesh network which seems suitable to serve as scaffolding for the basement membrane. (Rauterberg et al., 1986; Miller and Gay, 1987).

Type VI collagen has been previously reported as microfibrillar protein 1 (MFP 1), gp140 and collagen-like glycoprotein (CLGP; see Miller and Gay, 1987 and references within). It has the smallest collagenous domain among collagens (~ 30 % of the whole molecule) and in fact it can be said to be more a glycoprotein than a collagen. Many cell types, including fibroblasts, synthesize this molecule and type VI collagen is by no means confined to basement membranes.

There are three subunits, alpha1(VI), alpha2(VI) and alpha3(VI), each of M_r ~ 140,000 and the collagen type VI molecule can be an alpha1$(VI)_3$ or

an assembly of the three different subunits. The assembled molecule has a M_r of 340,000-400,000 and consists of two large globular domains at both ends of the linear triple helix rod, which is ~ 105 nm in length (see figure 5).

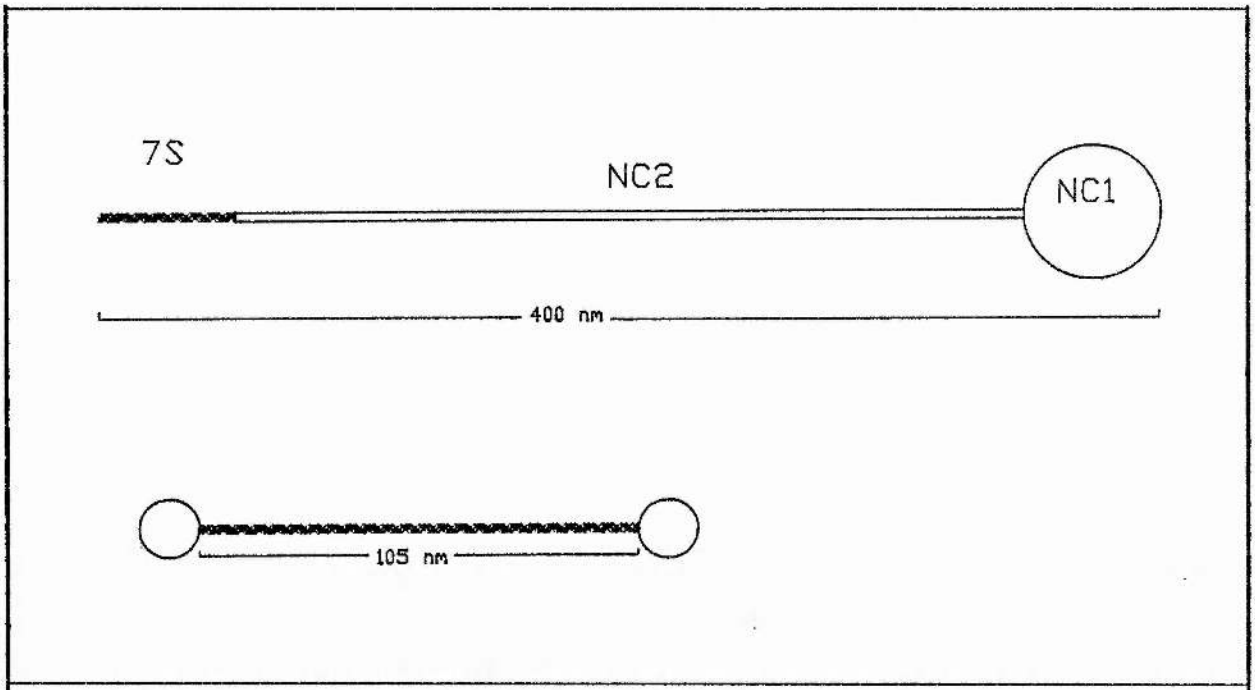


Figure 5: Structure of collagen type IV (top) and type VI (bottom).

Staggered association of two monomers which intertwine with each other forms a dimer; side to side assembly of four monomers into a tetramer gives the basic unit of the fibrils of collagen VI, which are end to end associations of tetramers via their globular domains. The fibrils are ~ 3 nm in diameter and have an axial periodicity of ~ 120 nm (Rauterberg et al., 1986; Miller and Gay, 1987).

Glycosaminoglycans

Glycosaminoglycans (GAGs) consist of a carbohydrate backbone made of repeating disaccharide units. Such carbohydrate chains are negatively charged and may possess sulphated groups, which contribute to rendering the molecule polyanionic in nature. Proteoglycans are GAGs which possess

a core protein and one or more carbohydrate chains. With the exception of hyaluronic acid, proteoglycans are the predominant form of GAGs in the tissues. GAGs, which are classified according to their repeating disaccharide units, are heterogeneous for number and location of sulphated groups, length of the carbohydrate chains and relative ratio of the saccharide components, and they also exhibit differences in the core proteins (Hook et al., 1984). For these reasons extreme diversity in properties and functions can be expected.

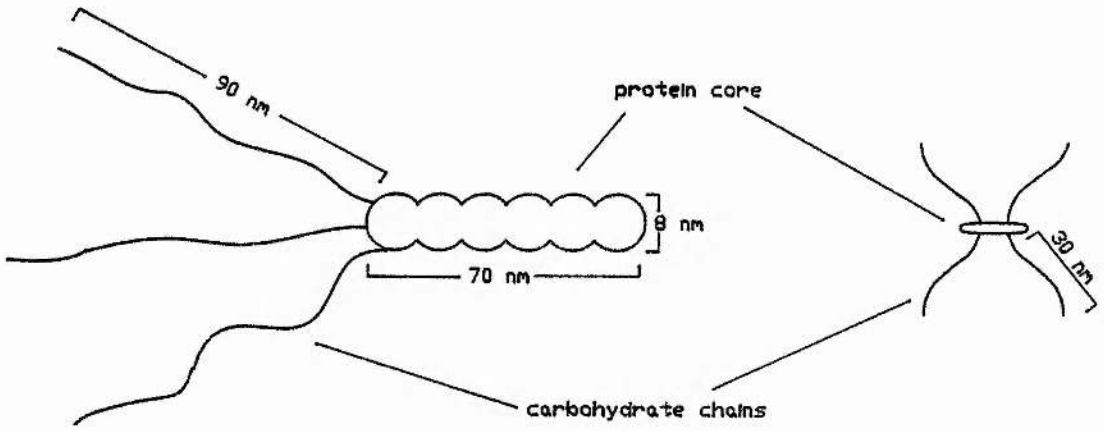


Figure 6: Model of high density (right) and low density (left) heparan sulfate proteoglycans of the extracellular matrix of EHS tumour (from Paulsson et al., 1986, modified).

The high density form has a small core protein (M_r 5,000-12,000) to which an average of 4 carbohydrate chains (M_r ~ 29,000) are attached. The low density form has a very large core protein (M_r ~ 400,000) with globular domains and connecting rods and its carbohydrate chains are ~ 3 times longer than in the low density form (Fujiwara et al., 1984; Martin and Timpl, 1987); Ruoslahti, 1988.

Hyaluronic acid is a very large molecule, usually exceeding a M_r of 10^6 and there is evidence that suggests that it is assembled extracellularly at the plasma membrane (Hook et al., 1984).

Heparan sulphate is present in plasma membranes and basement

membranes and its M_r is extremely variable (75,000-450,000) as is the size of the core protein (M_r 5,000-300,000) (Fujiwara et al., 1984; Paulsson et al., 1986). It is associated with the plasma membrane either as an integral or a peripheral membrane component (Hook et al., 1984).

Peripheral membrane GAGs are non-covalently attached to membrane components, mostly phosphatidylinositol (Yanagishita, 1989). Additionally there are membrane molecules that serve as receptors for the peripheral membrane GAGs. The binding to such receptors is reversible, saturable, can be specifically inhibited and does not cause any internalization or degradation of the ligand. It is not known whether the association of GAGs with their membrane receptor conveys a signal intracellularly. The M_r of the core proteins seems smaller than in the case of integral membrane GAGs and the molecule contains less carbohydrate. It is possible that some peripheral GAGs are derived from integral membrane components by proteolytic cleavage (Hook et al., 1984).

GAGs of the matrix and of the cell surface are in very close proximity and it is difficult to clearly separate them. Cells have binding sites for hyaluronic acid, each molecule probably binding simultaneously to multiple sites at the cell surface and perhaps being involved in important cell functions such as phagocytosis (Hook et al., 1984). GAGs associated with the cell membrane can function as acceptors for other molecules. Although these interactions may be quite aspecific due to the polyanionic nature of GAGs, the bindings with other matrix components such as structural glycoproteins and collagen are of interest. Interactions with the structural glycoproteins are probably very important for the formation and maintenance of the extracellular matrix. These interactions are described in detail in the sections on each of these molecules.

GAGs can bind to collagen either directly or by using a structural glycoprotein as a bridging element and they can modulate (inhibiting or

stimulating according to conditions) the formation of collagen fibers, having an effect on their thickness and ultimately on the properties of the matrix. GAGs derived from specific sites seem to have specificity only for collagens originating from the same tissue compartment, a specificity that seems attributable to the core protein (Hook et al., 1984).

All GAGs are influenced by the microenvironment, which determines the spatial orientation of each repeating disaccharide unit and, as a result, the long sugar chains can fold up or extend. Using infrared spectroscopy, it is possible to detect such changes in vitro in response to varying temperature or the presence of certain ions (Grant et al., 1989). Since GAGs present on the cell surface project for some distance away from the membrane, this property could be very important in presenting the cell with a representation of the distant environment and a working hypothesis on the role of these molecules on the cell surface is that they act as sensing devices (Gallagher, 1989).

The structural glycoproteins

The structural glycoproteins characteristically have asparagine- or serine-linked carbohydrate side chains and a structure composed of globular domains linked by rod like flexible regions. A description of the known structural glycoproteins of the sub-endothelial matrix follows.

Fibronectin.

Fibronectin is a large glycoprotein present as fibres in the extracellular matrix, as a surface glycoprotein on the cell membrane and in soluble form in body fluids. Although the name was originally coined to describe the trophism of this molecule for collagen and fibrin (nectere - to connect, fibres), lately it has become apparent that fibronectin is closely involved at a cellular level in a wide variety of adhesive

phenomena that go beyond its interactions with fibrous proteins.

There are various forms of fibronectin, the two main ones being plasma and cellular fibronectin, and indeed some authors refer to them as a family of closely related glycoproteins, all of which share the same structure where two similar but not identical subunits (each with a M_r of 220,000-250,000) are linked to each other asymmetrically at their COOH termini via two disulfide bridges. Strong similarities in structure and function exist among fibronectins but they differ in solubility, molecular weight, capacity to form multimers, sugar content and sulfated chains (Yamada, 1983; Ruoslahti et al., 1982; Yamada et al., 1984; Hynes, 1985; Mosesson and Amrani, 1980).

Electron micrographs of substratum bound fibronectin show a molecule that has a definite V shape with no large globular regions and possessing sites of increased flexibility, probably corresponding to the polypeptide sections between structured domains. In its prevalent conformation fibronectin is probably a folded and asymmetrical molecule (Engel et al., 1981; Hynes and Yamada, 1982). Each subunit has 9 structured regions or domains, connected by flexible regions and the dimer they form is an extended molecule with two long arms (~ 60 nm each) usually folded but capable of expanding when electrostatic interactions are disrupted (Hasty et al., 1986; Hynes and Yamada, 1982; Yamada, 1983; Hynes, 1985).

Plasma fibronectin can undergo a limited amount of self-association, particularly at low ionic strength at 4° C and will form filaments 2 nm or more in thickness, resembling fibronectin fibrils observed in vivo. In the collagen binding region there may be a self-association site for a complementary COOH terminal site. This polymerization is enhanced by polyamines and by heparin (which induces polymerization at physiological ionic strength). Factor $XIII_a$ or disulphide bonding cause polymerization at the cell surface, promoted by proteoglycans. It is thought that multimeric

fibronectin is required in cell-cell adhesion (Yamada, 1983; Grinnell, 1981).

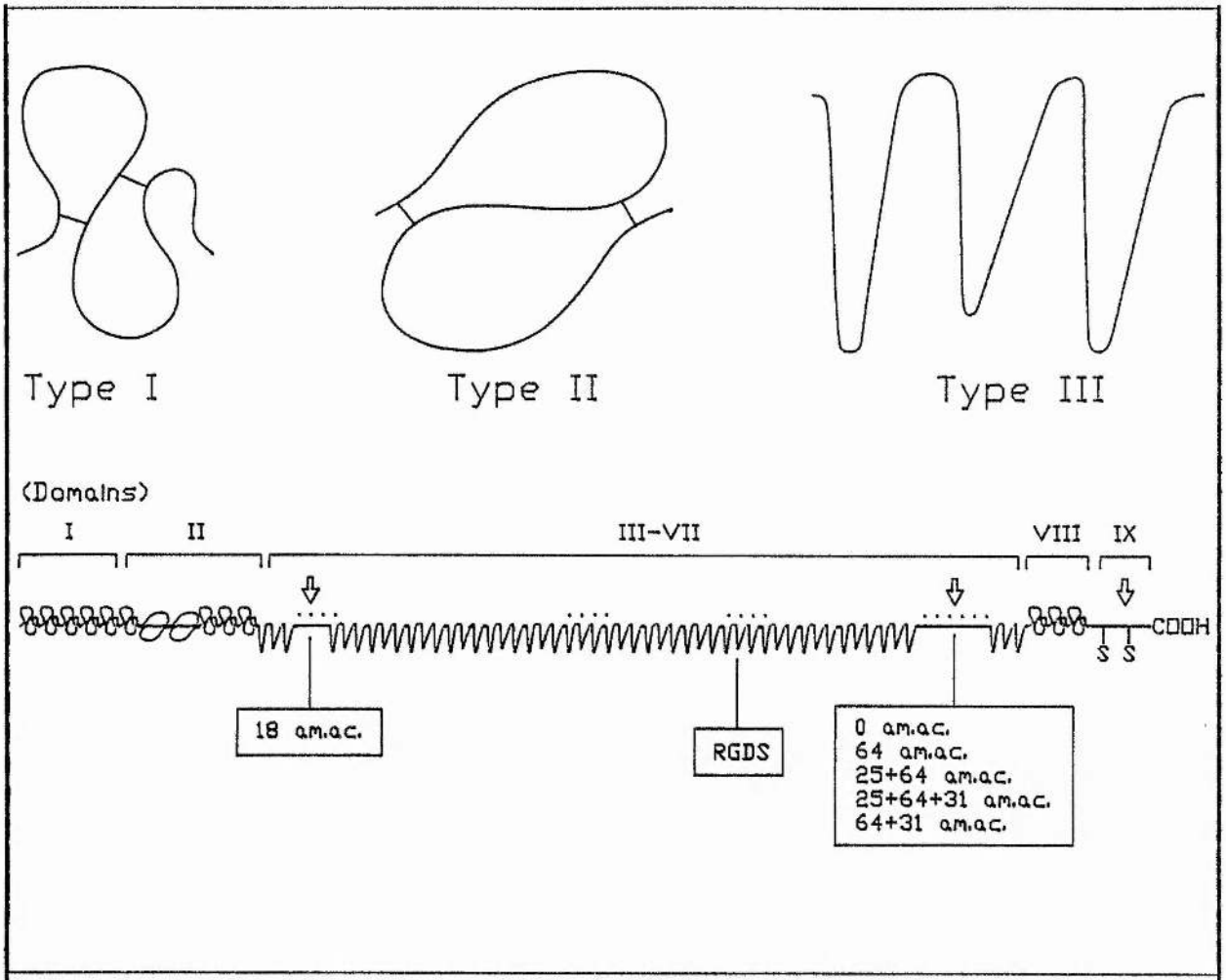


Figure 7: Structure of the fibronectin molecule.

Secondary structure of the three types of homologous repeats of fibronectin (top) and mapping of the homologous and non homologous (arrows) repeats in the fibronectin subunit (bottom). Regions of heterogeneity are marked by dotted lines: there is a 18 aminoacid fragment in the III domain, the 8th and 14th type III repeats may be absent and there is a further variable segment of up to 120 amminoacids in the VII domain. All differences between fibronectins seem due to post-translational changes of the nRNA. A fibronectin molecule (dimer) is usually formed by two subunits of unequal lenght.

As shown in figure 7, more than 90 % of the primary structure of fibronectin is made of a series of repeats of three different homologous sequences known as type I, type II and type III repeats (Hynes, 1985). It is believed that alternative splicing of nRNA accounts for most of the differences in mobility on SDS PAGE, in pI, in solubility and in the

capacity to form multimers and fibrils that fibronectin subunits and fibronectin molecules as a whole show (Yamada, 1983; Hynes, 1985).

Carbohydrates represent between 5 and 9.5 % of the weight of the molecule. Unglycosylated fibronectin is assembled and secreted normally and is fully functional so the main role of the carbohydrate chains seems to be stabilization of specific regions of the molecule against proteolytic attack (Yamada, 1983; Hynes, 1985; Mosesson and Amrani, 1980).

Fibronectin has numerous effects on cell morphology and function. It promotes phagocytic activity, mediates cell attachment and spreading on various substrata and stimulates cell movements as well as participating in fibrillogenesis within the extracellular matrix and fibrin gel (Mosesson and Amrani, 1980; McCarthy et al., 1985; Grinnell, 1981; Schwartz et al., 1985; Doolittle, 1984). All these activities are the result of interactions between various ligands and discrete domains within the molecule. The mapping of these domains has been achieved by proteolytic fragmentation of the entire molecule. The subdivision of the molecule into domains is a purely functional one and is not related to gene architecture. Indeed a domain may be envisaged as a part of the molecule resistant to proteolytic digestion, probably due to a highly structured tertiary conformation of the peptide, connected to other domains by flexible and protease sensitive segments (Hynes and Yamada, 1982; Yamada et al., 1984). A map of the domains, their relationship to the primary structure and the binding sites is shown in figure 8.

Fibronectin has two heparin/heparan sulfate binding sites, one in the first and one in the seventh domain. The binding of these ligands at the first domain is of lower affinity and can be inhibited by 0.25 M NaCl or physiological concentrations of Ca^{2+} (but not of other divalent cations). The binding site of domain VII is of higher affinity, is not modulated by divalent cations and requires 0.5 M NaCl to cause dissociation from its

ligands (Hayashi and Yamada, 1982; Yamada, 1983; Ruoslahti and Engvall, 1980). The binding of heparin stimulates the fibronectin-mediated uptake of gelatinized particles by macrophages and both heparin and heparan sulphate enhance the affinity of fibronectin for denatured collagen types I and III. Heparin-binding of fibronectin in cold plasma results in precipitation of fibronectin-fibrinogen complexes (Ruoslahti and Engvall, 1980; Yamada, 1983; Ruoslahti et al., 1982). Heparin binds to fibronectin with a K_d between 10^{-7} and 4×10^{-9} M and although the interactions seem to be electrostatic in nature, the binding is competitively inhibited only by heparin and heparan sulphate. Macromolecular heparin promotes the release of fibronectin from tissues and of fibroblasts from collagen (Yamada, 1983).

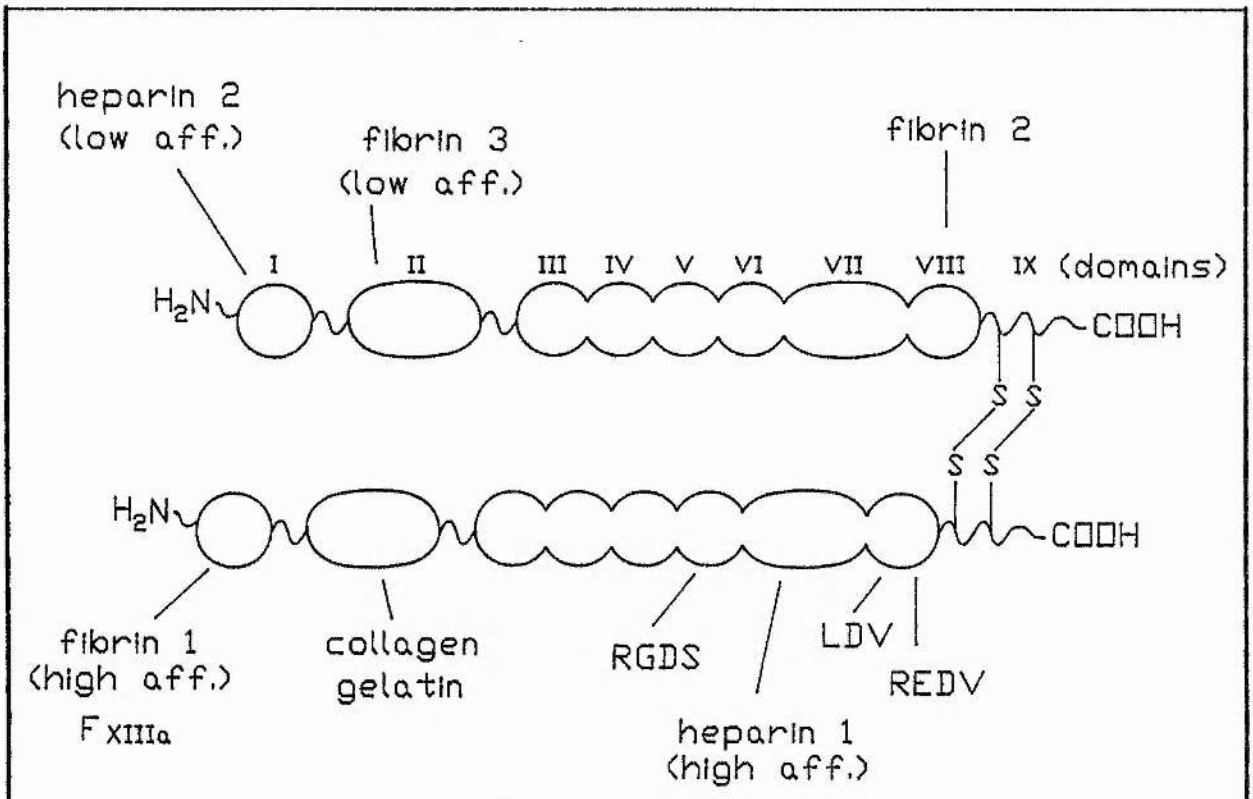


Figure 8: Map of the functional domains of fibronectin (from Hynes, 1985, modified).

In addition fibronectin may bind actin, myosin, tropomyosin, alpha-

actinin and vinculin although with such low affinity as to call into question the significance of these interactions. It is likely that fibronectin has a role in the clearance of cytoskeletal proteins and aggregates by scavangers (Yamada, 1983).

Fibronectin also binds to bacteria and is of key importance for the opsonization of microorganisms and foreign bodies. In human plasma there are 5 fibronectin isomers having the same molecular weight but differing in their isoelectric points (pI between 5.6 and 6.1). Only the isomer with the highest pI seems capable of binding to bacteria and inducing their opsonization. Purified fibronectin contains ~15 % of the pI 6.1 isomer and there is a marked loss of this isomer upon storage in the cold (Boughton and Simpson, 1984; Boughton, 1987).

There are three binding sites for fibrin on each fibronectin subunit. The binding site in the first domain possesses the highest affinity. In the eighth domain there is the second binding site for fibrin, but of lower affinity than that of the first domain. In the second domain there is a third and very weak binding site for fibrin adjacent to the collagen binding site and quite sensitive to proteases. Neither of the two major binding sites for fibrin is of particularly high affinity, at least at 37° C, and factor XIIa mediated crosslinking is quite important in stabilizing the interactions with the fibrin gel, which are otherwise relatively labile. Nevertheless, the affinity of fibronectin for fibrin increases at lower temperature (Hynes and Yamada, 1982; Yamada, 1983). Similarly, fibrinogen is not a ligand of fibronectin at 37° C but binds to it at 4° C, causing precipitation of the complex. This property gave fibronectin the name of cold insoluble globulin (CIG), because fibrinogen contaminant produced precipitates when the preparation was stored in the cold (Grinnell, 1981; Mosesson and Amrani, 1980).

The second domain contains the collagen binding site. Fibronectin

binds to a site on the collagen alpha-1(I) chain between residues 757 and 791 and a similar site in the alpha-2(I) chain. These are the least stable regions of the collagen triple helix and in fact contain the only site of attack of vertebrate collagenases (Yamada, 1983). Fibronectin also binds to denatured non helical collagen but with a somewhat higher affinity when compared to the corresponding native collagen and the K_d for denatured collagen varies between 2 and 5 x 10^{-9} M, depending on the type of collagen. For the type I collagen this is a significant 200 fold increase, while in the case of type III collagen the difference is minimal (2-6 fold) (Yamada, 1983). After binding to this domain of fibronectin, collagen can be covalently crosslinked to the first fibronectin domain by factor $XIII_a$ (Mosesson and Amrani, 1980).

There are at least 3 cell-binding sites on fibronectin: one is a RGDS sequence placed in a hydrophilic loop in the sixth domain (Hynes, 1985); the other two, LDV and REDV, are found in the 8th domain (Humphries et al., 1990). It is probable that these are not the only sites used by fibronectin in its interactions with cells. In fact, membrane bound GAGs are probably very important as fibronectin ligands, and there are reports of the existence of at least two different cell-binding sites on the fibronectin molecule (McCarthy et al., 1986). Interestingly, the RGDS-containing domain has also one of the three regions of variability of the fibronectin molecule in the form of the 14th type III repeat.

Fibronectin binds to hyaluronic acid with moderate affinity (K_d= 10^{-7} M) via a binding site different from that used to bind to heparin and this binding is not inhibited by 2 M NaCl. The localization of the domain responsible for this interaction has not been possible since fibronectin probably needs to be in its polymeric form to bind effectively to this ligand, suggesting the presence on each subunit of a low affinity site. In human fibronectin this activity is present only in cellular fibronectin

(Hynes and Yamada, 1982; Yamada, 1983). Hyaluronic acid interferes with some enhancement effects exerted by heparin on fibronectin function. Chondroitin sulfate proteoglycan (but not the free chondroitin sulfate chains) interacts with fibronectin and enhances fibronectin binding to collagen type I (Yamada, 1983). Overall it seems that proteoglycans may act as modulators of fibronectin-cell and fibronectin-extracellular matrix interactions (Yamada, 1983).

Laminin

Laminin is the most abundant glycoprotein present in basement membranes and has a M_r between 850,000 and 10^6. It consists of three disulphide linked subunits which are synthesized from different mRNA but show regions of similarities. There is one A-chain (or beta-subunit) with M_r 350,000-440,000 and two B-chains (or alpha-subunits) with M_r~ 220,000. Two different types of B-chains can be distinguished: a B1-chain with M_r 225,000 and a B2-chain with M_r 205,000 (Martin and Timpl, 1987; von der Mark and Kuhl, 1985; Paulsson et al., 1986; Ekblom et al., 1986; Yamada, 1983).

Although laminin has been reported to exist in many different forms, a typical complete laminin molecule (as for instance isolated from tumour tissue or placenta) is an A-B1-B2 assembly. Electron micrographs of substratum adsorbed, rotary shadowed preparations of laminin show a cross-shaped structure with one long arm and three short ones (Engel et al., 1981; Martin and Timpl, 1987; McCarthy et al., 1985; von der Mark and Kuhl, 1985; Yamada, 1983).

Digestion studies and analysis of cDNA clones reveal that laminin has a multidomain structure (Martin and Timpl, 1987). A map of the domains of the B1 chain is shown in figure 9. Domains I and II are alpha-helical coils and between the first and second domain there is a 30 amino acids

segment (segment alpha), cystein-rich, which represents a region of difference between B1 and B2 chains. Domains III and V are formed by repeats of homologous cysteine-rich segments, each ~50 amino acid residues long. Both the sixth (NH_2 terminal) and fourth domain are ~250 amino acid residues long and have a globular structure. The B2 chain possesses the same overall structure but is shorter than the B1 chain by ~100 amino acid residues, lacking the segment alpha and having a lower number of repeating homologous sequences in domains III and V. Data on the structure of the A chain are incomplete but it is believed that the NH_2 terminal segment (the third short arm of the laminin molecule) is similar to the correspective segments on the B chains; at the COOH terminus the A chain has a large globular domain, known as the "foot", which is formed by five loops or "toes" (Martin and Timpl, 1987; von der Mark and Kuhl, 1985; Paulsson et al., 1986).

An A-B1-B2 assembly has the NH_2 terminus of each chain forming the three short arms. The chains are then linked by disulphide bonds at the centre of the cross where the A chain probably runs within a coiled-coil alpha-helix ~ 50 nm long made by the two B-chains. The B chains are then linked at their COOH termini by a single disulfide bond while the COOH terminus of the A chain forms the globular structure at the end of the long arm (Martin and Timpl, 1987; Yamada, 1983; McCarthy et al., 1985; Paulsson et al., 1986; von der Mark and Kuhl, 1985).

Carbohydrate content is ~ 13 % w/w and the oligosaccharide chains are all asparagine-linked. Their role is unknown since they do not seem to confer resistance against proteolytic attack, they are scarcely involved in ligand interactions, and they do not appear to be important for the assembly or the secretion of the mature molecule (Martin and Timpl, 1987; Yamada, 1983).

Laminin is now regarded to be a molecular family of similar molecules

that are separate gene products. One of this, called S-laminin, is thought to be a macromolecular assembly ($M_r > 10^6$) of a single polypeptide of M_r 190,000 homologous to the B1 chain (Hunter et al., 1989). Another member of this family, called merosin, is very similar to the traditional laminin molecule but is a B1-B2-M assembly, the M chain having much homology with the A chain and a very similar tertiary structure (Leivo and Engvall, 1988). The laminin produced by invertebrates has a long arm of ~ 110 nm. Schwann cells and oocytes produce only B chains which are assembled in a Y-shaped dimer. Only after the two cell stage, do embryos contain laminin with A chains (Martin and Timpl, 1987; von der Mark and Kuhl, 1985; Yamada, 1983; Kleinman et al., 1984).

There is an heparin/heparan sulfate binding site in the large globular domain at the end of the long arm (Yamada, 1983; von der Mark and Kuhl, 1985; Martin and Timpl, 1987; McCarthy et al., 1985). It is known that the glycan chains of high density heparan sulfate bind to laminin, but the binding is very weak ($K_d > 10^{-6}$ M). Low density heparan sulfate also binds laminin but there are no data on the strength and sites of these interactions (Martin and Timpl, 1987).

Collagen type IV binds to the globular domains at the end of the short arms of the laminin molecule and an additional binding site is present in the globular domain at the end of the long arm. For the binding, the triple helical structure of collagen must be intact (Martin and Timpl, 1987; Yamada, 1983; McCarthy et al., 1985; von der Mark and Kuhl, 1985).

A binding site for nidogen (see page 44) is found in one of the globular domains of the short arms near the centre of the cross. The interaction with nidogen is very strong ($K_d < 10$ nM) and is dependent on divalent cations (chelating agents readily dissociate this binding). The role of this interaction is unknown, but it is thought that nidogen helps in stabilizing the structure of the basement membrane and it may also

modulate the activity of a cell-binding site present in the central part of the laminin molecule (Martin and Timpl, 1987; von der Mark and Kuhl, 1985). Nidogen is present in an equimolar ratio with laminin in the basement membranes of some tumours as well as those of normal cells (von der Mark and Kuhl, 1985; Paulsson et al., 1986).

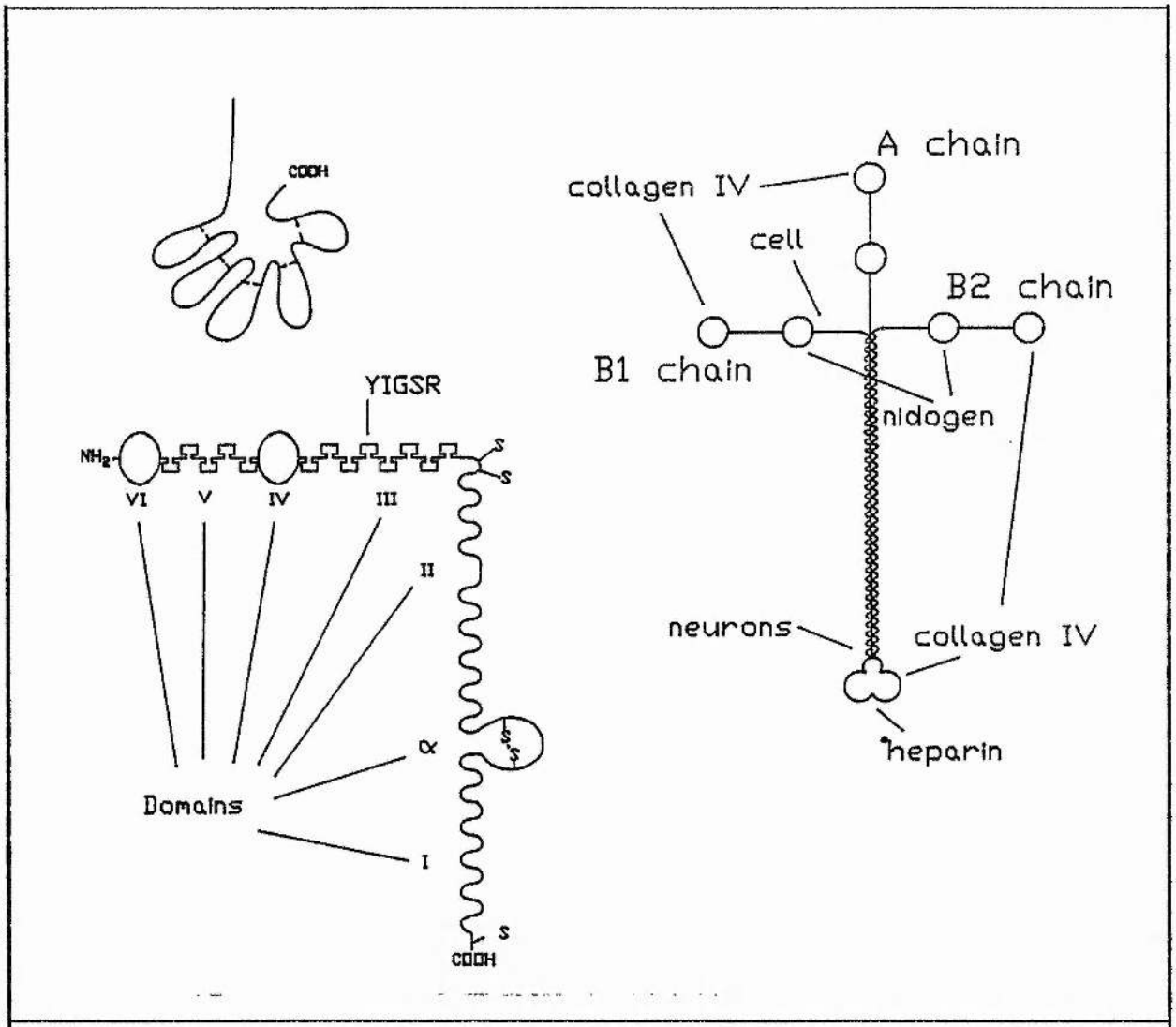


Figure 9: top left: the A chain "foot"; bottom left: a map of the B1 chain; right a model of laminin. (from Martin and Timpl, 1987, modified)

Laminin also binds a small glycoprotein (M_r= 40,000) present in basement membranes and referred to as BM-40, osteonectin or SPARC (Martin and Timpl, 1987; Paulsson et al., 1986). The binding to laminin is divalent cation independent although BM-40 is a calcium-binding protein

and calcium causes changes in the alpha helical conformation of this molecule (Martin and Timpl, 1987). Other ligands of laminin include the complement components C3 and $C1_q$, plasminogen, plasminogen activator, sulfatides and gangliosides although there are no indications on the physiological significance of these interactions (Martin and Timpl, 1987; Yamada, 1983).

Laminin is capable of forming large polymers that possess a definite structure. Dimers and trimers of laminin can be assembled even in the presence of chelating agents but the presence of divalent cations seem necessary for further polymerization. There are two regions for self association: one involves domains at the end of the short arms, a second one occurs between the globular domains of the long arms (Martin and Timpl, 1987).

So far two different cell-binding sites have been identified on the laminin molecule. One is the pentapeptide Tyr-Ile-Gly-Ser-Arg (YIGSR), located in the third domain of the B1 chain. the other is a RGD sequence located in the rod-like region near the centre of the cross (Aumailley et al., 1990). A receptor for the YIGSR sequence has been isolated from various cell types (Martin and Timpl, 1987). The receptor molecule is an integral membrane protein with a hydrophobic domain. It has, depending on the cell of origin, a M_r between 67,000 and 70,000 and is extractable from different sources using different procedures (Malinoff and Wicha, 1983; Rao et al., 1983; Martin and Timpl, 1987; von der Mark and Kuhl, 1985). Despite these differences indicating heterogeneity in the receptor structure, cells seem to possess comparable numbers of free acceptor sites for laminin (between 5 and 10 x 10^4 per cell) and their receptors have similar dissociation constants (K_d= 1.5-2 x 10^{-9} M) (Martin and Timpl, 1987; von der Mark and Kuhl, 1985). In normal epithelial cells this receptor is found only along the basal side of the membrane while its distribution in

tumour cells is less organized (Martin and Timpl, 1987). The solubilized receptor appears not to have high affinity for its ligand nor high specificity, showing some binding to immobilized fibronectin and collagen type I or IV. Nevertheless, when reconstituted into liposomes (or even when adsorbed onto nitrocellulose), it acquires specificity as well as an affinity for laminin comparable to that exhibited by whole cells (von der Mark and Kuhl, 1985; Ekblom et al., 1986). The receptor has been shown to bind actin and induce its polymerization (Martin and Timpl, 1987; von der Mark and Kuhl, 1985) but, because of the large number of molecules that can bind actin, the existence of a transmembrane link between extracellular matrix laminin and the cytoskeleton can, at the moment, only be postulated. What is known is that on the internal surface of the membrane the receptor is in association with, or actually contains, a 5' nucleotidase whose activity is stimulated several fold upon binding of laminin to the receptor, a fact which supports the hypothesis of the existence of a mechanism to translate laminin binding into an intracellular signal that could result in shape changes as well as triggering other cellular events such as differentiation (von der Mark and Kuhl, 1985).

Nidogen

This molecule is a single chain protein capable of binding laminin and collagen type VI and is probably identical or very similar to entactin, a M_r 158,000 sulphated protein (Ekblom et al., 1986). In its intact form nidogen has a M_r of ~150,000 but a series of fragments with M_r 130,000 100,000 80,000 and 40,000 are produced as a result of stepwise degradation at the NH_2 terminal end of the protein by the tissue proteases (Paulsson et al., 1986).

In rotary shadowed preparations of nidogen, the molecule possesses a dumbell shape with a large and a small globular region connected by a

thin rod. The region responsible for the interaction with laminin seems to reside either in the small globular region or in the rod-like segment proximal to it (Paulsson et al., 1986). Nidogen also binds to the globular NC1 domain of collagen type IV and this binding only occurs when the NC1 domain is rendered divalent, either by crosslinking of purified NC1 fragments or by the use of intact crosslinked collagen IV (Paulsson et al., 1986).

It is probable that this molecule serves as a stabilizer of the basement membrane structure by binding to both laminin and collagen IV and, since nidogen seems much more sensitive to proteolysis than any other basement membrane protein, it perhaps allows cells to manipulate the supramolecular structure of the basement membrane by means of proteolytic enzymes (Ekblom et al., 1986; Paulsson et al., 1986). Nidogen also contains a RGD and a LDV sequence which could be used to interact with cells (Humphries et al., 1990; Nagayoshi et al., 1990)

Thrombospondin

Thrombospondin was first identified by Baezinger and co-workers (1971) as a molecule released by platelets upon thrombin exposure. It is a large glycoprotein with a M_r of 450,000 involved in cell-cell and cell-matrix interactions. As shown in figure 10, thrombospondin is composed of three identical subunits and is capable of interactions with a number of molecules including collagen type IV, plasminogen, laminin, fibrinogen, fibronectin, histidine-rich glycoprotein (HRGP) and heparin (Lawler, 1986; Frazier, 1987). Thrombospondin is a true multifunctional protein, with independent domains mediating various activities, but a striking feature of this molecule is its Ca^{2+} dependent structure. Thus, changes in the concentration of the cation in the microenvironment can lead to modulations of its shape and function (Lawler et al., 1985, Lawler, 1986;

Frazier, 1987).

It has been shown that thrombospondin production is not limited to platelets and megakaryocytes: endothelial cells, vascular smooth muscle cells, fibroblasts from a variety of sources, alveolar type II epithelial cells, monocytes, peritoneal macrophages, as well as cells from many tumours, including B16 cells, all produce thrombospondin, secrete it and incorporate it into the extracellular matrix in variable quantity (Raugi et al., 1982; Mumby et al., 1984 a; Jaffe et al., 1985; Lawler, 1986).

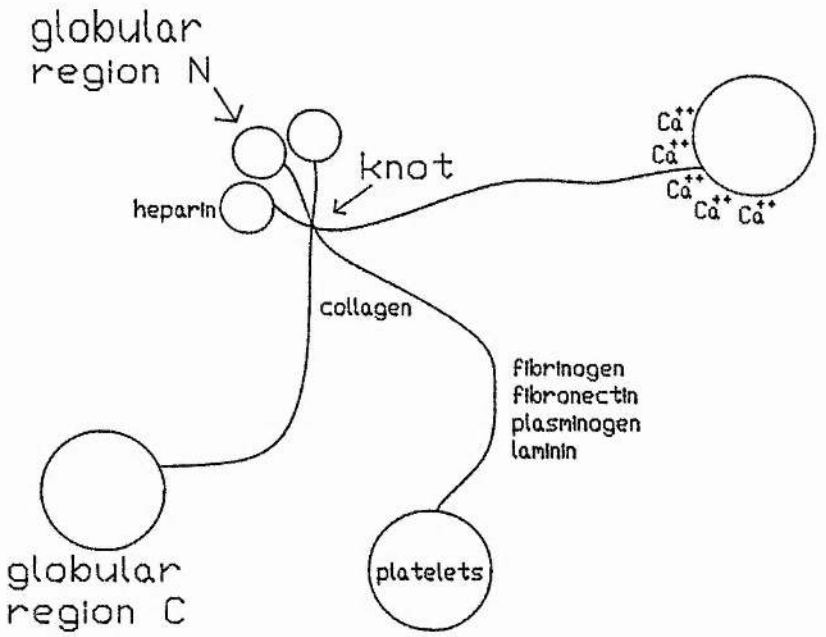


Figure 10: A model of thrombospondin.

Thrombospondin consists of three subunits, each of $M_r \sim 145{,}000$, joined together by disulfide bridges in a region close to the NH_2 terminus of these chains. Electron microscopy of surface adsorbed, rotary shadowed preparations reveals a structure composed of three large globular regions, three long, thin connecting rods, a knot where the three

chains are crosslinked and a small globular region close to it (Coligan and Slayter, 1984; Lawler et al., 1985; Lawler, 1986; Frazier, 1987).

Each large globular region is between 118 and 170 A in diameter and represents the COOH terminal part of each subunit and is in fact called globular region C. The small globular region consists of the NH_2 terminal ends of the three chains and is called globular region N. This globular region can sometimes be resolved into three separate smaller structures. The thin connecting rods are very flexible and may adopt a variety of orientations, furthermore their length, which in the presence of Ca^{2+} is between 160 and 290 A, increases to 383 A when the Ca^{2+} is chelated. This increase in length is related to the decrease in size of the globular region C (Margossian et al., 1981; Coligan and Slayter, 1984; Lawler et al., 1985; Frazier, 1987).

The heparin binding site on thrombospondin resides in the globular region N. Thrombospondin binds avidly to heparin and the binding is disrupted with high (0.45 M) concentrations of NaCl (Raugi et al., 1984; Dixit et al., 1984). Thrombospondin in the extracellular matrix has been demonstrated to be associated with fibrils containing GAGs, and both fibronectin and heparin can inhibit thrombospondin incorporation in the matrix (Majack et al., 1985).

Thrombospondin interacts with the surface of activated platelets by binding to fibrinogen. The affinity for this ligand has an in vitro $K_d = 3.4 \times 10^{-9}$ M and is inhibited by glucosamine, mannosamine and arginine, as indeed is the case for the lectin-like activity of platelets. The binding site for fibrinogen resides near the globular region C (Leung and Nachman, 1982; Frazier, 1987). Controversial reports indicate that the affinity of immobilized thrombospondin for soluble fibronectin is much lower than for soluble fibrinogen, but soluble thrombospondin has a comparable affinity for both these ligands when they are in solid phase

(Lahav et al., 1984). As well as by binding to fibrinogen, thrombospondin can interact with the cell surface via two known adhesive sequences, RGD and LDV (Humphries et al., 1990)

Thrombospondin also binds to collagen with a binding site located in a region of the connecting arms close to the interchain knot (Mumby et al., 1984 b). Monomeric collagens possess only one low affinity binding site so their interactions with thrombospondin in vitro are very weak and only type V collagen seems to have a second binding site, which is of much higher affinity (Mumby et al., 1984 b; Frazier, 1987). Such preferential affinity for collagen type V may not be representative of the in vivo interactions of thrombospondin with collagen in the matrix since thrombospondin would find a high number of binding sites (albeit of low affinity) in fibrillar collagen.

The interaction of thrombospondin with plasminogen, which occurs probably via the lysine binding site on this molecule, has a K_d of 3.5×10^{-10} M, while the binding of thrombospondin with HRGP has a K_d of 6.2×10^{-9} M. These molecules (thrombospondin, plasminogen and HRGP) can form a trimolecular complex that is capable of binding to heparin and that serves as a substratum for plasminogen activator (Silverstein et al., 1985; Leung et al., 1984). It is possible that, by forming this complex, thrombospondin enables the insertion of plasminogen into the fibrin clot and therefore serves as a regulator of plasmin dependent proteolysis within the clot.

Thrombin forms a stable complex with thrombospondin. This complex forms very rapidly and then is stabilized by thiol disulphate exchange (Danishefsky et al., 1984). Thrombospondin also binds laminin, von Willebrand factor and low density lipoproteins, as well as factor IX_a and factor X_a for which it can serve as a substratum (Lawler, 1986).

Vitronectin

Vitronectin is an alpha-1 globulin present in serum, amniotic fluid and urine as well as being found on the cell surface and in the extracellular matrix (Hayman et al., 1983; Barnes et al., 1985; Tomasini and Mosher, 1986). Its concentration in plasma is quite variable, ranging from 140 to 700 ug ml^{-1}. In plasma it is present in at least 2 forms, a native one, that has a M_r around 78,000 and its cleavage product, with a M_r of 65,000-70,000 , together with a M_r 10,000 fragment (Jenne and Stanley, 1985; Silnutzer and Barnes, 1984 a).

Vitronectin was first described two decades ago by Holmes (1967), but remained poorly characterized until it was shown not to be a fragment of fibronectin (Knox and Griffiths, 1979). Originally identified by its ability to mediate cell attachment and spreading and termed serum spreading factor, it was later named vitronectin because of its ability to mediate cell adhesion on glass surfaces and because of its affinity for glass (glass-bead chromatography was indeed used for its purification - Barnes et al., 1984). Lately it has been found that an independently characterized glycoprotein, named S-protein, with binding properties for the complement complex $C5_b$-7 has the same biological, immunological and physicochemical characteristics as vitronectin and comparisons of their primary sequences has confirmed that S-protein and vitronectin are one and the same (Suzuki et al., 1985; Tomasini and Mosher, 1986; Jenne and Stanley, 1985). The polypeptide backbone of vitronectin consists of 458 amino acid residues having a calculated M_r of 53,132. It possesses three potential sites for glycosylation and if the vitronectin carbohydrate content is confirmed to be in the region of 34 % w/w, the side chains would account for the difference between the experimentally determined and the calculated molecular weights (Suzuki et al., 1985).

Vitronectin binds to antithrombin III, to the complement lytic complex

$C5_b$-7..9 and to heparin (Ill and Ruoslahti, 1985; Pytela et al., 1985; Jenne et al., 1985; Hayashi et al., 1985; Preissner et al., 1985; Barnes et al., 1985). The amino acid sequence of vitronectin can be divided into regions of functional significance: the first 44 NH_2 terminal amino acids are the somatomedin B molecule. Somatomedin B was originally described as a growth hormone dependent polypeptide which stimulated glial cells to undergo mitosis, but recently it has been found that such activity was due to a small quantity of contaminant epidermal growth factor co-purified with somatomedin B. In view of this, the polypeptide should not qualify for such nomenclature but the term somatomedin B is still widely used to refer to this sequence (Jenne and Stanley, 1985). The somatomedin B region is rich in cysteine and contains four disulphide bridges which fold it into an independent domain (Jenne and Stanley, 1985; Suzuki et al., 1985).

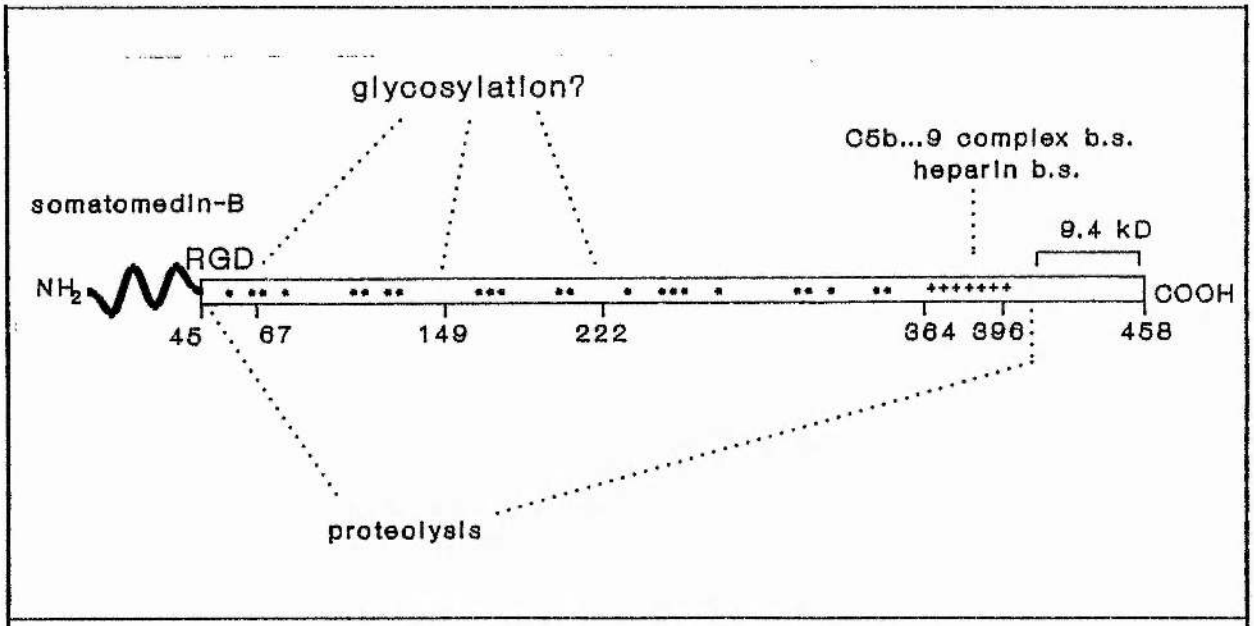


Figure 11: A map of vitronectin (+= positively charged amino acids, *= clusters of hydrophobic residues).

In positions 45-47 there is the familiar cell binding sequence RGD found in many other molecules (see section on fibronectin). Interestingly, the

somatomedin B polypeptide is generated by proteolytic cleavage at the R-G bond (in vivo probably the result of the action of thrombin which cleaves a similar R-G bond in fibrinogen) which depletes the molecule of its cell binding activity (Jenne and Stanley, 1985).

After the cell binding site there is a long (~ 300 amino acids) region which contains the three asparagine residues, in positions 67, 149 and 222, possible sites of attachment of N- linked sugars. This is a region rich in proline, with densely scattered hydrophobic residues and probably having an unusual secondary structure (Jenne and Stanley, 1985).

Between residues 364 and 396 there is the heparin binding site (Jenne and Stanley, 1985). Among these 32 residues there are 14 positive charges, two hydrophobic residues and no negative charges. This site could also be involved in the binding of vitronectin to the $C5_b$-7..9 complex and the ability of vitronectin to inhibit the heparin induced stimulation of the inactivation of thrombin by antithrombin III probably derives from interactions with heparin at this site (Jenne and Stanley, 1985; Preissner et al., 1985).

At position 402 there is a site for proteolytic cleavage that generates a COOH terminal fragment with M_r of 9,400. Partial vitronectin cleavage is undertaken in plasma where a fragment of M_r~ 10,000 from the COOH terminus is detected. In vitro, purified vitronectin is rapidly cleaved at this site by trypsin (Jenne and Stanley, 1985).

Although only one gene has been found for vitronectin, the existence of different length mRNAs and the heterogeneity in molecular weight that vitronectin preparations show upon electrophoresis with the presence of large bands, would suggest that vitronectin molecules of slightly different molecular weights originate from a unique nRNA by splicing (Suzuki et al., 1985; Jenne and Stanley, 1985).

Native vitronectin does not have the heparin binding site exposed,

becoming able to bind this ligand only when activated. This activation normally occurs during coagulation, and serum vitronectin (but not plasma vitronectin) expresses the heparin binding site (Tomasini and Mosher, 1986; Barnes et al., 1985; Ill and Ruoslahti, 1985; Hayashi et al., 1985; Silnutzer and Barnes, 1984 b). It has been shown that the presence of active thrombin is necessary for activation, even though it is not known whether the action of thrombin is a direct enzymatic one on the vitronectin molecule (Silnutzer and Barnes, 1984 b).

There are other agents that can expose the heparin binding site on vitronectin: treatment of the molecule with acids or short exposure to 100° C or 8.0 M urea, all activate the heparin binding site, although many other denaturing agents fail to do so (Hayashi et al., 1985). It is interesting to note that not all vitronectin molecules can be induced to bind heparin, but once the heparin binding site is exposed the molecule loses its ability to be adsorbed to the substratum (Barnes et al., 1985). Vitronectin in which the heparin-binding site is exposed is unable to support adhesion and spreading of cells in the usual assays, but only because of its lack of substratum binding. In fact the integrity of the cell binding site can be demonstrated if the molecule is anchored to the substratum by other means.

It seems that the two serum forms of vitronectin have different affinities for heparin, it having been reported that at physiological pH the binding of the M_r 78,000 form to heparin is dissociated by 0.12 M NaCl while 0.5 M NaCl is needed to disrupt the interactions of heparin with the M_r 65,000 form (Hayashi et al., 1985; Ill and Ruoslahti, 1985). This is likely to be related to different affinities of the two forms for the thrombin-antithrombin III complex (the M_r 65,000 form does not seem able to bind antithrombin III) and the influence of such complex on the heparin-vitronectin interactions.

Vitronectin binds to antithrombin III only after the latter has been activated and their interactions may be important in the regulation of the coagulation process (Ill and Ruoslahti, 1985; Jenne et al., 1985). Antithrombin III acquires affinity for vitronectin once it is complexed to thrombin. Adsorption to plastic or iodination has the same effect, suggesting the presence of a cryptic site for binding to vitronectin which is exposed by a change in the tertiary structure of the molecule (Ill and Ruoslahti, 1985). Accordingly, vitronectin is not associated with antithrombin III in plasma, while in serum it is found in a complex that usually includes also thrombin (Jenne et al., 1985; Ill and Ruoslahti, 1985). The binding to antithrombin III is independent of divalent cations and heparin does not influence either the formation or the stability of the complex (Ill and Ruoslahti, 1985).

The binding of vitronectin to the nascent thrombin-antithrombin III complex results in the suppression of the inhibition of thrombin, which is therefore still able to exert its amidolytic and proteolytic action, albeit at a somewhat slower rate. Vitronectin is also able to mitigate the enhancement of thrombin inactivation by antithrombin III induced by heparin. If vitronectin is added to an already formed thrombin-antithrombin III complex it will still bind to the complex but will not modulate the activity of antithrombin III (Ill and Ruoslahti, 1985; Preissner et al., 1985; Jenne et al., 1985). It is probable that the region of vitronectin involved in the binding to antithrombin III and the $C5_b$-7..9 complex is not readily available in the native molecule but becomes exposed during purification (Dahlback and Podack, 1985).

When vitronectin binds to the nascent $C5_b$-7 complex this cannot be inserted into the cell membrane. Membrane insertion of the complex is necessary for the inclusion of C8 into the complex which in turn promotes the insertion of C9 in the membrane and its polymerization, which leads to

membrane lesion (Podack and Muller-Eberhard, 1979; Preissner et al., 1985; Jenne and Stanley, 1985). When bound to an already formed $C5_b$-C9 complex, vitronectin also inhibits C9 polymerization and the dissociation of vitronectin from the lytic complex results in restoration of the lytic activity (Preissner et al., 1985). Vitronectin could represent a very effective protection for bystander cells from complement-mediated lysis and in fact its low constant of inhibition for the $C5_b$-7 complex (K_i= 0.55 uM) compares favourably with the concentration of vitronectin in plasma (~ 5 uM) (Dahlback and Podack, 1985; Preissner et al., 1985).

Von Willebrand factor

Von Willebrand factor is a large circulating, cell stored and matrix bound glycoprotein produced exclusively by endothelial cells and megakaryocytes (Hoyer, 1981; Reinders et al., 1985). It is composed of two apparently identical subunits assembled into a homodimer which can polymerize to form multimers containing up to 40 dimers or more. Von Willebrand factor exists therefore as an array of molecules of increasing M_r ranging from the 450,000 of the basic dimer to $2x10^6$ or more of the largest multimers (Wagner and Marder, 1984; Sporn et al., 1987).

Two important functions in the maintenance of haemostasis seem to be related to von Willebrand factor. The first concerns its association with the antihaemophilic factor VIII:C. Von Willebrand factor serves as a plasma carrier of VIII:C, to which it is non covalently bound and acts to significantly increases its circulatory half life (Ginsburg et al., 1985; Lynch et al., 1985). Such a circulating assembly is called FVIII complex (Hoyer, 1981). The other function is related to platelet adhesion and fibrin deposition during thrombus formation. Thus, von Willebrand factor is secreted from endothelial and platelet granules to promote interactions between the activated platelets and the damaged endothelium or the

exposed sub-endothelial matrix (Bockenstedt et al., 1986; Hada et al., 1986; Sporn et al., 1987).

The concentration of von Willebrand factor in human plasma is estimated to be 5-10 ug ml^{-1}, which is approximately 100 times greater than that of FVIII:C (Hoyer, 1981).

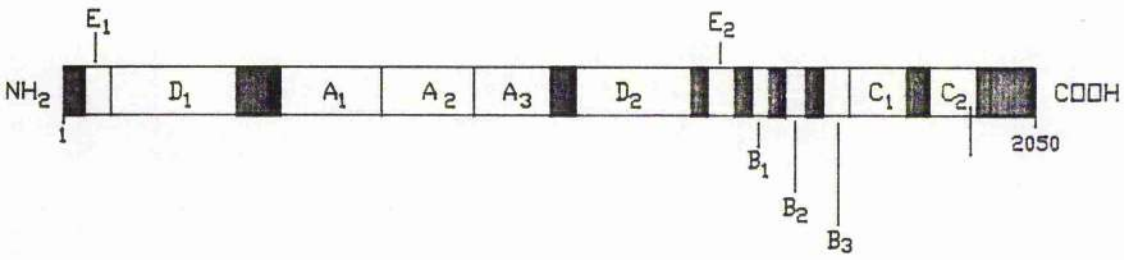


Figure 12: Distribution of the repeating sequences along a subunit of von Willebrand factor.

The mature von Willebrand factor subunit has a M_r of approximately 270,000 , as calculated from its primary structure, 18.7 % of which is carbohydrate side chains (Titani et al., 1986). The polypeptide backbone is made of 2050 amino acid residues, 75 % of which are arranged in repeating sequences, implying a complex evolutionary history of its gene with internal duplication, in a manner reminiscent of other characterized molecules involved in cell-matrix interactions. There are five types of repeating sequences, named A, B, C, D and E (Sadler et al., 1985; Shelton-Inloes et al., 1986), and their distribution along the subunit is shown in figure 12.

Von Willebrand factor is synthesized as pro-subunits containing the 2050 amino acid residues of the mature subunit, together with a NH_2 terminal sequence of approximately 700 amino acid residues known as pro-polypeptide (Titani et al., 1986). The pro-polypeptide, which is cleaved before secretion, is thought to encourage multimer formation (Sporn et

al., 1986; Wagner and Marder, 1984; Wagner et al., 1987). Von Willebrand factor is very rich in cysteine, with 169 half cysteine residues, all of which are engaged in inter- or intra-chain disulfide bonds (Titani et al., 1986).

In endothelial cells, after passing through the Golgi apparatus, von Willebrand factor is secreted, principally as a dimer (60 % of all von Willebrand factor secreted) or low molecular weight multimer (Sporn et al., 1986). A small proportion, estimated to be less than 10 %, is stored in specific structures called Weiber-Palade bodies (Sporn et al., 1986). Far less von Willebrand factor is produced in megakaryocytes. This is thought to be almost exclusively for storage and is found in the alpha-granules (Ginsburg et al., 1985).

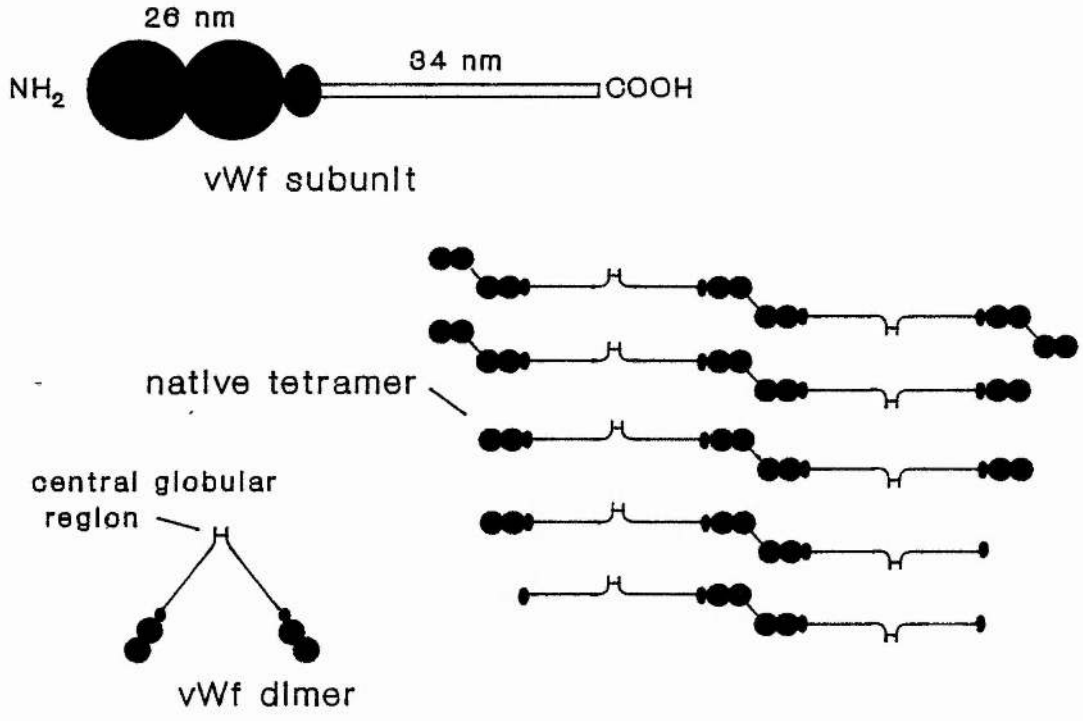


Figure 13: Subunit, dimer and different terminations of the 5 isomers of each multimer of von Willebrand factor (see text).

Although in megakaryocytes von Willebrand factor is stored together

with many other cell products, including fibronectin, thrombospondin and thrombin (Reinders et al., 1985), in endothelial cells there seems to be a specific mechanism for separating von Willebrand factor from other synthesized molecules. Weiber-Palade bodies contain only von Willebrand factor and von Willebrand antigen-II (the pro-polypeptide which, once cleaved from the rest of the molecule, has a M_r= 92,000-98,000 and is also present in plasma). This mechanism is very sensitive to slight shifts of the pH of culture medium (Reinders et al., 1985; Sporn et al., 1986; Wagner et al., 1987; Shelton-Inloes et al., 1986). Multimerization continues even after storage and in fact the stored material is rich in high molecular weight multimers (Sporn et al., 1986; Wagner et al., 1987).

Weiber-Palade bodies are specific granules of endothelial cells (Reinders et al., 1985). They are bound by a unit membrane and appear as rod shaped organelles of about 2 um in length and 0.5-1 um in width, of variable electron density, often clustered at the periphery of the cytoplasm and occasionally associated with the Golgi region from which they seem to originate (Sporn et al., 1986). Within them, Weiber-Palade bodies contain an average of 10-20 tubules 150 A in width, which are likely to be von Willebrand factor molecules in a highly condensed form. Indeed, they are similar to tubular structures present in certain regions of platelet alpha-granules known to contain von Willebrand factor (Sporn et al., 1986).

Analysis of quasi-elastic light scattering of von Willebrand factor in solution and of electron micrographs of substratum adsorbed von Willebrand factor indicate that the predominant native structure is that of a flexible polymer coiled upon itself. The shape of the coiled polymer is usually oval for the loosely coiled ones, with the long diameter measuring an average 200-300 nm. The more extensively coiled polymers have a shape that tends towards the sphere, with a somewhat smaller diameter.

Non-coiled von Willebrand factor seems to represent less than 15 % of the total and it is predominantly of low molecular weight (Fowler et al., 1985; Slayter et al., 1985).

Von Willebrand factor polymers are a linear assembly (no branching has ever been observed) of a repeating unit called a protomer. A protomer represents one dimer and its structure is outlined in figure 13 (Fowler et al., 1985). Polymers are end to end assemblies of protomers and there seems to be heterogeneity in the way in which they terminate. The extreme flexibility of the molecule at its COOH terminus allows the dimer the wide range of movements that are necessary for coiling of the polymer (Fowler et al., 1985; Slayter et al., 1985).

Electrophoretic separation of von Willebrand factor shows that each multiple of the simple unit (dimer) can be resolved into up to 5 different bands, separated by an overall difference in electrophoretic mobility corresponding to a M_r of approximately 150,000 (Fowley et al., 1985; Zimmerman et al., 1984). In fact multimers composed of the same numbers of protomers (isomers) but having different end morphology could account for the heterogeneity of molecular weight of each isomeric cluster (Fowler et al., 1985), as shown in figure 13.

The binding of von Willebrand factor to platelets occurs via two independent receptors on the platelet membrane designated glycoprotein Ib (gpIb) and glycoprotein IIb/IIIa complex (gpIIb/IIIa) on the basis of their electrophoretic mobility. Von Willebrand factor has a binding site for gpIb localized in a M_r 50,000 tryptic fragment, part of the large globular region. The binding of von Willebrand factor to gpIb is Ca^2 -independent (Shelton-Inloes et al., 1986; Houdijk et al., 1986). The RGDS sequence in von Willebrand factor is localized very near to or within the central globular domain. This sequence, being part of a very hydrophilic region, is most likely to be localized on the outer surface of the coiled molecule,

thus needing no activation step prior to binding (Shelton-Inloes et al., 1986; Titani et al., 1986) Von Willebrand factor also possess a LDV sequence (Humphries et al., 1990).

Although von Willebrand factor has the potential to bind to gpIIb/IIIa of activated platelets in vivo, the role that soluble von Willebrand factor secreted by endothelial cells plays in platelet aggregation (gpIIb/IIIa is responsible for this phenomenon) is likely to be very marginal if not irrelevant. In fact, all the molecules that bind to gpIIb/IIIa can displace each other in a competitive manner, all binding via the RGD sequence (Pytela et al., 1986). Because of the overwhelming molar excess of fibrinogen in plasma the binding of soluble von Willebrand factor to gpIIb/IIIa is minimal, if it occurs at all (Parker and Gralnick, 1986). Nevertheless von Willebrand factor released from platelet alpha-granules does bind to the platelet surface complex gpIIb/IIIa (Parker and Gralnick, 1986), probably because of the high concentration of von Willebrand factor localized at the platelet surface during degranulation. Similarly, the binding of Weiber-Palade body-derived von Willebrand factor to the surface of the endothelial cells, which express gpIIb/IIIa complex upon stimulation, probably happens because of the transient high concentration of von Willebrand factor near the cell membrane during secretion. The endothelium releases the content of Weiber-Palade bodies both towards the luminal side and into the sub-endothelial matrix, and a great part of released von Willebrand factor remains bound to the cells or to the collagenous matrix (Sporn et al., 1986; Sporn et al., 1987).

Collagen is indeed likely to be the main acceptor of von Willebrand factor in the basement membrane (Sadler et al., 1985). The collagen binding site in von Willebrand factor resides in a M_r 48,000 tryptic digest of the large globular region (Houdijk et al., 1986; Shelton-Inloes et al., 1986). Von Willebrand factor binds to collagen with high affinity and it is

not displaced by either fibronectin or fibrinogen. Although the binding to fibrillar collagen is Ca^{2+}-independent, the crosslinking that follows requires Ca^{2+} (Bockenstedt et al., 1986).

Unlike fibronectin, which actually has an increased affinity for denatured collagen, von Willebrand factor does not bind to gelatin (Bockenstedt et al., 1986). Von Willebrand factor is a substratum for a transglutaminase reaction catalysed by factor XIIIa and can be covalently crosslinked to the fibrin alpha-chain (Hada et al., 1986). To date there are no reports on the role of von Willebrand factor during the arrest of circulating cells, be it leukocytes/lymphocytes or metastasizing tumour cells.

Integrins

The interactions of cells with the extracellular matrix are mediated by a variety of cell surface molecules. Some of these are glycosaminoglycans, fibronectins and perhaps also other structural glycoproteins which may have an additional transmembrane domain or be bound to phospholipids and gangliosides of the cell membrane and that could provide a link with the extracellular environment because of their ability to bind a number of different ligands. Cells also possess an array of receptors of more conventional type which have a ligand binding site in their extracellular portion, a transmembrane domain and an intracellular part which may be linked with the cytoskeleton and mediate signals from the extracellular environment.

Fibrinogen, laminin and collagen receptors have been identified in the past. These are small molecules with M_r 30,000-80,000 and are unrelated to each other (von der Mark et al., 1984; von der Mark and Kuhl, 1985; Buck and Horwitz, 1987; Levesque et al., 1990) but, more recently, investigators from two separate fields (cell biology and immunology) have

independently uncovered the existence of a number of related receptors for the extracellular environment. In view of the structural and functional similarities shared by these molecules, it is believed that they are all members of a superfamily of cell surface receptors which have been termed "integrins".

In evolutionary terms the integrin family is very ancient, in fact in Drosophila there are homologous structures implicated in development called position-specific (PS) antigens (Kishimoto et al., 1989). The basic structure of integrins is an alpha-beta heterodimer, non covalently linked, with M_r usually approximately 250,000. The beta subunit has a high cysteine content and is composed of an extracellular domain, a transmembrane domain and a cytoplasmic domain; overall there is between 35 and 45 % aminoacid identity between beta subunits, with particularly high conservation in the transmembrane and cytoplasmic domains (Kishimoto et al., 1989). The alpha subunits are integral membrane proteins with a long extracellular domain, a hydrophobic transmembrane domain and a short cytoplasmic region; the polypeptide backbones are all of similar size and the differences in M_r could be due to the number of potential glycosylation sites; each alpha subunit contains three putative metal binding regions which are highly conserved. There is strong homology (>47 %) among the $alpha_L$, $alpha_M$ and $alpha_X$ and among the $alpha_V$, $alpha_{IIb}$ and $alpha_{1-7}$, but the homology drops to 27 % between these two groups. The alpha-beta heterodimer has a talin binding site in its cytoplasmic tail (Buck and Horwitz, 1987).

Probably the first of such molecules ever identified is the platelet membrane glycoprotein IIb/IIIa, whose discovery dates from the mid 1970' despites the fact that its functional identity was unveiled much later, but the work that lead to the present body of knowledge about integrins derived from a new approach used to search, in the early 1980', for the

cell receptor for fibronectin. The fibronectin molecule was digested into different fragments using a series of proteases and, after a small cell binding portion was identified and mapped, short synthetic peptides of this fragment were studied and it was found that cells specifically recognize and bind to a tripeptide sequence of the fibronectin molecule: arginine-glycine-aspartic acid (arg-gly-asp; RGD - Pierschbacher and Ruoslahti, 1984). Using affinity chromatography on immobilized cell binding fragments of fibronectin, a fibronectin receptor was isolated from cell membrane extracts (Pytela et al., 1985).

RGD	LDV
Fibronectin	Fibronectin
Thrombospondin	Thrombospondin
Nidogen[1]	Nidogen
von Willebrand factor	von Willebrand factor
Fibrinogen	Fibrinogen
Osteopontin	N-CAM
HLA-B associated (BAT-2)[2]	L-CAM
Vitronectin	C3
Laminin	Collagen $alpha_1$(I)
Tenascin	Collagen $alpha_1$(III)
Endoglin[3]	Collagen $alpha_1$(IV)
Collagen IV	Collagen $alpha_1$(XI)
BSP-2[4]	
VP-1 (foot and mouth disease virus)[5]	
Trigramin (snake venom)[6]	
TP (bacteriophages phi29 and M2)[7]	
Filamentous hemagglutinin (B. pertussis)[8]	

Table 1: adhesive molecules containing an RGD or LDV sequence. From : Humphries et al., 1990. Additional references: 1 Nagayoshi et al., 1989; 2 Banerji et al., 1990; 3 Gougos and Letarte, 1990; 4 Ruoslahti, 1988; 5 Fox et al., 1989; 6 Huang et al., 1989; 7 Kobayashi et al., 1990; 8 Relman et al., 1989.

The notion that three aminoacids were all that was needed for cells to specifically adhere to fibronectin was innovative and, searching through the data bank of already sequenced proteins, it was found that many molecules contained the adhesive sequence RGD in a position accessible for interactions with a receptor (a list of adhesive molecules containing this

sequence is shown in table 1). More RGD-binding molecules were being found, all quite similar to each other but many showing preferential binding to one or more RGD-containing ligands (Pytela et al., 1985b; Pytela et al., 1986; Pytela et al., 1987; Buck and Horwitz, 1987.). The emerging consensus was that RGD functions as specific "glue" while the adjacent aminoacids conferred the ligand specificity and the integrins molecules were termed RGD-binding proteins. Only later it became apparent that RGD is not the only sequence that can act as glue, since some of the integrins ligands like the ICAMs do not have this sequence (Warwryk et al., 1989) and that LDV and REDV are two other short sequences that can be recognized by some integrins (Humphries et al., 1990; Garcia-Pardo et al., 1990). In table 1 there is a list of some of the LDV-containing molecules implicated in cell adhesion; it is probable that other recognition and glueing sequences will be identified in the future.

To date 12 alpha and 6 beta subunits have been identified and so far the integrin superfamily consists of 18 different receptors, although it is very likely that these numbers will be much larger in a not too distant future. According to the current classification, integrins can be separated into six families, each with a common beta subunit, as shown in table 2. The $beta_1$ molecules, also known as the VLA (very late antigen) group, mediate cells to extracellular matrix adhesion. The $beta_2$ group molecules are also known as the leucocyte integrins or the CD18 cluster; these integrins mediate cell-cell interactions and are important in leukocyte function. The third family ($beta_3$) comprises of the vitronectin receptor and the platelet gpIIb/IIIa, both RGD ligands; a shorter $beta_3$ subunit has been recently demonstrated (Krissansen et al., 1990) and therefore this family has been divided into $beta_{3a}$ and $beta_{3b}$ subgroups. The fourth type of beta subunit ($beta_4$) is an unusual beta chain with a much higher M_r (400,000) and a much larger cytoplasmic domain than those of other

beta subunits; this beta chain has 4 type III repeats homologous to those of fibronectin and is linked to an $alpha_6$ chain to function as a laminin receptor (Suzuki and Naitoh, 1990). The fifth beta subunit ($beta_p$) is engaged with an $alpha_4$ subunit to form one of the two lymphocyte-Payer's patch adhesion molecules (LPAM-1) involved in cell adhesion to the postcapillary venules of Payer's patches; LPAM-2 is a member of the first family of integrins ($beta_1$ $alpha_4$). Lastly a $beta_5$ subunit, linked to an $alpha_v$ chain, forms the only member of the sixth group.

subunits $M_r \times 10^{-3}$		ligands recognition sequence	other names
β_1 130	$alpha_1$ 180	Co_I,Co_{IV},Lm	VLA-1
β_1 130	$alpha_2$	Co_I,Co_{II}[1],Co_{III}[1],Co_{IV},Co_{VI}[1],Ge[1],Lm,?Fn	VLA-2, gpIa-IIa
β_1 130	$alpha_3$	Co_I,Lm,Fn,Iv[2]	VLA-3
β_1 130	$alpha_4$ 150	Fn_{alt},VCAM-1[3] LDV or REDV	VLA-4, LPAM-2[4]
β_1 130	$alpha_5$ 140	Fn,Iv[2] RGD	VLA-5, FnR, gpIc-IIa
β_1 130	$alpha_6$ 140	Lm,Iv[2]	VLA-6, gpIc'-IIa
β_1 130	$alpha_7$ 200	Lm_{neu}[5]	
β_1 130	$alpha_V$ 150	Vn[6],Fn RGD	
β_1 130	$alpha_L$ 180	?	
β_2 95	$alpha_L$ 180	ICAM-1,ICAM-2	CD_{11a}/CD_{18},LFA-1
β_2 95	$alpha_M$ 170	F_X,$C3_{bi}$,Fb	CD_{11b}/CD_{18},Mac-1,Mo-1,OKM-1,CR3
β_2 95	$alpha_X$ 150	?	CD_{11c}/CD_{18},p150/95,CR4,LeuM5
β_{3a} 120	$alpha_V$ 150	Vn,Fb,Op/BSP-1,vWf,TSP[7] RGD	VnR
β_{3a} 120	$alpha_{IIb}$ 115	Fb,Fn,Vn,vWf,(TSP[7]) RGD	gpIIb/IIIa
β_{3b} 100	$alpha_V$[8] 150	?	(? β_5-alphaV)
β_4 400	$alpha_6$ 140	Lm	
β_p 130	$alpha_4$ 160	PP-PCVE	LPAM-1[4]
β_5	$alpha_V$[9] 150	Vn,Fn RGD	(? β_{3b}-alphaV)

Integrins not as yet fully characterized: SQM1[10], LRI[11], Gap b3[12].

Table 3: the integrin superfamily.

Abbreviations: BSP= bone sialoprotein; Co= collagen; CR3= complement receptor for C3; $C3_{bi}$= complement component 3_{bi}; Fb= fibrin(ogen); Fn= fibronectin; Fn_{alt}= Fn alternatively spliced; F_X= coagulation factor X; Ge= gelatin ICAM= intercellular adhesion molecule; Iv= invasin (Yersinia pseudotuberculosis); LFA= lymphocyte function associated; Lm= laminin; Lm_{neu}= neuron b.s. of laminin; LPAM= lymphocyte-Payer's patch adhesion molecule; Op= osteopontin; PP-PCVE= post capillary venule endothelium of Payer's patches; TSP= thrombospondin; VCAM= vascular cell adhesion molecule; VLA= very late antigen; Vn= vitronectin; VnR= vitronectin receptor; vWf= von Willefrand factor;

From :Ruoslahti and Giancotti, 1989; Kishimoto et al., 1989; Sonnenberg et al., 1990. Additional references: 1 Staatz et al., 1990; 2 Isberg and Leong, 1990; 3 Elices et al., 1990; 4 Holzmann and Weissman, 1989; 5 Ignatius and Reichardt, 1988; 6 Bodary and McLean, 1990; 7 Lawler and Hynes, 1989; 8 Krissansen et al., 1990; 9 Freed et al., 1990; 10 Wong et al., 1990; 11 Gresham et al., 1989; 12 Tsuji et al., 1990.

The extracellular matrix is thus an environment very rich in adhesive stimuli. However, most of the molecular components of the subendothelial matrix so far described can also be found on the surface of the endothelium and on other cells. Many molecules interact directly with the cell surface and, in fact, it is at times very difficult to understand whether these are part of the extracellular matrix or of the cell surface. To add to this, the same type of molecule can either form fibrils (the scaffolding of the matrix) or be an integral component of the plasma membrane. All these molecules are able to interact with each other in many different combinations and, when present on the cell surface, enhance the adhesive potential of the cell. Additionally, it is possible to envisage how each molecule present in the extracellular matrix may have a number of different sites specific for different cell surface receptors (integrins or non integrins) and mediating different and independent functions and messages. However, in spite of the fact that there are no distinct boundaries between cells and extracellular matrices and that the environment is full of an array of multivalent molecules, cell adhesion is a well disciplined phenomenon of fundamental importance. Future research should focus on understanding which of these interactions are of physiological importance and how they are regulated.

The B16F10 cell line

To investigate the process of metastasis various model systems have been developed in the past. Before the introduction of cell culture techniques, most of the work on metastasis was done using transplantable tumours. In those days there was little awareness of the immunological problems associated with non-syngeneic transplantation and indeed many experiments were based on models using human tumours in experimental animals. Additionally, some systems were based on chemically-induced tumours and the data obtained from them had a great deal of inherent variation.

It was only with the introduction of cell culture techniques and the understanding of the mechanisms underlying the rejection of transplants, that better model systems were developed. The B16 malignant melanoma is probably the most widely used model for investigating metastasis. The cell lines used in this model are derived from a transplantable malignant melanoma that arose spontaneously in a mouse of the C57Bl6/J strain in 1954 (Green, 1968). This tumour, called B16 melanoma, was later adapted by Fidler (1970) to in vitro growth.

Subsequently Fidler (1973) selected from the B16 population through repeated in vivo passages in syngeneic mice a series of sublines with differing abilities to form lung colonies when injected as single cell suspensions into the tail vein. From this original work two cell lines emerged which were used in a large number of studies. They were called B16F1 and B16F10 because they were obtained after 1 and 10 in vivo passages respectively (for review see Nicolson, 1982).

Although the B16F1 and B16F10 are established lines that were extensively manipulated during the selection procedure, they are widely used for their ability to preferentially colonize tissues according to determined patterns when injected intravenously into syngeneic mice. The cell lines have therefore been used as a tool to study the spread of metastases via the circulatory system.

In this respect many investigators have given the impression that the use of intravenous injections of B16 cells in syngeneic mice represents a model for metastatic spread via the blood (Fidler, 1975; Winkelhake and Nicolson, 1976; Rieber and Castillo, 1984; Poste and Nicolson, 1980; Hart et al., 1980; Liotta et al., 1980; Kimura and Xiang, 1986; Kawano et al., 1986; Miner et al., 1982; Poste et al., 1982). In reality this is merely an assay for colonization, since, during metastasis formation, tumour cells must somehow gain access to the circulation, while this important phase of the process is bypassed in the B16 model. Furthermore it is unlikely that a tumour suddenly releases a large number of single cells directly into the circulation (the standard inoculum consists of 10^5 cells), not to mention the artefactual systemic and localized effects of the significant haemodilution that arises from the sudden introduction of a volume of fluid corresponding to ~ $^1/_{10}$ to $^1/_{20}$ of the mouse blood volume. Stackpole (1981), in a carefully conducted study, demonstrated that the B16 population contains distinct metastasizing and colonizing phenotypes and suggested that within the sublines selected in vivo for lung colony formation (like the B16F10) there may be absence of the metastasizing phenotype.

When introduced directly into the blood stream as single cell suspensions, either by injection into the left ventricle or into the tail vein, the B16F10 has a high colonizing efficiency for the lungs, but rarely produces extrapulmonary foci. The B16F1 on the other hand, although

producing fewer lung colonies, disseminate more easily to various other sites such as the brain, the ovaries, the liver, the digestive system and the subcutaneous tissue (Fidler and Nicolson, 1976; D'Arrigo et al., 1985). Because the B16F1 cells, when injected into the systemic circulation, exhibit a propensity to establish foci of growth in many tissues, various authors have subsequently selected from their population a series of different sublines having enhanced ability to give a certain pattern of colonization. The selection procedures were in most cases adaptations of the original strategy used by Fidler (1973).

Sublines with enhanced colonizing ability to the liver (B16L - Tao et al, 1979), the ovaries (B16O10 - Brunson and Nicolson, 1979), the brain (B16B14b - Miner et al, 1982), as well as sublines with increased ability to penetrate some structures such as the bladder wall (B16BL6 - Poste et al., 1980) or blood vessel wall (B16BV8 - Poste et al., 1980) are examples of some of the sublines selected.

It has been shown that these colonization patterns do not merely reflect the adaptation of some tumour cells to grow in specific environments but are rather the result of the selection imposed by the entire colonization process, which involves survival, arrest, invasion and growth at discrete sites (Nicolson, 1982). In fact attempts to obtain a B16F10-like subline from the parent B16 cells using an injection protocol that allows non-discriminating mechanical lodging in the lung circulation (as is the case in the work of Nicolson and Custead, 1982, who injected cells attached to microcarrier beads of 180 um of diameter) resulted in failure to select a subline with a high lung colonizing potential.

Within the B16F10 subline, as well as other sublines selected from the original B16 line, there is evident heterogeneity, not only for biochemical markers such as melanin content, but also for colonizing potentials and malignant phenotype (Elvin and Evans, 1985; Tao and Burger, 1977;

Stackpole, 1981). Even within an individual lung colony cells with different colonizing ability exist, although the clonal diversity found in each colony is substantially less than that found within the subline. This would suggest that each lung colony originates from a single cell and, in fact, Poste and co-workers (1982), using cells bearing resistance to various drugs, found that the majority of lung colonies (> 90 %) were monoclonal in origin.

After intravenous injection, B16F10 cells that colonize the lungs extravasate and are found growing within the parenchyma of this organ. This is in contrast with their colonizing habits in other sites. If these cells are injected in the portal vein a widespread colonization of the liver results but the colonies are rather the consequence of intravascular growth and tumour cells do not migrate through the endothelium, at least not within the first few days (Nicolson, 1982).

When cloned, tumour cells from either the original B16 line or from any of the selected sublines may be relatively stable in short term cultures but with time they diverge to reform a heterogeneous population. Within the population there seem to be mechanisms to regulate the growth of each different phenotype, as was shown by Miner and co-workers (1982) who found that three clones with brain colonizing ability were unstable following in vitro culture, losing their ability to colonize the brain, while retaining this colonizing property when co-cultured.

That the reason for the B16F10 lung colonizing habits resides on the surface of these cells can be shown by transferring fragments of cell membrane from B16F10 cells to a subline with lower incidence of lung colonies, such as the B16F1. This can be done by fusing purified vesicles shed by B16F10 cells (shedding occurs spontaneously both in vivo and in vitro) to B16F1 cells, as done by Poste and Nicolson (1980), who showed that the lung colonies formed by B16F1 cells with integrated fragments of

B16F10 membrane were significantly increased. Such an effect is nevertheless transient and correlates with the natural turnover of membrane components.

Fidler (1975) showed that the adhesion to host lymphocytes was more pronounced within the B16F10 population than within the B16F1. When the B16F10 cells were selected for survival against lymphocyte killing, he was able to obtain a subline, called B16Lr6, which was no longer susceptible to lymphocyte-mediated cytotoxicity (Fidler et al., 1976). The cells of this line exhibited reduced lymphocyte binding and limited ability to form lung colonies.

Cells from B16 sublines also show an increased propensity to establish heterotypic adhesion with cell suspensions from the preferred organs of colonization and in some cases it has been possible to demonstrate enhanced adhesion to endothelial monolayers derived from target organs (Nicolson, 1982; Roos, 1984). Investigations of the specific density of the cells forming the populations of various B16 sublines, as determined by isopycnic density gradients of colloidal silica (Percoll), indicate that a correlation exists between lung colonizing ability and cell density. B16F1 cells have a higher mean density than the B16F10 and the population of the original B16 line shows a broad distribution of density, with cells from low density fractions exhibiting a higher ability to form lung colonies than cells from the higher density fractions (Baniyash et al., 1981).

B16F10 cells produce a variety of enzymes that can degrade components of the sub-endothelial matrix. Matrix-bound fibronectin and perhaps also laminin are dissociated from the matrix by means of proteolytic activity by these cells, and heparan sulphate and other sulfated glycosaminoglycans are solubilized in greater quantities by B16F10 cells than by B16F1 cells (Nicolson, 1982).

B16F10 cells produce and release several lysosomal enzymes such as

acid phosphatase, many glycosidases, cathepsin D and cathepsin B. Cathepsin B is present in quantities three to four times higher in the B16F10 than in the B16F1 cells and its secretion has been shown to be increased in malignant neoplasms of the breast when compared to benign tumours or normal breast tissue (Nicolson, 1982). Cathepsin B seems to activate procollagenases and helps in the degradation of proteoglycans. The B16F10 cell population is also a source of collagenases against various types of collagens, of endoglycosidases, of plasminogen activator, a serine protease that converts plasminogen into plasmin, as well as of an inhibitor of such an activator (Liotta et al., 1980; Hart, 1979; Nicolson, 1982).

Prostaglandins are produced in different amounts by different sublines: PgD_2 is produced more abundantly by B16F1 cells than by B16F10 which also produce PgE_2 and PgF_{2a} (Fitzpatrick and Stringfellow, 1979). Production of PgD_2 has been implicated in influencing the colonizing phenotype because of its anti-thrombogenic action (Stringfellow and Fitzpatrick, 1979). It has been suggested that platelet aggregation and thrombus formation to cover the tumour cells which have arrested in the pulmonary circulation is important in the establishment of a focus of growth (Stringfellow and Fitzpatrick, 1979).

Macromolecules such as fibronectin and thrombospondin are produced and promptly released by these cells. It has been calculated that within 48 hours cultured B16 cells secrete ~ 36% of their newly synthesized macromolecules and shed ~ 44% of tumour associated antigens (Nicolson, 1982). The rapid release of synthesized material, especially macromolecules, is not peculiar to tumour cells but seems to be also a characteristic of normal cells.

Concluding remarks

The arrest phase at the metastatic site is probably one of the most critical steps of the spread of tumours via the blood stream. The mechanism of arrest is not known. The circulating tumour cell could form a thrombus with platelets or an embolus with other tumour cells and arrest simply by lodging in a smaller diameter vessel. On the other hand the phase of arrest could rely on specific tumour cell-blood vessel wall interactions with an underlying mechanism similar to the one involved in lymphocyte recirculation (Bjerknes et al., 1986). This mode of arrest, as opposed to mechanical lodging, would rely on ligand-receptor interactions and can therefore be called specific arrest. If specific arrest does occur, tumour cells must possess a machinery that enable them to adhere to the surface of the vessel wall in a very short time and with enough strength to resist detachment by the flowing blood.

This study was an attempt to establish whether tumour cells have adhesive properties of the kind required for specific arrest. In order to establish this it was decided to use an in vitro assay to assess rapid adhesion. It would be impossible to recreate with any accuracy in an in vitro assay the hydrodynamic forces and the environment that tumour cells encounter during the arrest phase. In this sense, any in vitro assay to investigate this phenomenon has the limitation that some of the parameters of the assay may not compare closely enough to the in vivo situation. Since the assessment of adhesion is strongly dependent on factors like the timescale available to the cells to establish contact with the substratum or the quality and quantity of the forces that act towards

the disruption of adhesion, in vitro assays will always represent an approximate assessment of the phenomenon. On the other hand, in vitro work allows freedom to interfere with the system and manipulate it under carefully controlled conditions, and the generation of interpretable data represent at this stage a reasonable compromise.

The second purpose of this study was to assess the suitability of various components of the blood vessel wall to promote the adhesion of tumour cells. A third aim was to introduce into the assay system factors that would enhance or diminish the adhesion of tumour cells and study their effects on various substrata in order to establish whether the adhesion of tumour cells to the endothelium can be attributed to any of the adhesive molecules produced by the endothelial cells.

Since the in vitro adhesion assay gives indications only on interactions that occur in the first few minutes of the process, this study utilized also a second in vitro assay to monitor the long term fate of the adhesive interactions. This second assay assessed the ability of tumour cells to flatten and spread on various substrata. Spreading results in an increase of the number of adhesion points that a cell establish with the substratum (or in an increase in the area in which these points are contained) and this may be accompanied by secretion of extracellular material and enzymatic modification of the substratum. The suitability of a substratum for such activity may be relevant to the phenomenon of specific arrest.

The final part of this study was the extraction, partial purification and characterization of an endothelial cell-derived adhesion factor. The adhesive properties of the extracted material was studied and compared with other adhesive substrata using in vitro adhesion and spreading assays.

The cells used in this work were the F1, F10 and BL6 variants of the B16 mouse malignant melanoma and the BAE cells (bovine aortic endothelial

cell). The use of BAE cells gave us the convenience of working with continuous cell lines, avoiding the need to establish primary cultures periodically. There are some inherent complications associated with the use of primary cultures, such as less reproducible results because of inevitable differences between batches, the increase in the risk of occult infections that would render the interpretation of the results very difficult and the increased work load. The tumour cells lines were chosen because they were known to possess suitable characteristics for use in our assay systems. In fact these cells are capable of arresting in the microcirculation when injected in vivo. Moreover single cell suspensions can be prepared without resorting to the use of enzymes which could denude the cell surface of important molecules involved in the binding to the endothelium. Above all our laboratory has years of experience in the manipulation and behaviour of all these cell lines.

Materials and methods

Plastic and glassware

Tissue culture grade plastic Petri dishes of 3 cm (cat.no. 1-53066), 6 cm (cat.no. 1-50288A), 9 cm (cat.no. 1-50350A) and 14 cm in diameter (cat.no. 1-68381A), tissue culture grade plastic multidish clusters with 24 wells (cat.no. 1-46485A) and tissue culture grade plastic microdish plates with 72 wells and Terasaki configuration (cat.no. 1-36528A) were all Nunc products and purchased from Gibco.

Two litre borosilicate glass roller bottles with a surface area of 750 cm^2 (Wheaton, USA) were obtained from Flow Laboratories (cat.no. 60-210-00).

Screw cap 1.8 ml polypropylene tubes for storage of cells in liquid nitrogen were purchased from Gibco (cat.no. 3-63401A). Sterile centrifuge tubes with a conical base, screw cap and a capacity of 10 ml (polystyrene, cat.no. 144AS), 25 ml (polystyrene, cat.no. 128A) and 50 ml (polypropylene, cat.no. 36050) were purchased from Sterilin.

Polypropylene microcentrifuge tubes with integral caps and a capacity of 1.5 ml (cat.no. LW 2075) and 0.5 ml (cat.no. LW 2072) were purchased from Alpha Laboratories, UK.

Sterile polypropylene 2 ml microtubes with a conical bottom, flat base and screw cap with O-ring were purchased from Sarstedt, West Germany (cat.no. 72.694.006).

Sterile flat bottom polystyrene tubes with screw cap and a capacity of 5 ml (cat.no. Z6744) and 10 ml (cat.no. Z9944) were purchased from Teklab Medical Laboratories, UK.

General procedures and chemicals

Sterilization by filtration was achieved in a system pressurized with inert gas (nitrogen) using durapore (polyvinylidene difluoride) membrane filters (Millipore Co.) with pores of 0.22 um in diameter. Glassware was dry heat sterilized in an oven at 250° C for 4 hours. Glass bottles, filter membranes and pipette tips were sterilized in an autoclave at 121° C for 40 minutes.

All chemicals were of analytical grade, unless otherwise stated, and were obtained from BDH or Sigma Chemicals. The water used throughout this study was glass double distilled from deionized tap water and sterilized by filtration.

Minimum Essential Medium (MEM) of Eagle (modified), with Earle's salts was purchased from Flow Laboratories (cat.no. 10-101-24) as premixed powder. It was reconstituted with the appropriate volume of distilled water, supplemented with 2 g/l $NaHCO_3$ and buffered with CO_2 to pH 7.2. It was then sterilized by filtration and stored in 100 ml aliquots at 4° C. Each batch of medium was tested for sterility and used within 60 days.

Phosphate buffered saline (PBS) and Ca^{2+} and Mg^{2+}-free PBS (D^{-}) were prepared according to Dulbecco's formulae (see Flow Laboratories, 1987 catalogue, page 57) to a pH of 7.2, sterilized by filtration, stored in 100 ml aliquots at 4° C and used within 60 days.

A 100 times concentrated solution of non-essential amino acids for Eagle's MEM of was purchased from Flow Laboratories (cat.no. 16-810-49), stored in 1 ml aliquots in 5 ml Teklab tubes at 4° C and used within 60 days.

L-glutamine was purchased from Flow Laboratories (cat.no. 15-801-15), dissolved in PBS to a concentration of 200 mM, filter sterilized, stored in 1 ml aliquots in 5 ml Teklab tubes at -20° C, and used within 3 months.

Hepes was purchased from Sigma Chemicals as free acid (cat.no. H 3375) and as the sodium salt (cat.no. H 7006). A 1 M solution with a pH of 7.4 at 37° C was prepared, sterilized by filtration, stored in 1 ml aliquots in 5 ml Teklab tubes at 4° C, and used within 6 months.

Insulin was purchased from Sigma Chemicals (cat.no. I 5500), prepared to a concentration of 12.5 IU ml^{-1} in PBS, sterilized by filtration, stored in 5 ml Teklab tubes in 1 ml aliquots at 4° C, and used within 60 days.

A 100 mM solution of Na-pyruvate was purchased from Flow Laboratories (cat.no. 16-820-49), stored in 1 ml aliquots in 5 ml Teklab tubes at -20° C, and used within 3 months.

A 100 times concentrated solution of vitamins for MEM of Eagle was purchased from Flow Laboratories (cat.no. 16-014-49), stored in 1 ml aliquots in 5 ml Teklab tubes at -20° C, and used within 3 months.

Benzylpenicillin-Na BP (Glaxo, UK) and streptomycin sulfate BP (Evans, UK) were dissolved together in PBS at a concentration of 5000 IU ml^{-1} and 5 mg ml^{-1} respectively, sterilized by filtration, stored in 1 ml aliquots in 5 ml Teklab tubes at -20° C, and used within 3 months.

Endothelial cell growth supplement was purchased from Sigma Chemicals (cat.no. E 2759), dissolved in PBS at a concentration of 0.5 mg ml^{-1}, stored in 1 ml aliquots in 5 ml Teklab tubes at -20° C, and used within 3 months.

Mycoplasma and virus-free fetal calf serum (FCS), sterilized through a membrane filter with pores 0.1 um in diameter, was obtained from Gibco (cat.no. 011-6290), and stored in 10 ml aliquots in 10 ml Teklab tubes at - 20° C. Because of the variability even within the same batch with regards to the ability to support the growth of BAE cells, an aliquot from each 500 ml bottle received from Gibco was tested for this purpose and, if

found positive, used to supplement BAE cell medium.

Ethylenediaminetetra-acetic acid, disodium salt (EDTA) was purchased from BDH (cat.no. 10093) and dissolved in $D^{=}$ at a concentration of 2 mM. The solution was filter sterilized, stored in 9 ml aliquots in 10 ml Teklab tubes at 4° C, and used within 60 days. This solution is here referred to as EDTA-$D^{=}$.

Bacto-trypsin was obtained from Difco and dissolved in $D^{=}$ at a concentration of 0.5 % w/v. The solution was filter sterilized, stored in 1 ml aliquots at -20° C, and used within 3 months.

Bovine serum albumin (BSA) was purchased from Sigma Chemicals (cat.no. A 7906) and dissolved in distilled water at a concentration of 21 % w/v. The solution was then sterilized by filtration through an assembly of five membranes with mean pore diameter decreasing from 8 um to 0.22 um. The concentration of BSA was estimated by reading the optical density (O.D.) of the solution in a spectrophotometer (Pye Unicam SP 1800) at 280 nm in quartz cells of 1 cm light path (Hellma type 6000-Blue), knowing from published work (Larson et al., 1986) that a BSA solution of 1 % w/v has an $E_{1cm,280}$= 6.7 . The concentration of the solution was then adjusted to 20 % with distilled water. One ml aliquots were stored in 2 ml Sarstedt tubes and stored at 4° C. Working solutions of BSA of lesser strength were prepared by diluting this BSA solution in an appropriate buffer.

Laminin was purchased from Bethesda Research Laboratories (cat.no. 6260 LA). Purified polyclonal anti-laminin antibodies (IgG), raised in rabbits, were purchased from Collaborative Research Inc. USA (cat.no.40023).

Polyclonal antibodies against human fibronectin, a gift of Scottish Antibody Production Unit (cat.no. S037-205), were raised in sheep. The antibody preparation was purified by gel filtration on a 24 ml column

(12 cm x 2 cm^2) of Sephadex G-75 M using PBS as eluent. The preparation was sterilized by filtration, the protein content was determined according to Bradford (1976), and samples were stored at 4° C.

The synthetic pentapeptide GRGDS (L-glycyl-L-arginyl-L-glycyl-L-aspartyl-L-serine) was obtained from Sigma Chemicals (cat.no. G 4391), reconstituted in 20 mM tris-HCl buffer containing 150 mM NaCl at pH 7.4, filter sterilized, stored at 4° C, and used within 30 days.

A monoclonal antibody against vitronectin was the gift of Dr. Ruoshlati (La Jolla Cancer Research Institute, La Jolla, USA).

Tunicamycin was purchased from Sigma Chemicals (cat.no. T 7765). It was dissolved in 0.01 M NaOH at 10 ug ml^{-1}, sterilized by filtration, stored in 1 ml aliquots in 5 ml Teklab tubes at -20° C and used within 30 days. Leupeptin was purchased from Sigma Chemicals (cat.no. L 2884).

Gelatin from bovine skin was purchased from Sigma Chemicals (cat.no. G 9382), dissolved in PBS at a concentration of 1 mg ml^{-1}, and sterilized by filtration. A solution of 95 % collagen type I and 5 % collagen type III extracted from bovine derma and suitable for tissue culture was prepared by TM Collagen Corporation and obtained from Flow Laboratories (Vitrogen 100TM, cat.no. 60-000-49). Collagen type IV was purchased from Collaborative Research Inc. (cat.no. 40233).

Von Willebrand factor purified from human blood was the gift of Dr. Nick Hunter (MRC SNBTS Blood Components Assay Group, Edinburgh).

Chondroitin sulfate from bovine trachea (cat.no. C 8529), dermatan sulfate from porcine skin (chondroitin sulfate type B, cat.no. C 4259), heparin-Na from porcine intestinal mucosa (cat.no. H 7005), heparan sulfate from bovine kidney (cat.no. H 9637), keratan sulfate from bovine cornea (cat.no. K 3001) and hyaluronic acid from human umbilical cord (cat.no. H 1751) were all purchased from Sigma Chemicals.

Single stranded deoxyribonucleic acid (DNA) from calf thymus (cat.no. D

8899) and ribonucleic acid (RNA) from calf liver (cat.no. R 7250) were obtained from Sigma Chemicals.

Haemoglobin from bovine erythrocytes was purchased from Sigma Chemicals (cat.no. H 2625); it was dissolved in PBS, and sterilized by filtration. The protein concentration was determined according to Bradford (1976), and the preparation was stored at 4° C.

Cell culture

For the model systems the source of endothelial cells were two continuous cell lines, both established from the endothelium of bovine aorta. The first line, a clonal derivative called BAED, was the gift of Dr. Jean Starkey (University of Washington, Washington, DC, USA). BAED cells produce FVIII and form confluent monolayers in vitro with the morphologic attributes of the endothelium. The second endothelial cell line, called BAES, was established by Dr. Schwartz (University of Washington, Washington, DC, USA) and obtained from Dr. Starkey (Gajdusek and Schwartz, 1983).

B16F10 and B16F1 cell lines were used as sources of tumour cells. These were obtained from Dr. I. J. Fidler (Frederick Cancer Research Centre, Frederick, MD, USA).

All cells were routinely handled in a horizontal sterile laminar air flow hood using sterile glass pipettes, a pipette pump and an aspirator. All plasticware was disposable and sterile.

B16F10 and B16F1 cells were routinely cultured on a three day cycle. Only cultures which had been submitted to less than 20 passage cycles were used. Samples containing cells with low passage number were kept in a liquid nitrogen freezer in 1 ml aliquots containing 2×10^6 cells in complete medium (see below) + 10 % v/v dimethyl sulfoxide (BDH Chemicals). Freezing and thawing were performed according to published procedures (Freshney, 1983).

The medium used for routine cultures was Eagle's MEM, supplemented with 10 % v/v of FCS, 1 mM Na-pyruvate, 2 mM L-glutamine, 50 IU ml^{-1} benzylpenicillin-Na B.P., 50 ug ml^{-1} streptomycin sulfate B.P., 1 % v/v MEM

non-essential aminoacids and 1 % v/v MEM vitamins. This supplemented medium is here referred to as complete Eagle's medium.

Cells were harvested according to the following routine. The spent medium was aspirated and the dish was rinsed with an equivalent volume of $D^{=}$. The rinsing solution was aspirated and 0.8 or 2 ml of detachment medium (EDTA-$D^{=}$) were inoculated into Petri dishes of 6 or 9 cm in diameter respectively. After 1 minute of incubation at room temperature, the action of the detachment medium was neutralized by addition of 2.5 volumes of complete Eagle's medium and the cells were dislodged from the dish by gently squirting the medium onto the adhering cells using a 5 ml pipette. The cell suspension was transferred to a 10 ml centrifuge tube and submitted to 200 x g for 3 minutes in a refrigerated centrifuge (MSE Chilspin). After centrifugation the supernatant was discarded and the pellet resuspended in complete Eagle's medium using 1 ml of medium for every estimated 10^6 harvested cells. A single cell suspension was obtained by passing the cells through the orifice of a 5 ml pipette and a series of 10 such passages was enough to obtain a suspension with > 90 % single cells. Cell concentration was determined by an electronic count of a 40 ul sample diluted in 20 ml of isotonic solution (Isoton II, Coulter Electronics, U.K.). Two readings of the number of suspended particles contained in 0.5 ml of the isotonic cocktail were performed using a Coulter Counter (model ZB 100, Coulter Electronics, U.K.) mounted with a probe having an orifice of 100 um diameter. In order to maximize the accuracy the following settings were used: attenuation = 64, aperture current = 0.177, upper threshold = 86, lower threshold = 10. The distribution of the counted particles was analyzed using a channelyzer (model C1000, Coulter Electronics, U.K.) set on a base channel threshold of 5 and a window width of 100. This procedure was used both to eliminate subthreshold counts in order to obtain a more accurate evaluation of the density of the

cell suspension and to check the size distribution of cells within the sample. Cells were seeded in Petri dishes of 6 or 9 cm of diameter, containing 3.5 or 10 ml of complete Eagle's medium respectively, at a concentration of 3.6 x 10^3 cells cm^{-2}. The plates containing the cell inocula were gently shaken to disperse the cells in the medium and then placed in a Heraeus incubator (type B5060 EK/CO_2, Heraeus GmbH, West Germany) at 37° C in a humidified atmosphere containing 95 % air and 5 % CO_2.

BAE cells were routinely cultured in a variety of tissue culture plastic dishes and in pyrex roller bottles. The cells were kept on a 3 days cycle and used for up to 20 passages. Storage, freezing and thawing of these cells were as described for the B16 cell line. The medium used for routine cultures was complete Eagle's medium (containing FCS which could support BAE cell growth) further supplemented with 5 ug ml^{-1} of endothelial cell growth supplement and 0.125 IU ml^{-1} of insulin. This medium is called complete BAE medium.

BAE cells were harvested following a routine similar to that used for the B16 cells, the only differences being that the monolayer of BAE cells was incubated with the detachment medium for 3 minutes at 37° C and that such detachment medium also contained 0.05 % w/v of trypsin. This detachment medium is referred to as T-EDTA.

BAE cells were plated at a concentration of 9 x 10^3 cell cm^{-2} in 9 or 14 cm diameter Petri dishes or in 750 cm^2 roller bottles containing 10, 25 and 117 ml of complete BAE medium respectively. Petri dishes were placed in an Heraeus incubator, as for the B16 cells, while bottles were gassed with a mixture of 95 % air and 5 % CO_2 and incubated in a hot room at 37° C rolling at 0.7 rpm.

BAE cells to be used in adhesion assays were seeded 3 days prior the assay in 24-well plates at the somewhat higher concentration of 2.5 x 10^5

cells cm^2 in order to obtain a confluent monolayer. Briefly, cells, harvested as per usual, were resuspended at a concentration of 5×10^5 cells ml^{-1} in complete BAE medium and 1 ml of this suspension was inoculated into each well of the 24-well plate. The plates were then placed in a Heraeus incubator as described previously. After 48 hours the medium was carefully aspirated and substituted with an equal volume of fresh complete BAE medium containing 10 mM Hepes buffer and the plates returned to the incubator where cells were allowed to grow for a further 24 hours.

Cell viability was measured using the trypan blue dye exclusion test. Trypan blue solution (Sigma Chemicals, cat.no. T 9520) was diluted in PBS to a concentration of 0.2 % w/v. A chamber was constructed by attaching two strips of proprietary adhesive tape parallel to the shorter side of a glass slide leaving a gap between each strip of ~ 1.25 cm. Twenty ul of dye solution were mixed on the glass slide with 20 ul of cell suspension. A cover glass was then placed on top of the liquid and, using a light microscope with a 200 x magnification, at least 200 cells were scored for their ability to exclude the dye. In isotonic medium and at physiological pH, trypan blue diffuses freely across cell membranes but viable cells actively transport it extracellularly. Failure to do so results in intracellular accumulation of the dye and therefore non viable cells are stained blue (Freshney, 1983).

Adhesion assay

B16 cells were cultured as described earlier and harvested as per usual, when subconfluent, after three days of growth. Cells were resuspended in complete Eagle's medium containing 10 mM Hepes and diluted in a 25 ml centrifuge tube to a concentration of 4 x 10^5 cells ml^{-1} in a volume of 4 ml. Before the assay, the cell suspension was incubated at 37° C for 10 min in order to equilibrate the temperature of the suspension to that used during the assay. Monolayers of BAE cells grown to confluence in a 24-well plate, were transferred to a hot air cabinet heated at 37° C and spent medium was carefully removed with the help of an aspirator immediately before the addition of the tumour cell suspension. Inocula of 0.2 ml of B16 cell suspension were carefully and rapidly delivered using a repetitive syringe (Eppendorf, West Germany, Multipette mod. 4780, with a 5 ml syringe tip) into each well and into two vials containing isoton. These two vials were used to accurately determine the number of cells inoculated into each well.

Following inoculation, each well was sealed with an appropriately modified 10 ml plastic syringe bearing an inlet and an outlet tube (see figure 14). The inlet tube was connected to a reservoir containing an isotonic buffered salt solution (Hepes-HCl 20 mM, NaCl 0.9 % w/v, pH 7.4) and capable of delivering a constant volume per unit of time under the principle of the Mariotte flask. The reservoir was placed on a laboratory jack and flow rate was regulated by lowering or raising the reservoir relative to the outlet tube. After a set time each inlet flow valve was opened and the well flooded with isotonic solution, which was collected at

the end of the outlet tubes in a set of plastic coulter vials (Coulter Electronics, U.K.) of uniform weight. The volume of isotonic solution contained in each vial was determined by weight. The cell concentration was determined by electronic counting using the same apparatus and settings described in the section on cell culture techniques.

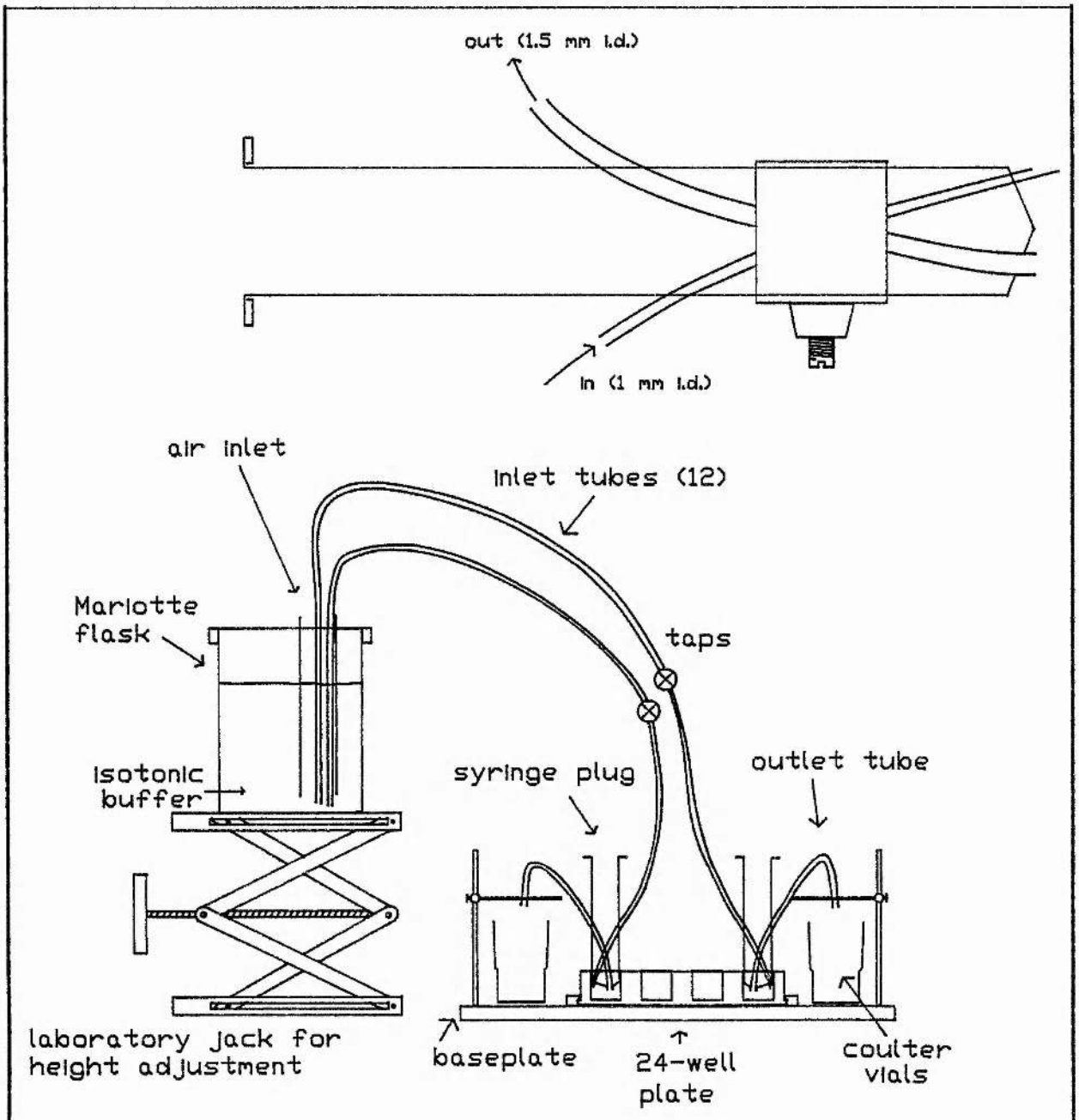


Figure 14: Apparatus for the adhesion assay. A syringe plug is shown at the top.

For each experiment a well was inoculated with 0.2 ml of medium not containing cells and, by processing it as per usual, the number of endothelial cells detaching from the monolayer was determined Furthermore, a vial containing isotonic solution plus medium alone was counted so as to take into account any background noise introduced into the assay and this result was subtracted from all measurements obtained.

Vials were counted twice and the overall number of cells contained in each of them determined according to the volume of isotonic solution collected. The total number of cells inoculated into each well was estimated by averaging the number of cells inoculated directly into two vials at the start of the assay. The percentage of adhering cells was determined according to the formula:

$$\% \text{ adhering cells} = (T - NA)/T \times 100$$

where T=number of cells inoculated and NA=number of non-adhering cells.

Each experiment typically used a row of 6 wells, 5 of which received an inoculum containing tumour cells while one received medium alone. The five wells were sequentially washed at 1 minute intervals for 30 seconds with 15 ml of isotonic solution and a time-dependent curve of the adhesion kinetics was constructed. In each curve all point measurements were obtained during the same experiment. Alternatively, end point measurements were performed by washing non-adhering cells after 5 minutes incubation. The apparatus was later modified so that 12 wells could be washed at the same time, thereby speeding up the procedure of screening for the adhesive fractions purified from the material extracted from the endothelial monolayer.

When testing the adhesion properties of selected molecules or extracted material rather than endothelial monolayers, the wells of the plates were incubated at 37° C with 0.2 or 0.5 ml of the solution to be tested. An incubation time of 1 hour was chosen in order to achieve adsorption of

the molecules under test to the plastic substrata. Following this incubation the solution was aspirated and the remaining free sites on the plastic were saturated by addition of 0.5 ml of a 1 % w/v solution of BSA in PBS and incubated at 37° C for a further hour. Following this treatment the BSA solution was aspirated and the wells washed three times with PBS before being used for the assay, which was performed as per usual.

Antibodies and peptides:

In some experiments antibodies were used in order to try to block the adhesiveness of endothelial monolayers or molecular substrata. For this purpose, the substrata were adsorbed with the adhesive molecules and the remaining free sites on the plastic were blocked with BSA, as already described. Alternatively, endothelial monolayers were seeded and grown to confluency as described previously. The supernatant was then gently aspirated and replaced with a medium containing 1 mg ml^{-1} of the antiserum to be tested. Following an incubation period of 1 hour the antiserum was removed and the well rinsed with three volumes of medium before the adhesion assay was performed as per usual.

In other experiments the competitive effect of the fibronectin-derived pentapeptide GRGDS were studied. For this purpose, immediately before the addition of the tumour cells suspension, 100 ul of GRGDS solution was inoculated into each well, quickly followed by an equal volume of a suspension of 8×10^5 tumour cells ml^{-1}.

Tunicamycin, leupeptin and trypsin:

When the role in adhesion of the glycosylated proteins present on the surface of tumour cells was evaluated, B16F10 cells were cultured in the presence of the antibiotic tunicamycin. This substance inhibits the

transfer of N-acetyl-glucosamine-1-phosphate to the dolichol monophosphate unit at the end of the carbohydrate side chains of proteins during the processing of newly translated molecules, thereby blocking the glycosylation process (Duksin and Mahoney, 1982). Cells were cultured in their usual medium and 3, 6, 12, 18, 24, 30, 36, and 48 hours before harvesting (that is 69, 66, 60, 54, 48, 42, 36 and 24 hours respectively after plating) 450 ul of tunicamycin solution were added to the medium to give a final concentration of 0.45 ug ml^{-1}. The plate was then returned to the incubator. In other experiments cells were incubated in the presence of tunicamycin for 36 hours as described, after which the medium was carefully aspirated and substituted with complete Eagle's medium. The cells were then allowed to recover from the exposure to tunicamycin for a period of 6, 12, 18 and 24 hours before the adhesion assays. In further experiments cells were incubated with tunicamycin for 36 hours as described before together with the enzyme inhibitor leupeptin (acetyl-L-leucyl-L-leucyl-L-argininal) which was present in the culture medium at the final concentration of 100 uM, 200 uM, 400 uM, 600 uM and 800 uM. Following this treatment, adhesion assays were performed as per usual.

When the ability of trypsin to digest active molecules on the tumour cell surface was tested, B16F10 cells were harvested as per usual and resuspended at a concentration of 8 x 10^5 cell ml^{-1}. Four ml of this cell suspension was transferred to a 25 ml centrifuge tube and washed three times with PBS or $D^{=}$. The pellet was then resuspended in 1 ml of a solution of trypsin 0.05 % w/v in PBS or $D^{=}$ and incubated at 37° C for various lengths of time. The reaction was stopped by the addition of 7 ml of warm complete Eagle's medium containing 10 mM Hepes and the adhesion assay performed as per usual.

Formaldehyde, low temperature and EDTA:

In some experiments either the tumour cells or the endothelial monolayer were fixed prior to the assay. To achieve this, B16F10 were harvested as per usual, resuspended in formol saline (4 % w/v formaldehyde 0.9 % w/v NaCl, BDH cat.no. 23077 7A) and left for 45 minutes at room temperature, after which cells were centrifuged as per usual and resuspended in complete Eagle's medium with Hepes 10 mM. To obtain substrata of fixed endothelium, the medium was aspirated from BAES monolayers ready for the assay and an equal volume of formol saline was added to the wells. After incubation at room temperature for various lengths of time the fixative was removed and complete BAE medium with Hepes 10 mM was added. Assays were then performed as usual.

When the adhesion kinetics of B16F10 cells on various substrata were studied at a temperature of 4° C, the apparatus for the assay was transferred into the cold room beforehand and both the cell suspension and the substrata were precooled to 4° C.

In some experiments the effect of EDTA on the adhesion of tumour cells to various substrata was tested. B16F10 cells were harvested as per usual with the exception that the medium used was EDTA-D containing 0.05 % w/v BSA and that the monolayer was washed three times with D before exposure to EDTA. Cells were then resuspended in EDTA-D + 0.05 % BSA and the assay performed as usual.

Spreading assay

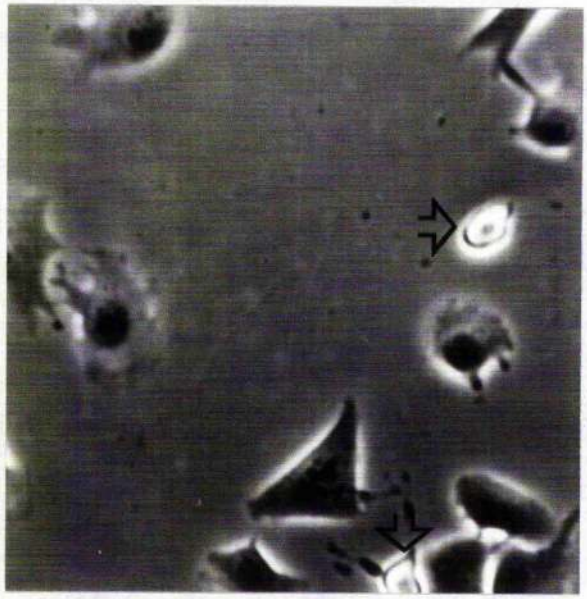
For this assay B16F10 cells, grown for three days in 6 cm diameter Petri dishes as described, were harvested as per usual but with the following modifications:

1 The dishes were washed three times with 4 ml of $D^{=}$ before addition of detachment medium.

2 The medium used for the assay was complete Eagle's medium without fetal calf serum and with 0.05 % w/v BSA. This medium was called complete BSA medium.

3 After dislodgement, cells were transferred to a centrifuge tube containing 0.2 ml of a 1 % w/v BSA solution in PBS.

Plastic tissue culture Terasaki plates were used in the spreading assay. The substratum was conditioned by the addition of 5 ul of solution containing the agent to be tested, followed by incubation for 1 hour at 37° C. At the end of the incubation the spent solution was removed, 10 ul of a solution of 20 % w/v BSA was added and the plate incubated for a further 1 hour at 37° C in order to block the remaining reactive sites on the plastic. After washing with excess PBS 3 times, the plate was ready to receive the cell suspension. This was at a concentration of 2 $x10^4$ cells ml^{-1} and was inoculated into each well in 10 ul aliquots. The plate was placed in a Heraeus incubator, as described, for 1 hour, after which the amount of spreading was determined by using a phase contrast inverted microscope (Olympus) and a magnification factor of 100x. With the help of a graticule,



Microphotograph of round (arrow) and spread cells.

all cells at the bottom of each well were counted and scored subjectively as spread or round. For examples of spread (arrows) and round cells see the photograph on page 93. Parameters used included: surface area occupied by the cell and its shape, nucleus to cytoplasm ratio, presence of perinuclear or cytoplasmic glare, nucleoli and cytoplasmic opacity. Results were expressed as percentage of spread cells.

When agents that could interfere with cell spreading (such as specific antibodies) were tested, three different procedures were followed after adsorption and blocking of the plastic substratum and subsequent washing of the wells:

1 Five ul of the test agent solution were added to the wells and incubated for 1 hour at 37° C, following which the solution was aspirated, the wells washed three times with PBS and cells added as per usual.

2 After incubation with 5 ul of test agent for 1 hour at 37° C, the solution was left in the wells and 5 ul of cell suspension were added. Since the presence of ~ 200 cells per well was required for the assay, the cell suspension was prepared at 4×10^4 cells ml^{-1}.

3 In some cases it was desirable to observe the effects of the test agent when added at the same time as the cells. In such cases, 5 ul of test agent solution and 5 ul of a suspension of 4×10^4 cells ml^{-1} were added simultaneously.

For all of these three methods, after the inoculation of the cell suspension the assay was carried out as described before.

When the kinetics of cell spreading were investigated, the assay was performed as per usual but the incubation period was reduced accordingly. In this case, for each experiment data from several plates were used, since determining the number of spread cells after a set time interval involved interrupting the incubation.

Extraction from the endothelial cell monolayer

For the extraction, endothelial monolayers were grown for 3 days in roller bottles as described before.

Usually the extract from 6 roller bottles was prepared at the same time. Medium was decanted and each bottle was gently washed 3 times with an equal volume of warm Ca^{2+} and Mg^{2+} -free Tris buffered saline (Tris-HCl 20 mM, NaCl 150 mM, pH 7.5, here called $T^{=}$), then 10 ml of a solution of 2 mM EDTA in $T^{=}$ were added and the bottles were returned to the hot room and incubated rolling as per usual. After 30 min the extracted material was transferred to a 25 ml conical tube and centrifuged at 200 x g for 5 minutes in a refrigerated centrifuge (MSE Chilspin) in order to pellet detached cells. The supernatant was then transferred to a second conical tube and centrifuged as before but at 1500 x g for 30 minutes. The pellet was discarded and the supernatant sterilized by filtration and stored at 4° C.

Production of extract from medium.

For this extraction, bottles, seeded according to the procedure used to obtain endothelial monolayers but without the addition of cells, were processed as described for the preparation of the endothelial extract. The extract from the medium constituted in effect a control for the endothelial extract.

Liquid Chromatography

For preparative and experimental chromatography a low pressure system was used. The system was assembled around the liquid chromatography controller of a fraction collector (Fraction collector FRAC-100, Pharmacia, Sweden) and comprised an optical gradient maker (Ultrograd 11300 gradient mixer, LKB, Sweden), a fixed wavelength (280 nm) photometer with a quartz flow cell (Single path monitor UV-1, Pharmacia, Sweden) and a plotter (Recorder REC-481, Pharmacia, Sweden). Fluid drive was obtained by means of a peristaltic pump (Pump P-1, Pharmacia, Sweden) or by gravity feed using Mariotte flasks (LKB, Sweden).

Borosilicate glass columns of three different cross sectional areas (0.78, 2.0 and 5.3 cm^2) and various lengths, featuring adjustable end pieces in order to enable the reversing of the direction of flow, were obtained from Pharmacia (C-column system, Pharmacia, Sweden).

The gel filtration media Sephadex G-15, Sephadex G-25 M, Sephadex G-25 F, Sephadex G-50 M, Sephadex G-75, Sephadex G-100, Sephadex G-150, Sephadex G-200, and the ion exchange media Q Sepharose fast flow and S Sepharose fast flow were all obtained from Pharmacia, Sweden. For affinity chromatography Affi-Gel-Gelatin (Bio-Rad Laboratories, cat.no. 153-6125), Heparin-Agarose (Sigma Chemicals, cat.no. H 5380) and poly-L-lysine-Agarose (Sigma Chemicals, cat no. P 6893) were used. All these media were hydrated, handled and packed under flow according to the manufacturers' instructions and flow rates and operating pressures never exceeded the recommended values.

All eluents were filter sterile and degassed by vacuum and a 5 um nylon mesh filter (obtained from Henry Simon Ltd, UK) was mounted in line at the inlet of the column.

Columns packed with gel filtration media were equilibrated in the buffer of choice and a standardization run was performed to determine the volume of the stationary phase and the void volume of each column. For such runs a mixture of high M_r (2×10^6) dextran (Blue Dextran, Sigma Chemicals, cat.no. D 5751) and tyrosine (Sigma Chemicals, cat. no. T 3754) was applied to the top of the column, typically at a concentration of 0.1 mg ml^{-1} in an appropriate buffer. The elution profile of these standards allowed us to check that the chromatographic medium was correctly packed and that the experimentally determined values were in accordance with the estimated ones. Column parameters were calculated according to published procedures (Gel filtration, theory and practice, Pharmacia Fine Chemicals, Sweden).

Fibronectin purification

Fibronectin was purified from human plasma. Forty ml of peripheral blood were obtained by venipuncture in the antecubital fossa and rapidly mixed with 4 ml of anticoagulant containing EDTA-Na_2 40 mM and Tris-HCl buffer 200 mM, pH 8.3. Plasma was obtained by centrifugation of the anticoagulated blood for 20 min at 1500 x g in a refrigerated centrifuge (MSE Chilspin). The supernatant layer of plasma was carefully removed using a plastic pipette and applied to a 25 ml column (4.5 cm x 5.3 cm^2) of Affi Gel-Gelatin previously equilibrated with Tris-HCl buffer 20 mM pH 8.3. Application flow rates never exceeded 5.5 ml cm^{-2} h^{-1}. The column was first washed with application buffer and then with the same buffer containing NaCl 1.0 M. Fibronectin was eluted, after flow inversion, with Na-acetate buffer 50 mM, NaBr 1.0 M, pH 5.0 at a flow rate of 5.5 ml cm^{-2} h^{-1}.

The eluted material was collected and applied, with a flow rate of 225 ml cm^{-2} h^{-1}, to a 150 ml column (28 cm x 5.3 cm^2) of Sephadex G-25 M previously equilibrated with Tris-HCl buffer 20 mM, pH 7.5. In order to exchange the buffer, tris-HCl buffer 2 mM, pH 7.5 was used for the elution.

After buffer exchange the eluted material was applied with a flow rate of 225 ml cm^{-2} h^{-1} to a 16 ml column (8 cm x 2 cm^2) of Q Sepharose fast flow previously equilibrated with Tris-HCl buffer 20 mM, pH 7.5. The column was washed with the same buffer until the O.D. returned to within 2 % of the baseline. The material retained in the ion exchange column was eluted with NaCl 0.15 M in the same buffer. The elution of a sharp peak

followed the change in eluent and once the O.D. at the outlet returned to within 2 % of the baseline the flow was reversed and the remaining material retained in the column was eluted with Tris-HCl buffer 20 mM, NaCl 0.25 M, pH 7.5 at a flow rate of 1.1 ml cm^{-2} h^{-1}.

The material eluted with 0.25 M NaCl was filter sterilized through a 0.22 um pore diameter durapore filter (Millipore Co), protein concentration was estimated according to the dye binding assay (Bradford, 1976) and, if necessary, the solution was diluted to less than 0.5 mg ml^{-1}. Aliquots were stored in 2 ml Sarstedt microtubes at 4° C and the activity of the preparation tested in spreading and adhesion assays.

All fractions were tested for the presence of fibronectin in immuno-dot blots on 0.45 um pore diameter nitrocellulose membrane (Millipore Co., cat.no. HAHY0010) as reviewed by Towbin and Gordon (1984). A template (the top margin of a 96-well cluster, Nunclon, Gibco) was pressed on nitrocellulose membranes in order to obtain a pattern of separate rings. The samples were applied to the centre of each ring in volumes of 2 ul and the dots were allowed to dry at 37° C. The membrane was blocked with a solution of BSA 2 % w/v for 30 minutes at 37° C after which the membrane was rinsed 3 times in TBS, allowing the buffer to equilibrate for 5 minutes during each rinse. A working solution of sheep polyclonal antibodies anti-human fibronectin in TBS, prepared at the lowest dilution recommended in the batch instructions, was incubated with the membrane for 30 minutes at 37° C. The membrane was then rinsed 3 times as before and incubated for 30 minutes with a solution of horseradish peroxidase-conjugated donkey polyclonal antibodies anti-sheep IgG (a kind gift of the Scottish Antibody Production Unit) in TBS, prepared at the lowest dilution recommended in the batch instructions. After rinsing in TBS, the membrane was stained with a solution of 3 mg ml^{-1} of 4-chloro-1-naphthol (Sigma Chemicals, cat.no. C 8890) in methanol (BDH Chemicals, Aristar,

cat.no. 45102) to which 5 volumes of a 0.018 % solution of H_2O_2 (BDH Chemicals, cat.no. 28519) in cold TBS were added. The colour was allowed to develop for 10 minutes at room temperature, after which the reaction was stopped and the membrane washed several times in distilled H_2O. Results were visually evaluated immediately after development against dilutions of positive and negative controls.

Results

Purification of fibronectin

Part 1: Background

Although fibronectin is now commercially available from many sources, purity of the preparation and functional activity can be quite poor. For this work it was decided therefore to undertake purification of the molecule in our own laboratory.

Plasma from human peripheral blood was chosen as source of fibronectin for convenience and availability and a protocol of purification was devised to overcome the pitfalls of various published methods. The purification technique exploits the property of fibronectin to bind to denatured collagen. Investigations on the nature of this interaction have shown that the binding is inhibited even at low ionic strength (27 % inhibition in the presence of 50 mM NaCl at pH 7.5), but once established it is not disrupted even when the NaCl concentration is increased to 2.0 M (Vuento et al., 1982). However, molar concentrations of specific cationic compounds readily dissociate the complex (Vuento and Vaheri, 1978; Dessau et al., 1978).

The formation of the gelatin-fibronectin complex has an optimum pH between 7.0 and 9.0, with very low binding at pH below 6.0 and with a sharp fall off above pH 9.5, with the highest binding occurring at pH 8.5. Furthermore the reaction, even in the presence of excess gelatin, is not rapid, reaching equilibrium after 15 to 30 minutes (Vuento et al., 1982).

EDTA was chosen as the anticoagulant in view of the fact that the plasma was used immediately after preparation, without storage. The use

of heparin or protamin-SH was avoided since heparin is a known modulator of the gelatin-fibronectin interation as it binds to fibronectin; there would also be the possibility that heparin or protamine-SH could be still present as contaminants in the final fibronectin preparation, with obvious risk of providing a source of bias in the work that followed since they could both be involved in modulation of cell adhesion. The use of acid citrate dextrose was also avoided in order to maintain the plasma pH weakly basic and so maximize fibronectin binding to gelatin. To ensure this, the anticoagulant also contained Tris-HCl buffer at pH 8.3.

Plasma was slowly applied to a column of gelatin-agarose; the affinity ligand used had a capacity to bind 1 mg of fibronectin per ml of gel. The column was never theoretically loaded above 25 % of its maximum capacity, given a concentration of fibronectin in plasma of 0.3 mg ml^{-1}.

Part 2: Findings

When plasma was applied to the gelatin column no fibronectin could be detected immunologically in the not bound fraction (first peak of the trace on the left in figure 15). Following application of 1.0 M NaCl at pH 8.3, some material was eluted from the column (second peak in figure 15). This peak did not contain fibronectin as determined immunologically and did not possess any adhesion-promoting activity as determined in an adhesion assay. A peak containing fibronectin and adhesion-promoting activity was eluted using 1.0 M NaBr at a pH of 5.0 (third peak, left chart, figure 15).

This eluate was then applied to a column of Sephadex G-25 M for a rapid exchange of the buffer (figure 15, middle chart). To produce this figure and show the separation from low molecular weight material, a low

tracer with low M_r (tyrosine, 0.5 mg) was added to the NaBr eluate prior to application to the Sephadex column. The second peak of the middle chart of figure 15 was negative when immunologically tested for fibronectin, and contained no adhesion-promoting activity. The first peak, which represents the desalted NaBr eluate, contained both fibronectin and adhesion-promoting activity.

When this peak was applied to a Q-Sepharose column, some material was not retained by the column (figure 15, first peak of the chart on the right); such material was not fibronectin as determined immunologically nor did it contain adhesion-promoting activity. The column was then washed with 0.15 M NaCl which caused the elution of a peak (second peak, same chart) containing material which was not immunologically related to fibronectin nor had adhesion-promoting activity. The application of 0.25 M NaCl resulted in the elution of a large peak containing both fibronectin and adhesion-promoting activity. Gel electrophoresis on SDS containing polyacrylamide (5-20 % linear gradient) of this last peak showed the presence of three bands, one with an estimated M_r of 450,000 and two with M_r~ 200,000. As estimated with the dye binding test (Bradford, 1976) the total amount of protein contained in the last peak was 7.12 mg. The adsorption at 280 nm was 9.52 O.D. (0.272 O.D. ml^{-1}, 35 ml). The extinction coefficient for a 1 mg ml^{-1} solution of fibronectin is $E_{280\ 1cm}$=1.28 (Dessau et al.,1978), so the amount of fibronectin recovered would be 7.44 mg as deduced from its extinction coefficient. According to these calculations, the initial plasma sample used contained between 0.31 and 0.32 mg ml^{-1}, which is in general agreement with published estimates.

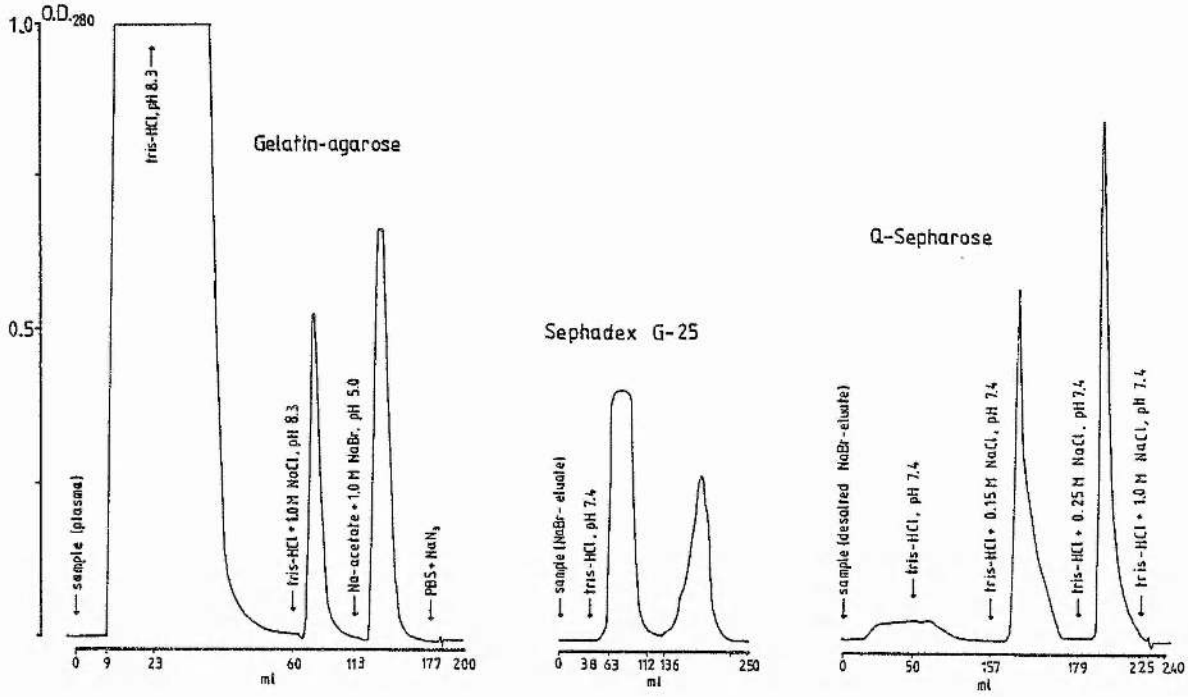


Figure 15: Purification of fibronectin from human plasma.

Plasma (23 ml) was applied to a column of gelatin (left). Bound material not containing fibronectin was eluted with 1 M NaCl at pH of 8.3. The remaining bound material, containing fibronectin, was eluted with 1 M NaBr at a pH of 5.0 and applied to a 150 ml column of Sephadex G-25 M for buffer exchange (centre). Such desalted material was then applied to a 16 ml column of Q-Sepharose (right); bound material not containing fibronectin was eluted with 0.15 M NaCl while fibronectin was eluted with 0.25 M NaCl.

Adhesion

Part 1: Background.

The purpose of this work was to investigate the molecular interactions between circulating tumour cells and endothelial cells which may occur during the vascular arrest of tumour cells at a distant metastatic site. To accomplish this task a convenient model system was developed based on the observation that B16 melanoma cells in suspension adhere to an endothelial monolayer when they are left to settle on it (Kramer and Nicolson, 1979).

The adhesion assay was developed from the collecting lawn assay of Walther and coworkers (1973). In their paper, these authors showed how a suspension of tumour cells adhered to a monolayer of endothelial cells in a time dependent fashion. To study and quantify this phenomenon the cells were radiolabelled. The non-adherent cells were washed away and, in order to determine the number of adherent tumour cells, the radioactivity retained by the monolayer was counted after solubilization.

Most authors working with similar assays measure adhesion after relatively long periods of incubation, sometimes even up to 2 hours or more (Tao and Johnson, 1982; Winkelhake and Nicolson, 1976). Long incubation times do not offer the opportunity to study adhesive phenomena of the kind that might allow a circulating tumour cell to arrest at a distant site in order to produce a metastasis. Such adhesion, in fact, needs to be established in a relatively short time (certainly not hours), since tumour cells may have perhaps only very brief contacts with the

endothelium before being forced to progress by the blood flow. Furthermore, during long incubation times cells usually secrete and deposit extracellular material on the substratum (Clark et al., 1986; Grinnell and Feld, 1979; Jaffe et al., 1983; Raugi et al., 1982), rendering it more suitable for adhesion. Alternatively, cells may modify the substratum using, for example, enzymes. All this would provide misleading information with regards to the role of the substratum under test in early adhesive interactions.

Part 2: Modifications to the adhesion assay.

In our hands this assay has been developed and made more reliable. The use of radiolabelled cells was avoided. Labelling of the tumour cells is a procedure that subjects the cells to unnecessary manipulation and introduces a possible source of artefact. Furthermore there are the added cost and safety disadvantages of working with radioactive material. To determine the number of adherent cells, the non-adherent cells (i.e. those remaining in suspension) were collected and counted using an electronic particle counter. This method proved to be extremely practical, enabling us to have the results within a few minutes of each experiment, as well as being reliable and precise.

Another modification of the original method was the way in which non-adhering cells were harvested once the incubation was completed. We recognized the need to wash the monolayer under controlled conditions with regards to the amount of fluid used, the flow rate and the duration of the wash: it is possible that tumour cells have a wide range of adhesive potential to the endothelium regarding the ability to resist dissociation and therefore even slight changes in the washing routine

would introduce significant variability between experiments. To perform a controlled wash, an apparatus was developed which was capable of delivering fluid in a sealed circuit under gravity feed. The coupling of electronic cell counting of non-adhering cells with the controlled washing of the monolayer proved very successful and made it possible to use this assay to study rapid adhesion.

Our assay gave us the opportunity to look at events occurring earlier than was possible with any other published method, being able to determine the number of adhering cells even after only 1 minute of incubation. We were able to select the parameters of the controlled wash so that the adhesion developed after 5 minutes by the majority of the tumour cells with the endothelium would be of sufficient strength to resist detachment induced by the flow of washing solution. The assay was subsequently adapted to measure the adhesion of tumour cells to an adsorbed layer of molecules. This enabled us to compare the endothelium and specific molecules with respect to their function as an adhesive support for the tumour cells.

Part 3: Findings.

a. Adhesion to endothelium.

In the initial experiments, the adhesion of B16F10 cells to a monolayer of endothelial cells was studied over a period of 60 minutes. As shown in figure 16, adhesion is time dependent and the kinetics has two phases. During the first 7-8 minutes there is a rapid increase in the number of adherent cells up to approximately 80 %. Following this, there is a second phase where the number of adhering cells slowly increases to almost 100 % in 25-30 minutes. Monitoring ahdesion for up to 60 minutes showed that

the initial adhesion was not short lived and that tumour cells remained attached to the endothelium for the duration of the assay. Visual inspection of the wells at the end of the assays, showed an intact endothelium with few tumour cells still visible over its surface, while a large proportion of the tumour cells had migrated through the monolayer and was lying between the endothelium and the bottom of the well.

Since this part of the study was related to the initial adhesion that circulating tumour cells may form with the endothelium in order to arrest, it was decided to monitor only the first five minutes of the adhesion kinetics. Indeed, if different substrata were to be proven more or less adhesive for tumour cells, it would be in this part of the curve (where the number of adhering cells increased rapidly) that the greatest differences would be detected.

Because of the nature of the experiments, it was not always possible to run adhesion assays in close succession and most of the work was spread out over a period of 12 months. Since it is essential to compare experiments that were performed many months apart, it is important to assess the internal consistency of the assay. For this purpose, adhesion experiments to unmodified endothelium which included a set of five time points (1-5 minutes) performed at 37° C in the presence of the normal medium performed in four different months (January, May, August and October) were grouped and statistically analyzed. A multivariate analysis of variance indicated that there is no significant difference between the four groups ($p= 0.49$), as shown in figure 17.

Some experiments were performed in order to characterize the nature of the adhesion of B16F10 cells to BAE monolayers. As shown in figure 18, this was dependent on the presence of divalent cations and could be inhibited by lowering the temperature of the assay to 4° C. Such temperature-dependent inhibition was reversed on return to 37° C. To

investigate the relative contribution toward adhesion of the two cell types, experiments were done using dead cells. As shown in figure 19, when the endothelium was killed by fixation, B16F10 cells were still able to adhere to it, although at approximately half the normal rate. When the tumour cells were treated with the fixative, there was virtually no adhesion. Although this pointed towards an active role of tumour cells during adhesion, it could be envisaged that live tumour cells are only needed to give the endothelium an initial trigger and that, once the endothelium is activated, it would conduct the adhesion process itself. If this was the case, probing the endothelium with a mixture of live and dead tumour cells would result in adhesion of all the cells used. In these experiments adhesion was exactly half that of the controls, indicating that only the live cells were adhering to the endothelium.

Exposure of either the endothelium, the tumour cells or both to EDTA for up to 15 minutes had no effect when adhesion was later tested in the normal medium (results not shown). Nevertheless exposure of tumour cells to trypsin resulted in modulation of adhesion. The extent of such modulation depended on the presence or absence of chelating agents and on the length of exposure to the enzyme. As shown in figure 20, trypsin-EDTA exposure for 10 minutes resulted in an almost total inhibition of adhesion, while 2.5 and 5 minutes exposure caused a slight decrease of adhesion. When trypsin was used without chelating agents, the reduction in adhesion after 10 minutes exposure was less marked and 5 minutes incubation had no significant difference over the untreated cells (see figure 21). Interestingly though and somewhat controversially, a much shorter exposure of the cells to (inactivated) enzyme for a nominal 0 minutes resulted in a significantly greater adhesion. Such increase in adhesion was not recorded when fibronectin was used as a substratum.

To investigate possible differences between different sublines of the

same original B16 population, the adhesion of B16F1, B16F10 and B16BL6 cells to endothelial monolayers was studied. As shown in figure 22, all tumour cell lines adhered rapidly to the endothelium and the kinetics were similar for all three cell lines.

b. Adhesion to molecular substrata.

When fibronectin was adsorbed to the plastic, it supported the adhesion of B16F10 cells in a concentration dependent fashion. As shown in figure 23, the kinetics of adhesion markedly improved as the density of this molecule on the substratum was increased from 1 to 4 ug cm^{-2}; slightly faster kinetics were obtained with concentrations of 5 ug cm^{-2}, while raising the concentration up to 9 ug cm^{-2} resulted in only a marginal increase in adhesion. No further improvement beyond this was noted even when concentrations as high as 20 ug cm^{-2} were used (results not shown). For this reason a concentration of 5 ug cm^{-2} was chosen for all further experiments. Similar to results from the experiments of adhesion to the endothelium, the adhesion of B16F10 cells to fibronectin was dependent on the presence of divalent cations and could be inhibited by lowering the temperature to 4° C, as shown in figure 24. No significant difference could be demonstrated when the adhesion of B16F10 and B16F1 cells to fibronectin (figure 25) was compared (multivariate analysis of variance, p= 0.21).

Figure 26 shows the adhesion of B16F10 cells to different concentrations of laminin. These results are similar to those obtained for fibronectin. There was about a two fold increase in adhesion after 5 minutes when the concentration of laminin was raised from 1.25 to 3.75 ug cm^{-2} but only a marginal increase when the concentration was raised from 3.75 to 6.25 ug cm^{-2} and to 10 ug cm^{-2}. Above 10 ug cm^{-2} the adhesion kinetics did not change significantly (results not shown) and a

concentration of 6.25 ug cm^{-2} was chosen for all further experiments. As already observed for fibronectin and endothelium, adhesion to laminin was dependent on the presence of divalent cations and could be inhibited by lowering the temperature to 4° C (figure 27).

When FCS was tested for its ability to support the adhesion of B16F10 cells, it was found to be a poorly adhesive substratum and even after increasing the concentration up to 500 ug cm^{-2} there was no detectable improvement. Figure 28 shows the adhesion kinetic of FCS and the inhibition brought about by the absence of divalent cations.

A wide panel of different molecular substrata was tested in adhesion assays and it was found that many molecules such as collagen type I (800 ug cm^{-2}), gelatin (100 ug cm^{-2}), GAGs (heparin, heparan sulfate, chondroitin sulfate types A and C, dermatan sulfate, keratan sulfate and hyaluronic acid, all used at 100 ug cm^{-2}), low molecular weight von Willebrand factor (5 ug cm^{-2}), RNA and DNA (100 ug cm^{-2}), anti-fibronectin antibodies (500 ug cm^{-2}) and haemoglobin (100 ug cm^{-2}), were not effective in supporting adhesion of B16F10 cells (results not shown).

In figure 29 the adhesion of B16F10 cells to BAE monolayers was compared to their adhesion to a variety of molecular substrata. Adhesion to FCS or collagen type I showed relatively slow kinetics, while the adhesive molecules fibronectin (5 ug cm^{-2}) and laminin (6.25 ug cm^{-2}) supported adhesion in a manner comparable to that of the endothelium.

Attempts were made to block the effectiveness of some substrata such as fibronectin, laminin and BAE monolayers in supporting the adhesion of B16F10 by using polyclonal antibodies against fibronectin and laminin. Antibodies were incubated with the substrata for 1 hour after which the wells were washed with buffer and the adhesion assays were performed. While the anti-fibronectin antibodies proved effective in blocking the adhesion of tumour cells to fibronectin substrata, they had no effects on

the adhesion to laminin substrata and only a moderate effect on endothelium (some of these results are shown in Figure 30). The anti-laminin antibodies had no effect on the adhesion of tumour cells to any of the three substrata (results not shown).

c. Effect of tunicamycin.

B16F10 cells were cultured in the presence of the antibiotic tunicamycin in order to inhibit glycosylation of newly synthesized proteins. When the adhesion of these cells to fibronectin and endothelium was then tested, no significant reduction of adhesion was caused by exposure to tunicamycin for 6-30 hours and only after 36 hours of exposure a decrease in the rate of adhesion was noted (see figures 31 and 32). Prolonging the incubation for a further 12 hours (48 hours in total) did not result in additional reduction of adhesion, as shown in figure 31.

Conversely, when tunicamycin treated B16F10 cells were used in adhesion assays on laminin substrata, a significant reduction in adhesion (the adhesion at 5 minutes decreased from 76.2 % to 25.2 %) occurred after only 24 hours of exposure to the antibiotic, as shown in figure 33.

In later experiments, B16F10 cells that were exposed to tunicamycin for 36 hours were allowed to recover in the normal medium not containing the antibiotic. These cells were then assessed for their ability to adhere to endothelium or fibronectin. As shown in figures 34 and 35, a recovery period of 18 hours was necessary to reacquire a level of adhesiveness comparable to that of cells not exposed to tunicamycin. Longer recovery times did not result in improved adhesion kinetics beyond the control levels.

The inhibitory effect of tunicamycin on cell adhesion could be due to proteolytic degradation of carbohydrate-stripped molecules, because unglycosylated proteins often have an increased sensitivity to proteolytic

digestion. Alternatively, the decrease in adhesion could be the result of the lack of the glycosylated moiety itself which could be directly involved in the phenomenon. In order to investigate this latter possibility, some experiments were conducted using the enzyme inhibitor leupeptin, which would block proteolytic degradation of the unglycosylated molecules. B16F10 cells were cultured in the presence of both tunicamycin and leupeptin for 36 hours and their adhesion to endothelium or fibronectin was assayed. Figure 36 shows that the presence of leupeptin did not reduce the adverse effect of tunicamycin on tumour cell adhesion to endothelial monolayers. On the other hand, as shown in figure 37, leupeptin countered the inhibitory action of tunicamycin on tumour cell adhesion to fibronectin; the value for 5 minute adhesion was raised from 47.8 % (no leupeptin) to 72.6 % (leupeptin 200 uM), close to that of cells not exposed to tunicamycin (76.5 %).

d. Adhesion to endothelial cell extract.

When partially purified endothelial cell extract was tested, it was found to support the adhesion of B16F10 cells extremely well. The extract was still very active (85.6 % adherent cells in 5 minutes) at a concentration of 6 ug cm^{-2}, as shown in figure 38. Extract from roller bottles exposed to the culture medium but that did not contain any endothelial cells had no adhesive activity (results not shown).

The pentapeptide GRGDS was used to inhibit the adhesion of B16F10 cells to endothelium, fibronectin or endothelial cell extract substrata. It was found that adhesion to fibronectin was unaffected by the presence of the soluble pentapeptide (up to 2 mg ml^{-1}), while the adhesion to endothelial cell extract was totally inhibited and the adhesion to endothelial monolayers was reduced to approximately 30 %. In further experiments adhesion of B16F10 cells to substratum-adsorbed GRGDS (up to

100 ug cm^{-2}) was tested but this substratum was not adhesive for the tumour cells. These results are summarized in figure 38. Finally, incubation of endothelial cell extract substrata with anti-fibronectin antibodies had no effect on the kinetics of adhesion (results not shown).

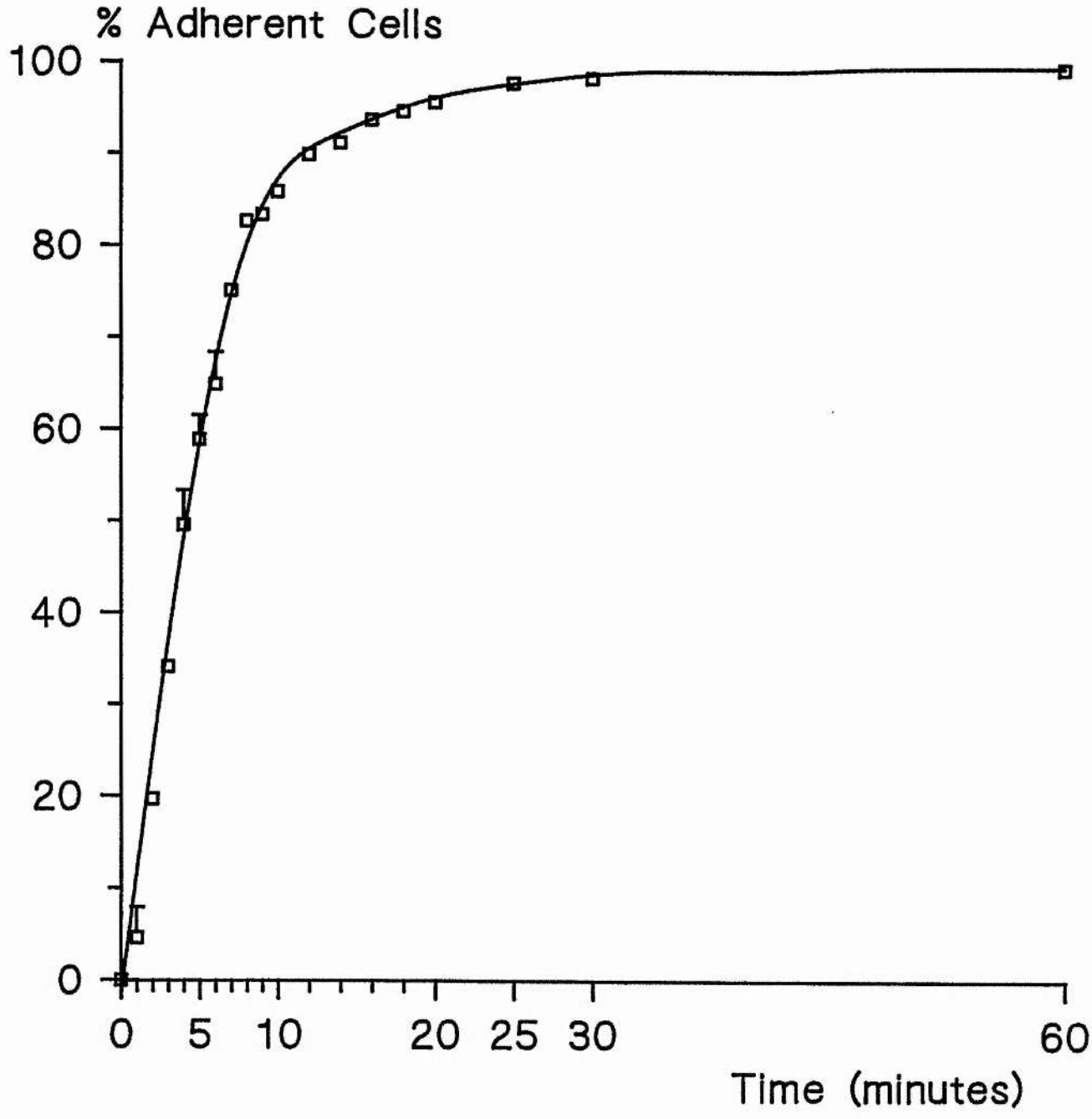


Figure 16: Adhesion of tumour cells to endothelial monolayers.

The adhesion of B16F10 cells to BAES monolayers was monitored over a period of 60 minutes; n= 5.
In this and all subsequent figures: bar = S.D.; all S.D. less than 2.5 are not represented; all curves represent cubic fits.

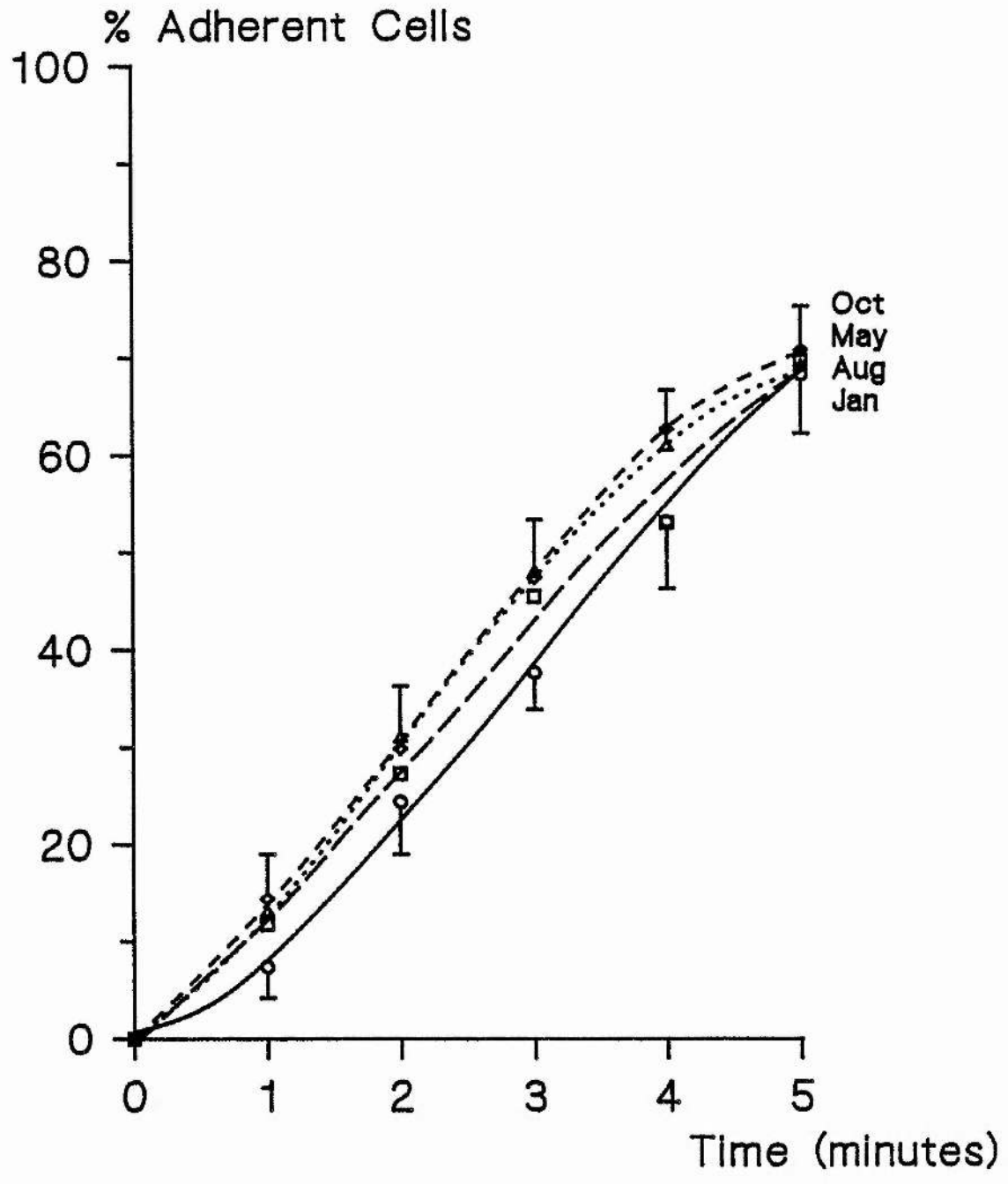


Figure 17: Control for internal consistency.

The adhesion of B16F10 cells to BAES monolayers assayed in four different months were groupped and statistically analyzed using a multivariate analysis of variance. The four groups were not statistically different (p= 0.49).
○ = January (n=7).
△ = May (n=12).
□ = August (n=7).
◇ = October (n=9).

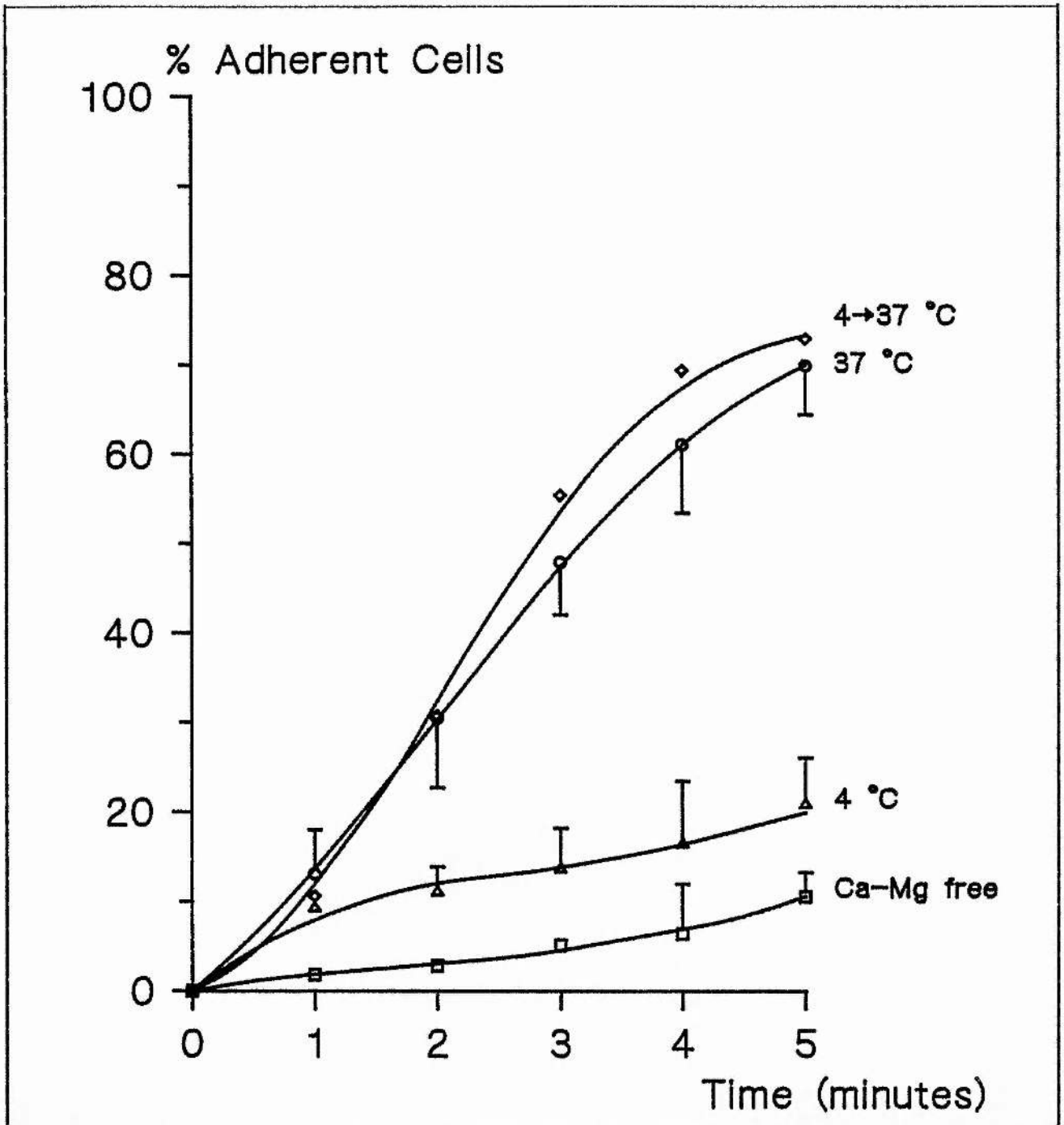


Figure 18: Adhesion of tumour cells to the endothelium: influence of temperature and divalent cations.

The ability of BAES monolayers to support B16F10 cell adhesion in a medium depleted of divalent cations or at various temperatures was tested.
○ = tumour cells and endothelium at 37°C (n=8).
△ = tumour cells and endothelium at 4°C (n=3).
◇ = tumour cells and endothelium cooled to 4°C for 15 minutes; 5 minutes before the assay the temperature was then raised to 37°C and kept at this value throughout the assay (n=1; 5 min n=3).
□ = tumour cells and endothelium at 37°C in a Ca^{2+} and Mg^{2+}- free medium (n=3).

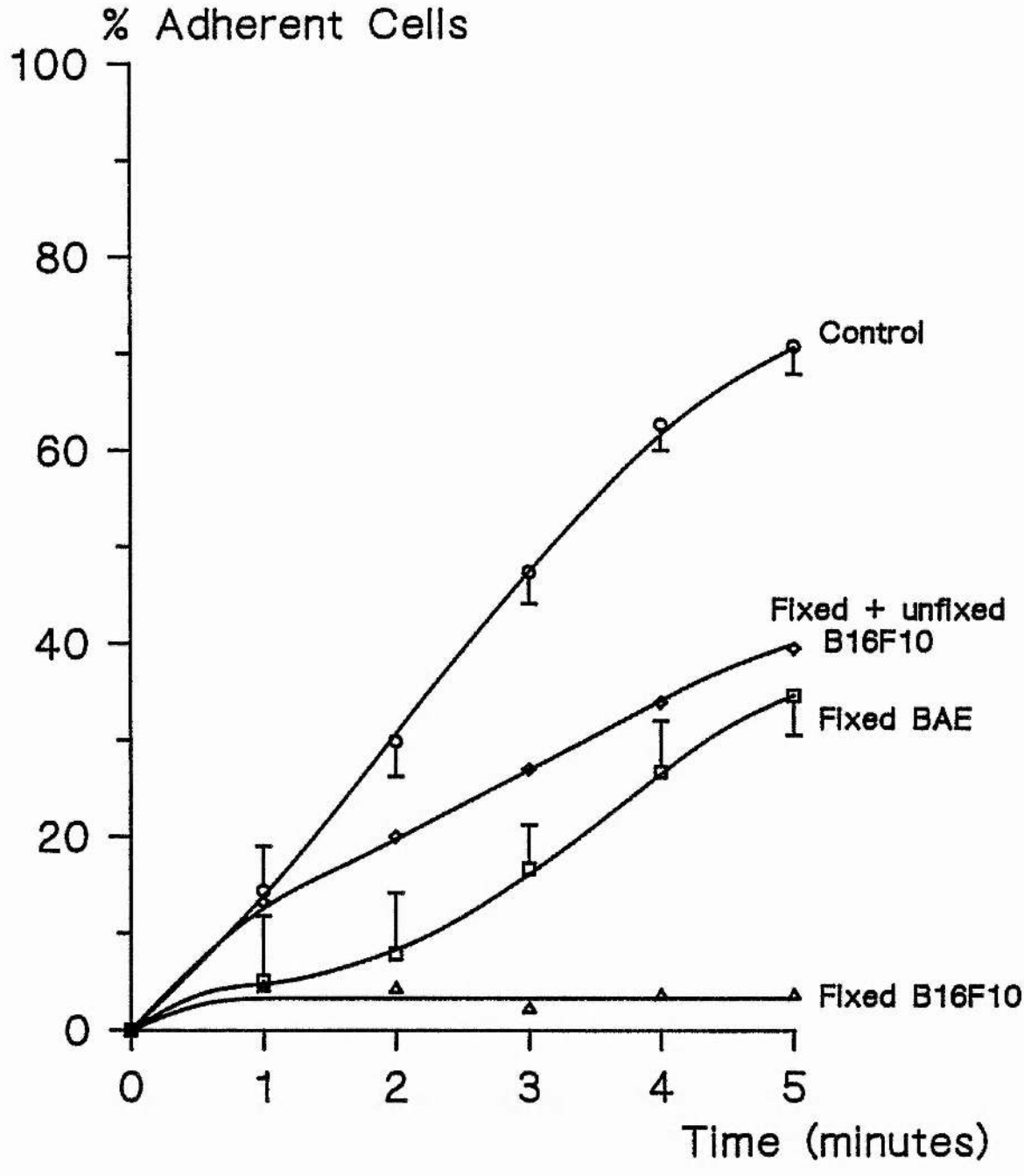


Figure 19: Adhesion of tumour cells to endothelium: effect of fixation.

B16F10 cells or BAES monolayers were fixed with formol saline for 45 minutes; subsequently the adhesion of tumour cells to BAES monolayers was tested in adhesion assays.

o = neither tumour cells nor endothelium exposed to fixative (n=5).
□ = endothelium exposed to fixative (n=3).
△ = tumour cells exposed to fixative (n=3).
◇ = 50 % of the tumour cells exposed to fixative (n=2).

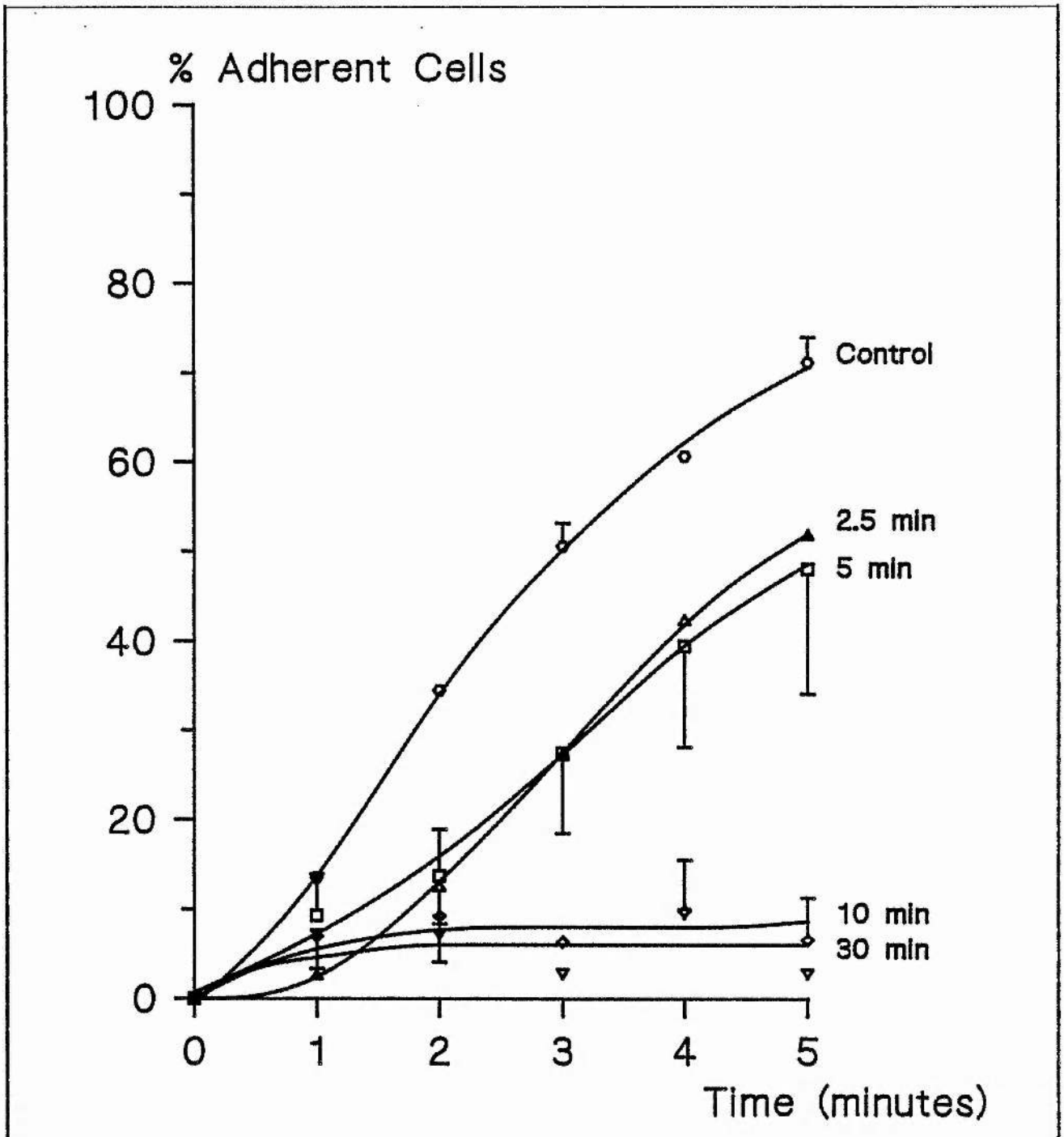


Figure 20: Adhesion of tumour cells to endothelium: effect of exposure of tumour cells to trypsin in the absence of divalent cations.

B16F10 cells were incubated for various lengths of time at 37° C with 0.05 % w/v trypsin in the presence of a chelating agent (EDTA 2 mM). The reaction was stopped by the addition of FCS containing medium and their adhesion to BAES monolayers was tested.

○ = tumour cells not exposed to trypsin (n=10).
△ = tumour cells exposed to trypsin for 2.5 minutes (n=2).
□ = tumour cells exposed to trypsin for 5 minutes (n=7).
◇ = tumour cells exposed to trypsin for 10 minutes (n=3).
▽ = tumour cells exposed to trypsin for 30 minutes (n=2).

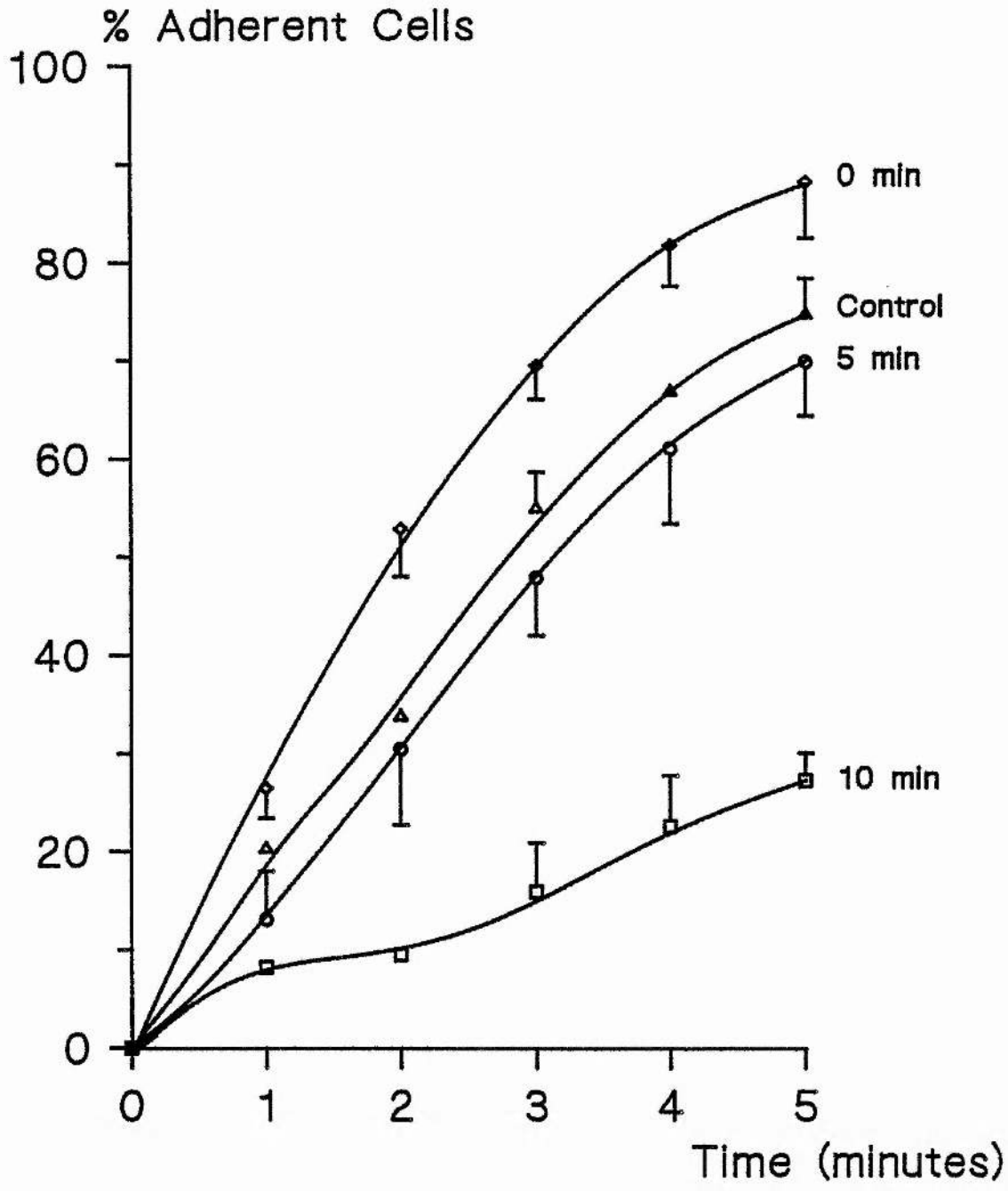


Figure 21: Adhesion of tumour cells to the endothelium: effect of exposure of tumour cells to trypsin in the presence of divalent cations.

B16F10 cells were incubated for various lengths of time at 37°C with 0.05 % w/v trypsin. The reaction was stopped by the addition of FCS containing medium and their adhesion to BAES monolayers was tested.
△= tumour cells not exposed to trypsin (n=9).
◇= tumour cells exposed to trypsin for 0 minutes (n=3).
○= tumour cells exposed to trypsin for 5 minutes (n=3).
□= tumour cells exposed to trypsin for 10 minutes (n=2).

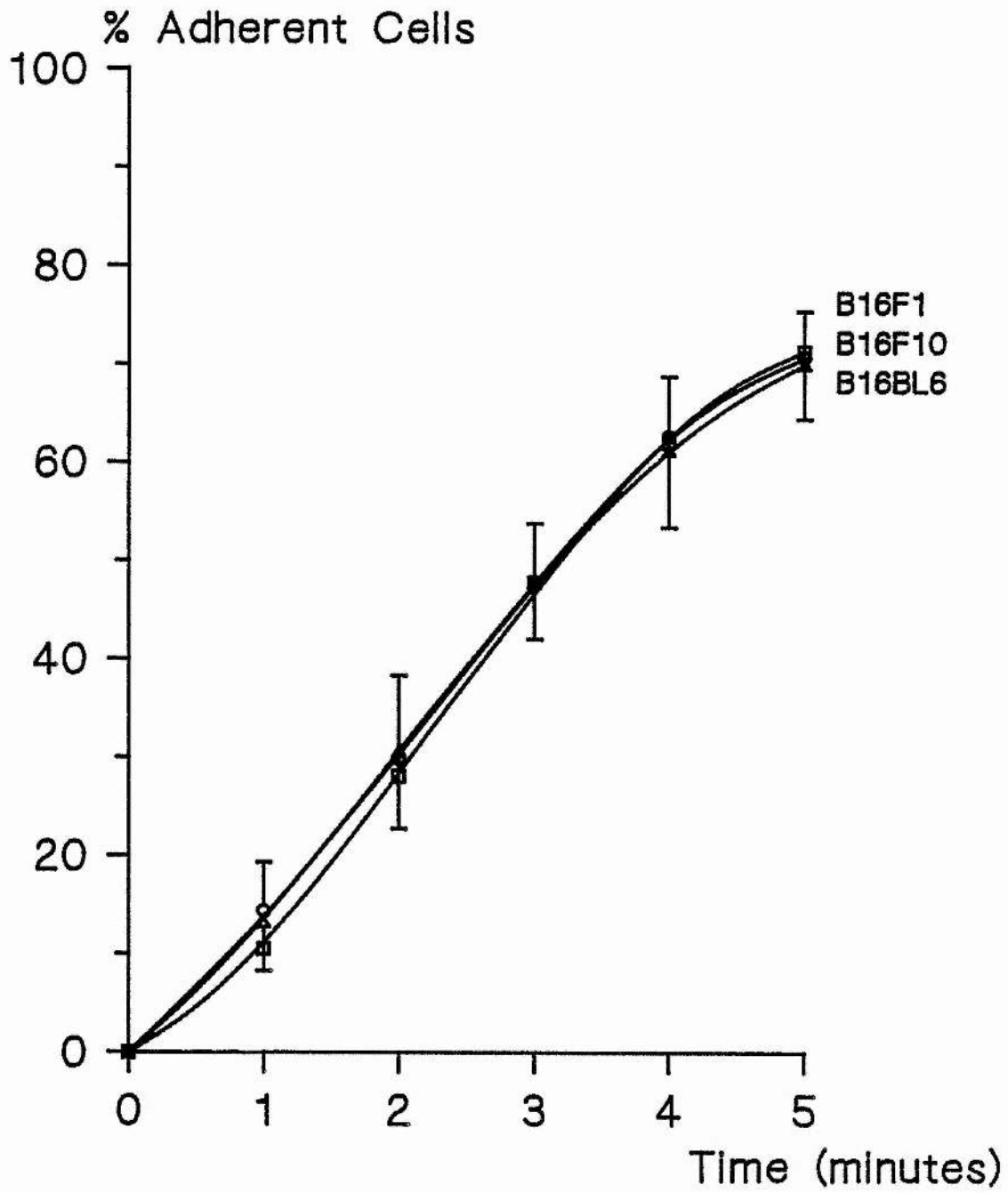


Figure 22: Adhesion of different tumour cell lines to endothelium.

The adhesion of B16F1, B16F10 and B16BL6 cells to BAES monolayers was compared.
□ = B16F1 cells (n=8).
○ = B16F10 cells (n=6).
△ = B16BL6 cells (n=3).

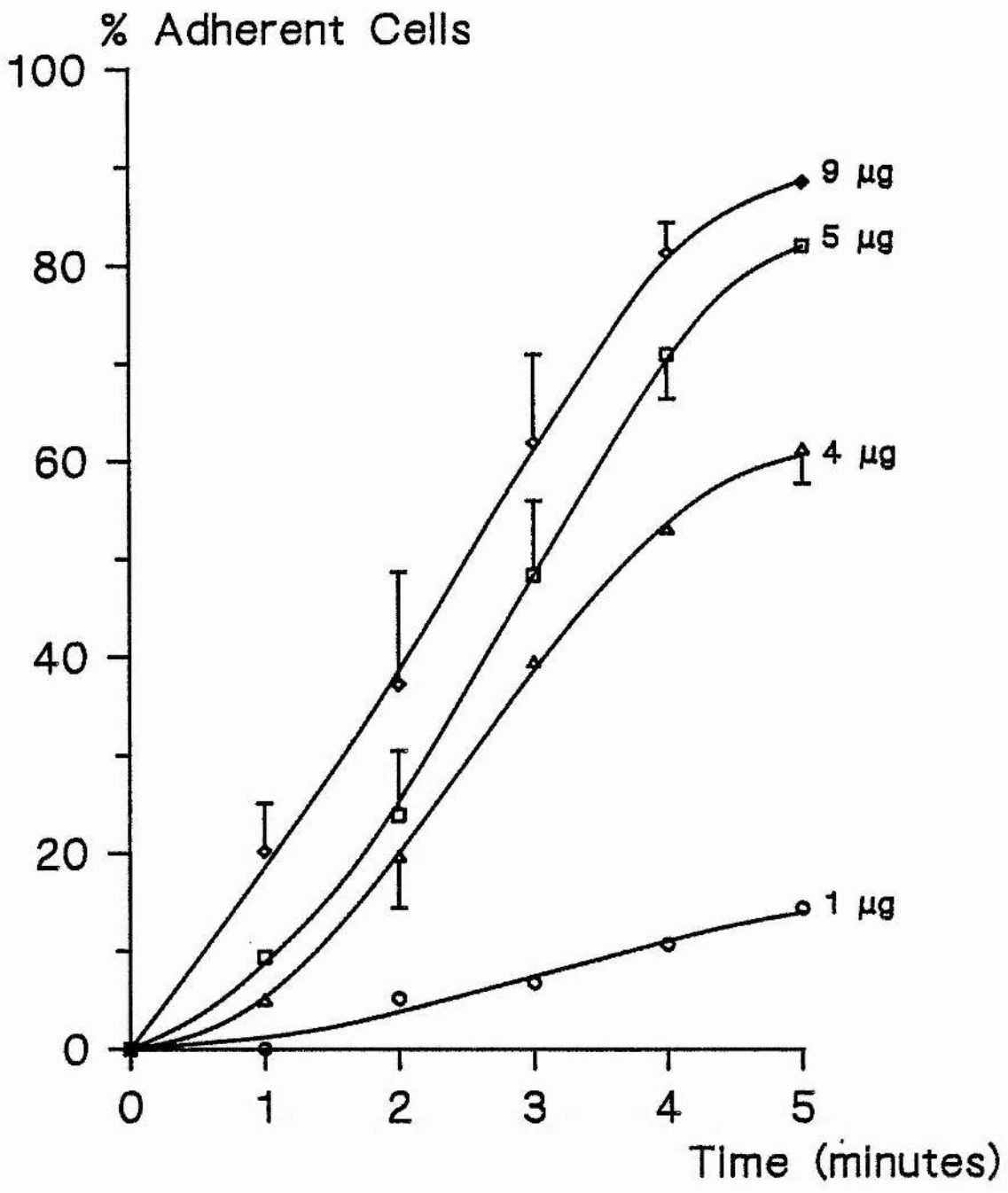


Figure 23: Adhesion of tumour cells to fibronectin: effect of ligand density.

Substrata with various amounts of adsorbed fibronectin were prepared and tested for their ability to suppor B16F10 cell adhesion.
○ = substrata containing 1 ug cm^{-2} of fibronectin (n=2).
△ = substrata containing 4 ug cm^{-2} of fibronectin (n=4).
□ = substrata containing 5 ug cm^{-2} of fibronectin (n=3).
◇ = substrata containing 9 ug cm^{-2} of fibronectin (n=3).

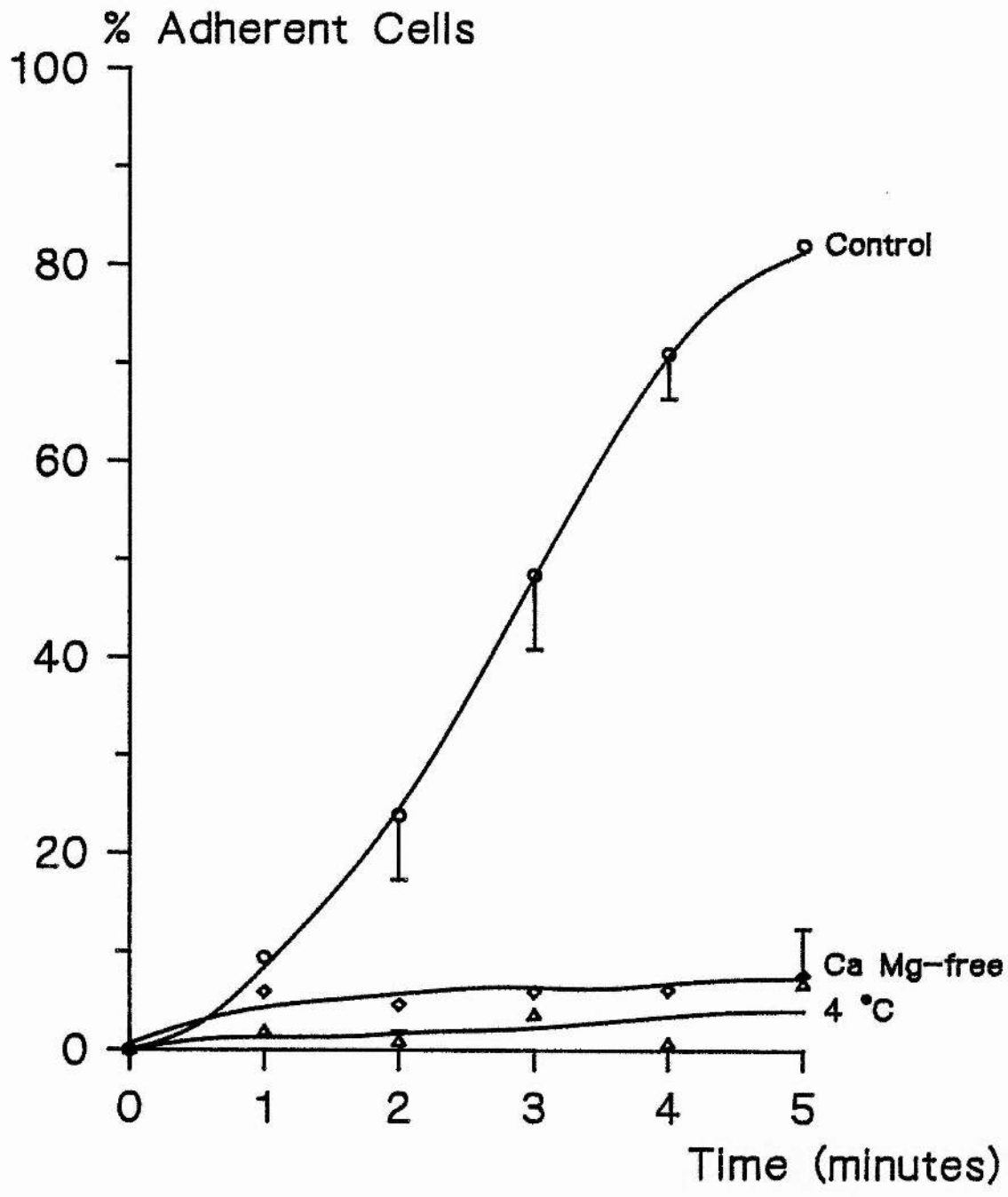


Figure 24: Adhesion of tumour cells to fibronectin: influence of temperature and divalent cations.

Substrata containing 5 ug cm^{-2} of fibronectin were prepared and their ability to support B16F10 cells adhesion in a medium depleted of divalent cations or at various temperatures was tested in adhesion assays.

○ = tumour cells and substrata at 37°C (n=4).

△ = tumour cells and substrata at 4°C (n=1; 5 min n=3).

◇ = tumour cells and substrata at 37°C and in a Ca^{2+} and Mg^{2+}-free medium (n=2; 5 min n=3).

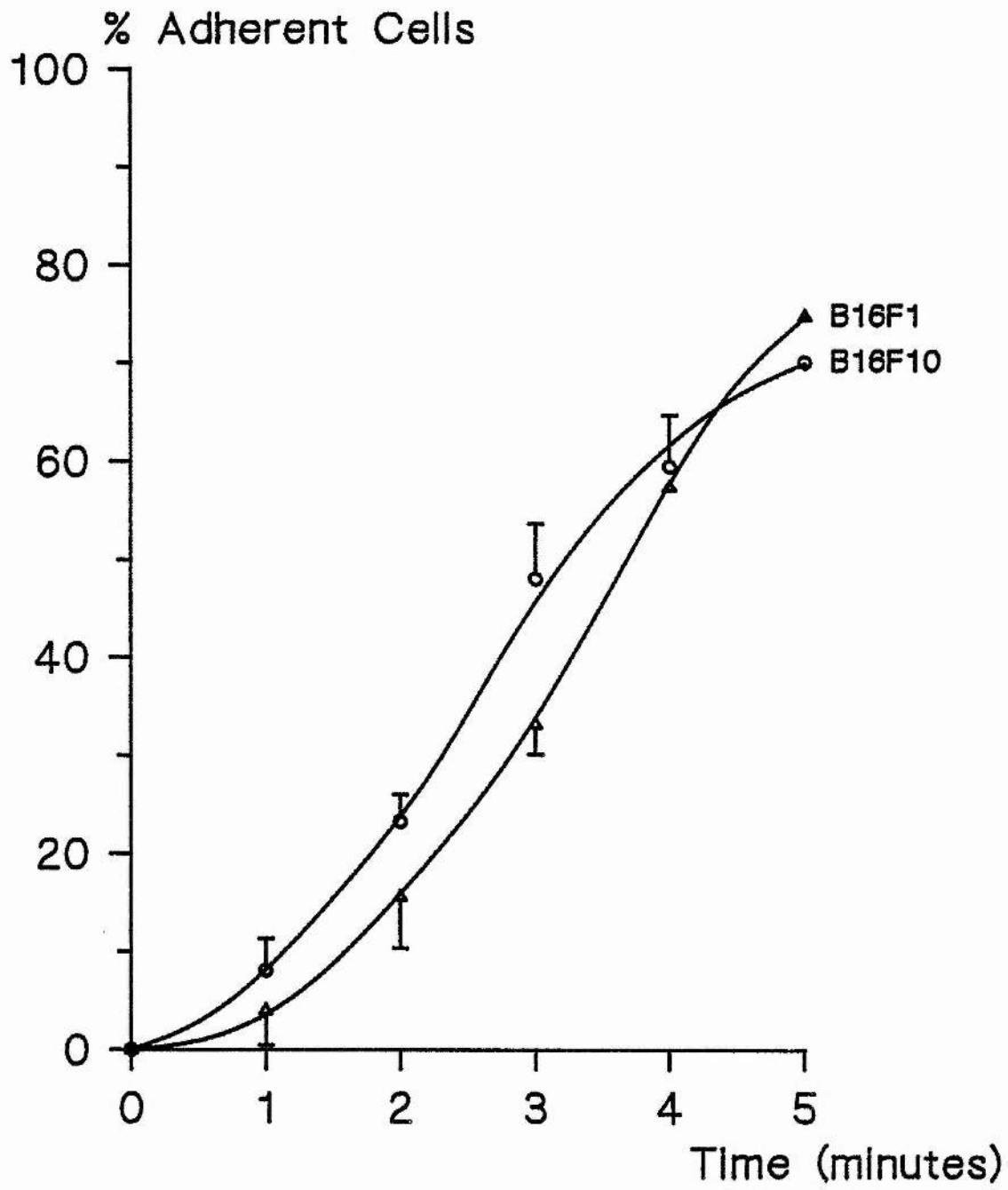


Figure 25: Adhesion of different tumour cell lines to fibronectin.

The adhesion of B16F1 and B16F10 cells to substrata containing 5 ug cm^{-2} of fibronectin was compared.
△ = B16F1 cells (n=3).
○ = B16F10 cells (n=4).

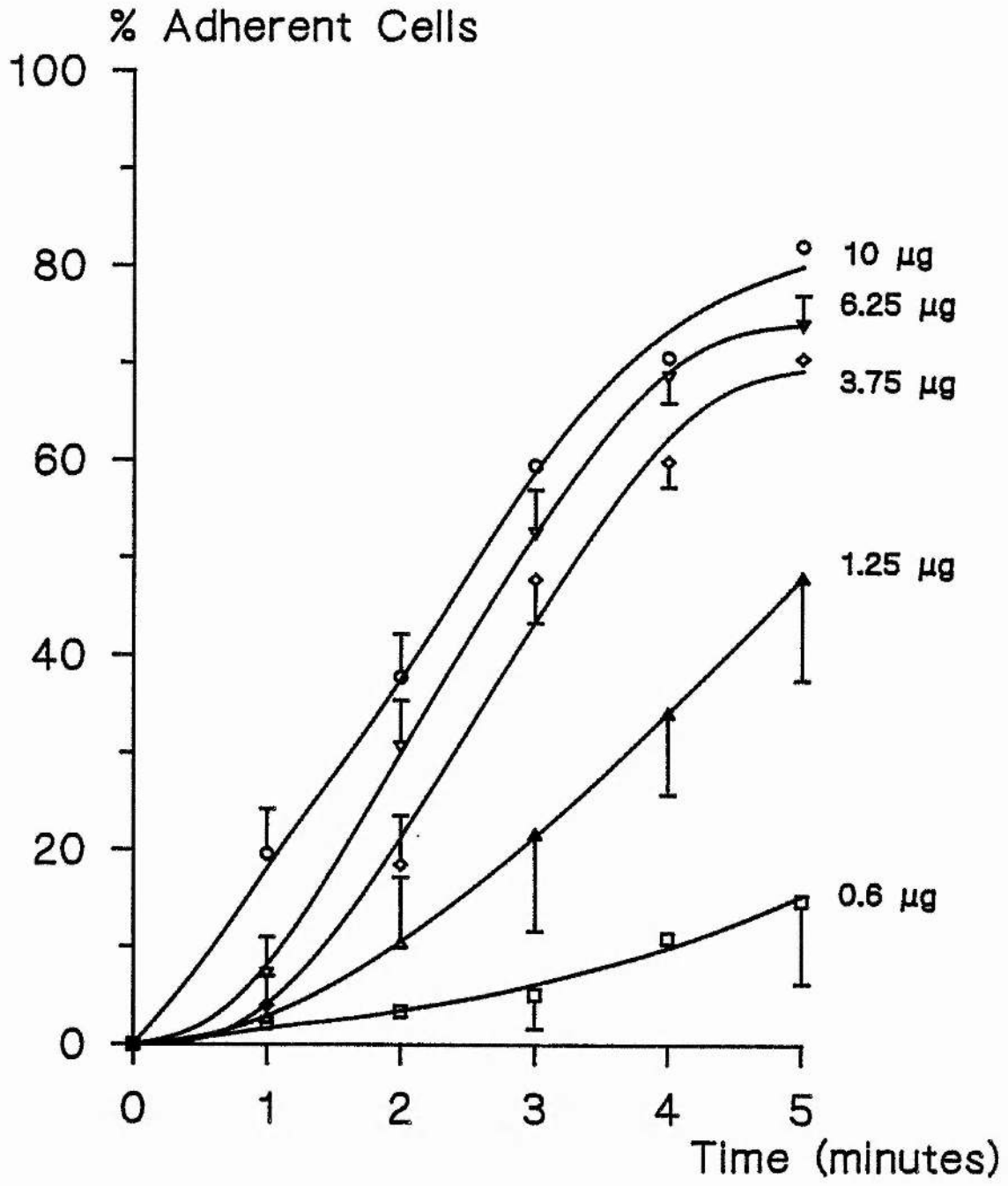


Figure 26: Adhesion of tumour cells to laminin: effect of ligand density.

Substrata with various amounts of adsorbed laminin were prepared and tested for their bility to support B16F10 cell adhesion.
□ = substrata containing 0.6 ug cm^{-2} of laminin (n=4).
△ = substrata containing 1.25 ug cm^{-2} of laminin (n=3).
◇ = substrata containing 3.75 ug cm^{-2} of laminin (n=3).
▽ = substrata containing 6.25 ug cm^{-2} of laminin (n=4).
○ = substrata containing 10 ug cm^{-2} of laminin (n=3).

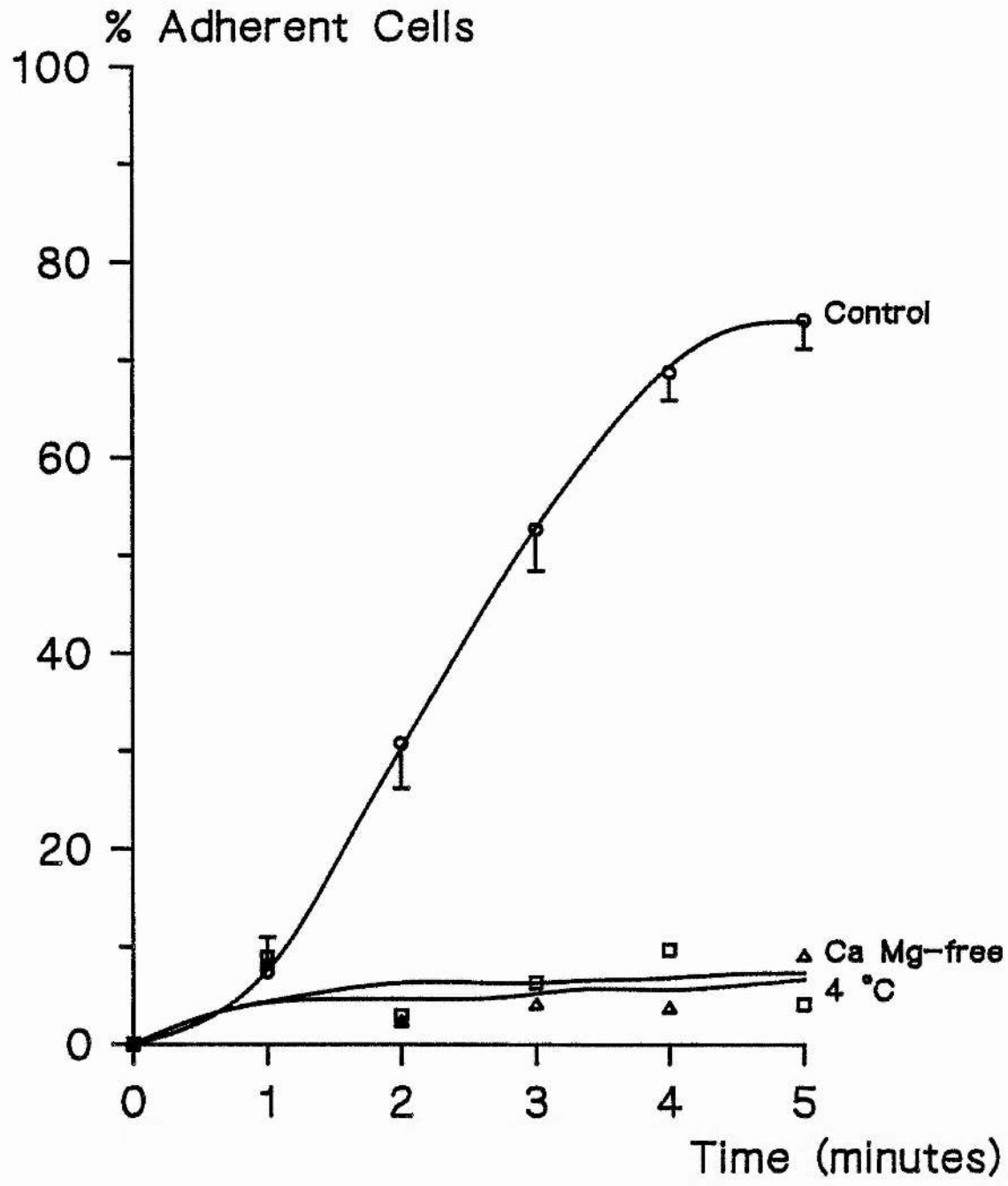


Figure 27: Adhesion of tumour cells to laminin: influence of temperature and divalent cations.

Substrata containing 6.25 ug cm^{-2} of laminin were prepared and their ability to support B16F10 cell adhesion in a medium depleted of divalent cations or at various temperatures was tested.

○ = tumour cells and substrata at 37°C (n=4).

□ = tumour cells and substrata at 4°C (n=1; 5 min n=3).

△ = tumour cells and substrata at 37°C in a Ca^{2+} and Mg^{2+}-free medium (n=1; 5 min n=3).

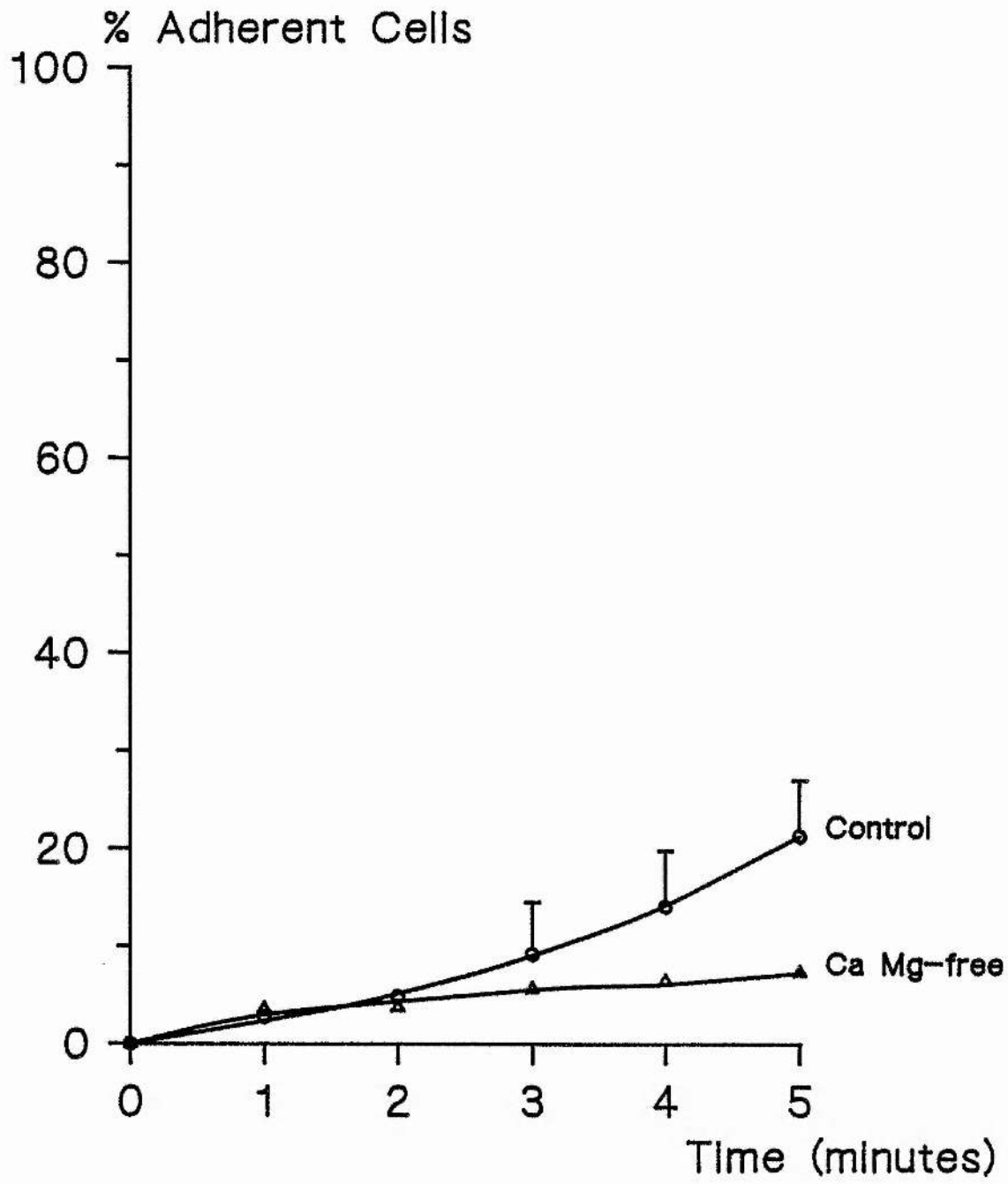


Figure 28: Adhesion of tumour cells to FCS: influence of divalent cations.

Substrata containing 100 ug cm^{-2} of FCS were prepared and their ability to support B16F10 cell adhesion in a medium depleted of divalent cations was tested.

○ = tumour cells and substrata in normal medium (n=5).

△ = tumour cells and substrata in a Ca^{2+} and Mg^{2+}-free medium (n=2; 5 min n=3).

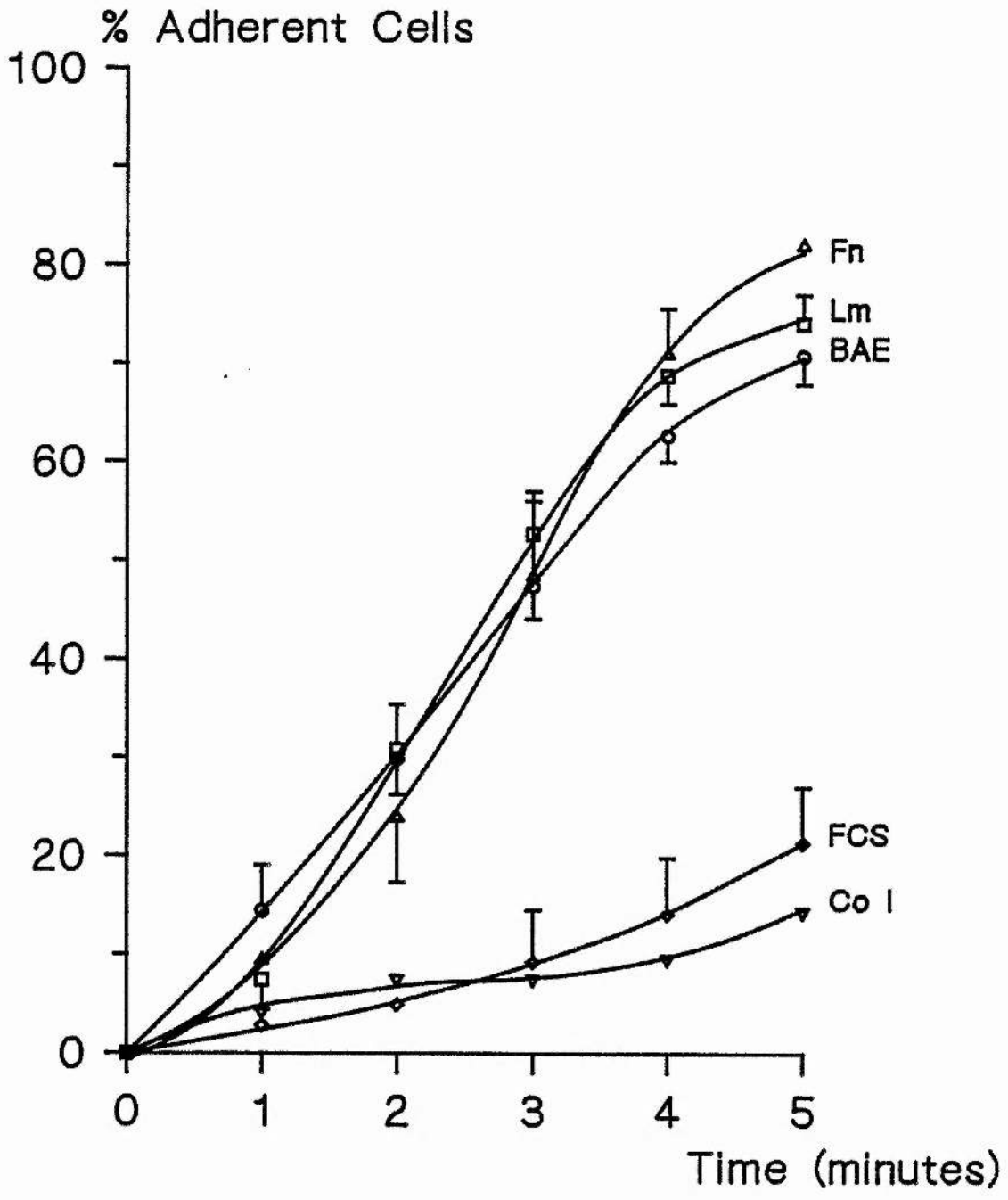


Figure 29: Tumour cells adhesion to different substrata.

Comparison of the ability of various substrata to support the adhesion of B16F10 cells.
△ = substrata containing 5 ug cm^{-2} of fibronectin (n=5).
□ = substrata containing 6.25 ug cm^{-2} of laminin (n=4).
○ = BAES monolayers (n=7).
◇ = substrata containing 100 ug cm^{-2} of FCS (n=5).
▽ = substrata containing 800 ug cm^{-2} of collagen type I (n=3).

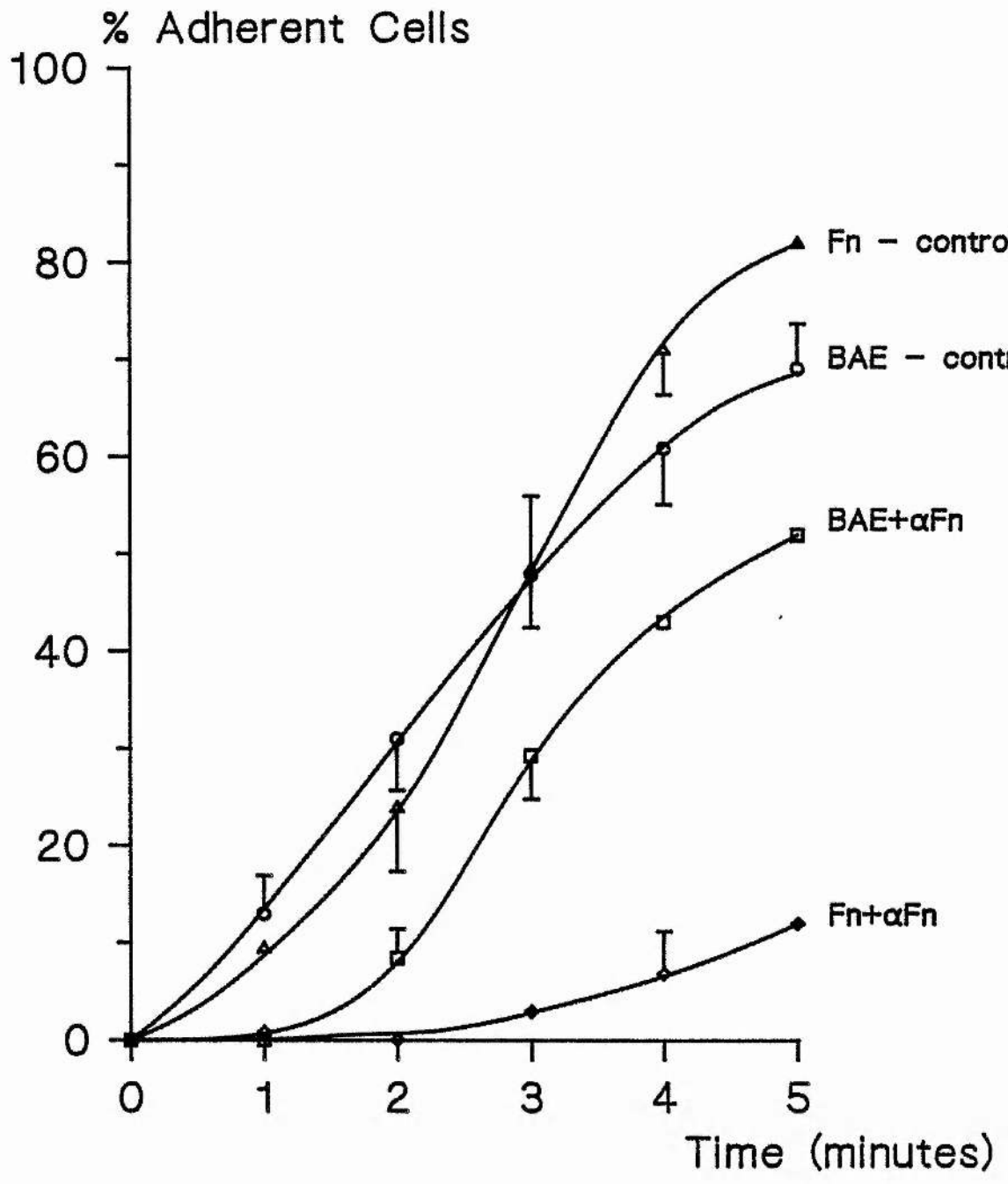


Figure 30: Adhesion of tumour cells to endothelium and fibronectin: effect of anti-fibronectin antibodies.

Substrata of fibronectin (5 ug cm^{-2}) or BAES monolayers were incubated for 1 hour with polyclonal anti-fibronectin antibodies, following which their ability to support the adhesion of B16F10 cells was tested in adhesion assays.
△ = fibronectin substrata not exposed to anti-fibronectin antibodies (n=3).
○ = endothelium not exposed to anti-fibronectin antibodies (n=4).
□ = endothelium exposed to anti-fibronectin antibodies (n=2).
◇ = fibronectin substrata exposed to anti-fibronectin antibodies (n=2).

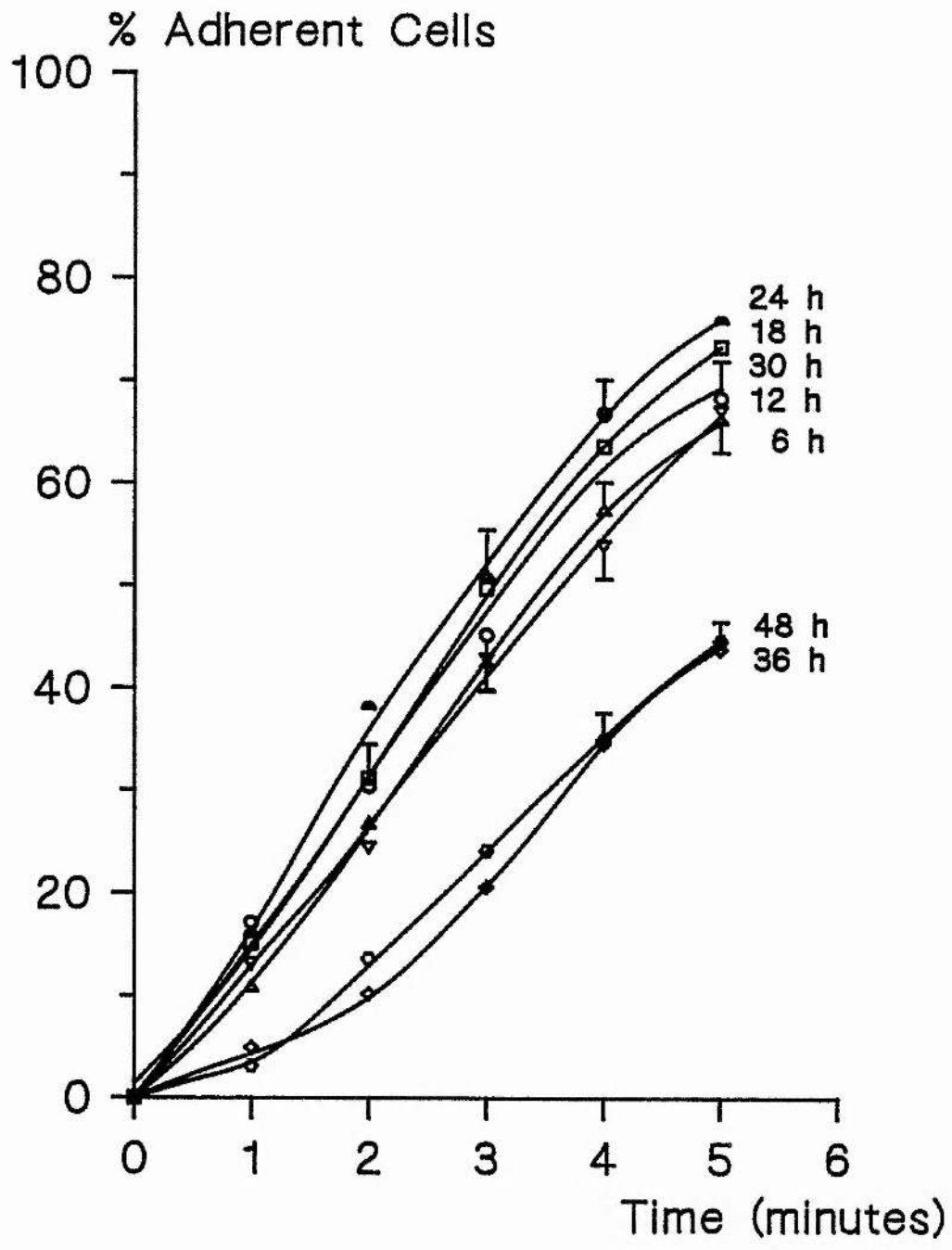


Figure 31: Adhesion of tumour cells to endothelium: influence of tunicamycin.

B16F10 cells were cultured in the presence of 0.45 ug ml^{-1} of tunicamycin for various lengths of time and their adhesion to BAES monolayers was subsequently tested.

△ = tumour cells exposed to tunicamycin for 6 hours (n=6).
▽ = tumour cells exposed to tunicamycin for 12 hours (n=6).
□ = tumour cells exposed to tunicamycin for 18 hours (n=5).
⌓ = tumour cells exposed to tunicamycin for 24 hours (n=4).
○ = tumour cells exposed to tunicamycin for 30 hours (n=4).
◇ = tumour cells exposed to tunicamycin for 36 hours (n=6).
○ = tumour cells exposed to tunicamycin for 48 hours (n=4).

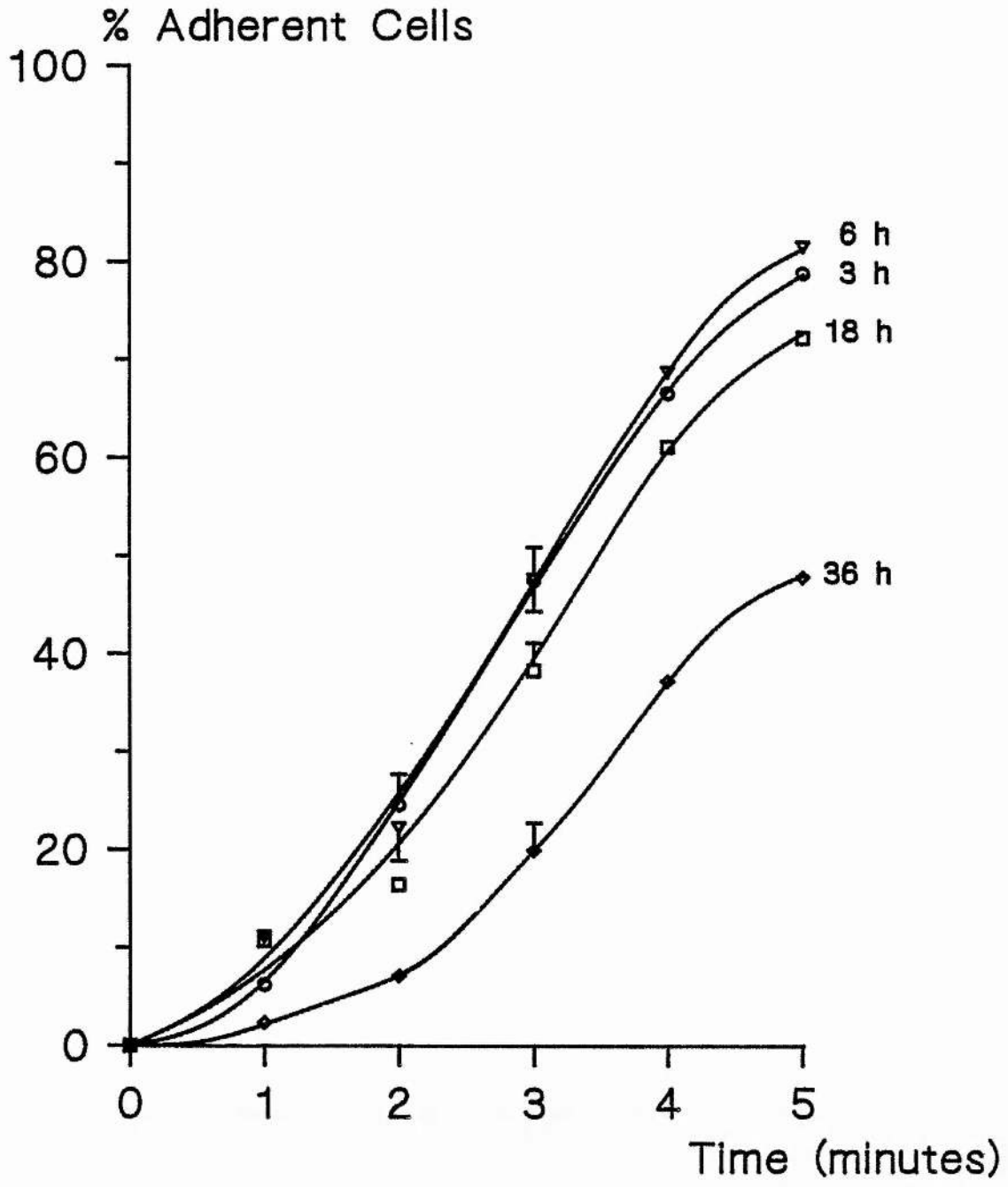


Figure 32: Adhesion of tumour cells to fibronectin: influence of tunicamycin.

B16F10 cells were cultured in the presence of 0.45 ug ml^{-1} of tunicamycin for various lengths of time and their adhesion to a plastic substratum coated with 5 ug cm^{-2} of fibronectin was subsequently tested.
○ = tumour cells exposed to tunicamycin for 3 hours (n=3).
▽ = tumour cells exposed to tunicamycin for 6 hours (n=3).
□ = tumour cells exposed to tunicamycin for 18 hours (n=5).
◇ = tumour cells exposed to tunicamycin for 36 hours (n=3).

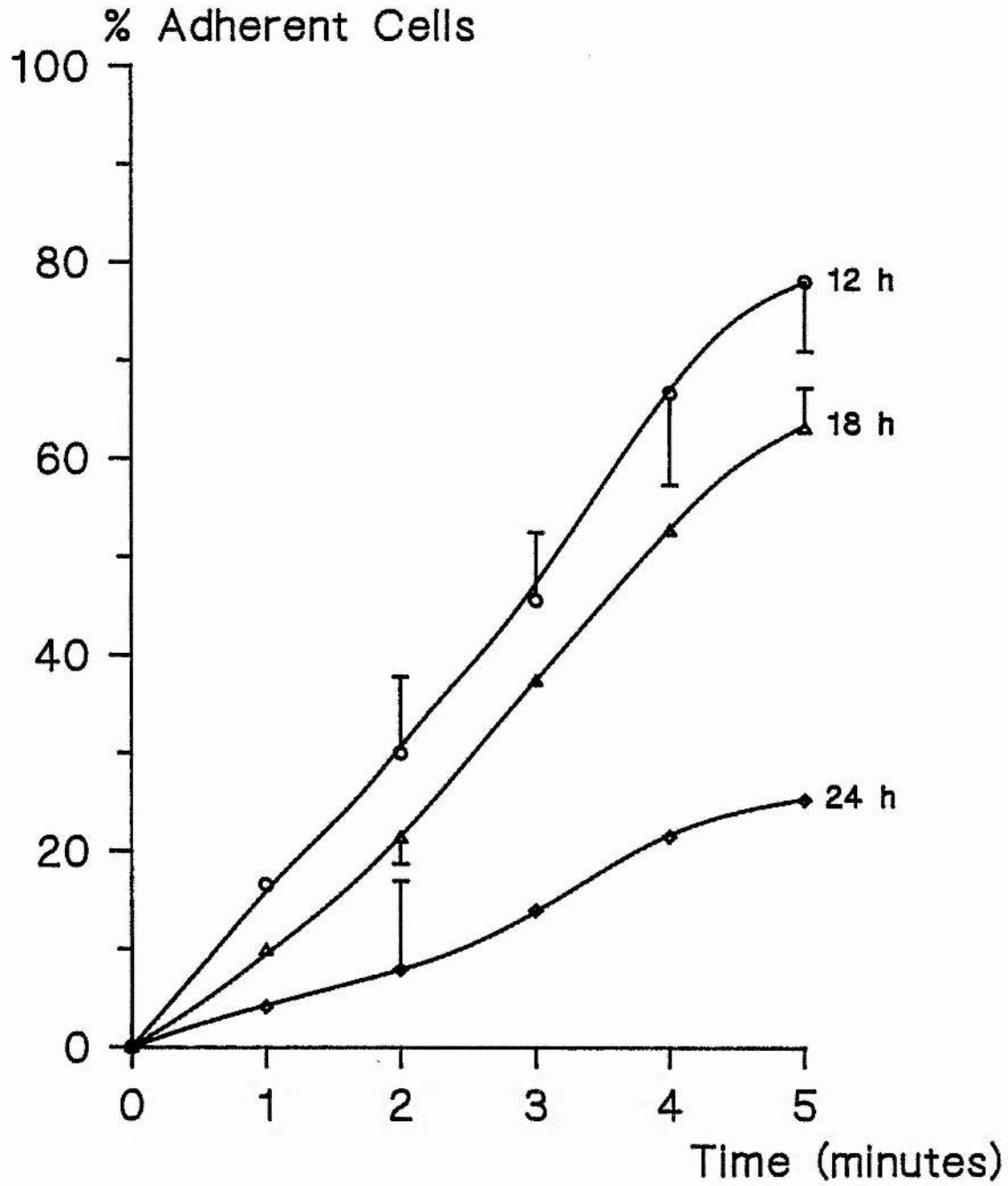


Figure 33: Adhesion of tumour cells to laminin: influence of tunicamycin.

B16F10 cells were cultured in the presence of 0.45 ug ml^{-1} of tunicamycin for various lengths of time and their adhesion to a plastic substratum coated with 6.25 ug cm^{-2} of laminin was subsequently tested.
○ = tumour cells exposed to tunicamycin for 12 hours (n=2).
△ = tumour cells exposed to tunicamycin for 18 hours (n=2).
◇ = tumour cells exposed to tunicamycin for 24 hours (n=3).

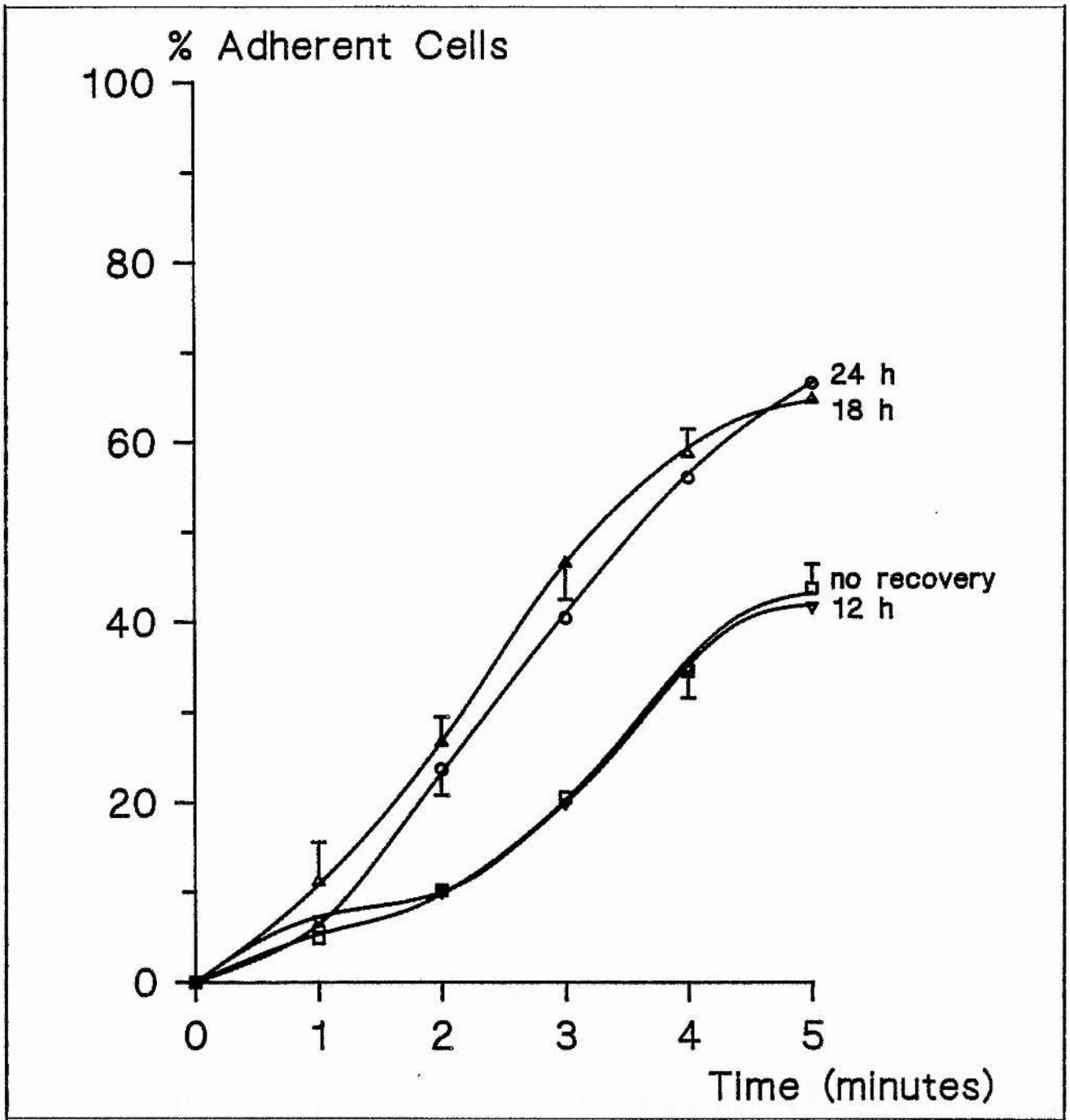


Figure 34: Adhesion of tumour cells to endothelium: recovery after exposure to tunicamycin.

B16F10 cells were cultured in the presence of 0.45 ug ml^{-1} of tunicamycin for 36 hours. Tunicamycin was then removed and the cells were allowed to recover for various lengths of time before testing their adhesion to BAES monolayers.

□ = tumour cells exposed to tunicamycin for 36 hours (n=6).
▽ = tumour cells exposed to tunicamycin for 36 hours and allowed to recover for 12 hours (n=5).
△ = tumour cells exposed to tunicamycin for 36 hours and allowed to recover for 18 hours (n=3).
○ = tumour cells exposed to tunicamycin for 36 hours and allowed to recover for 24 hours (n=4).

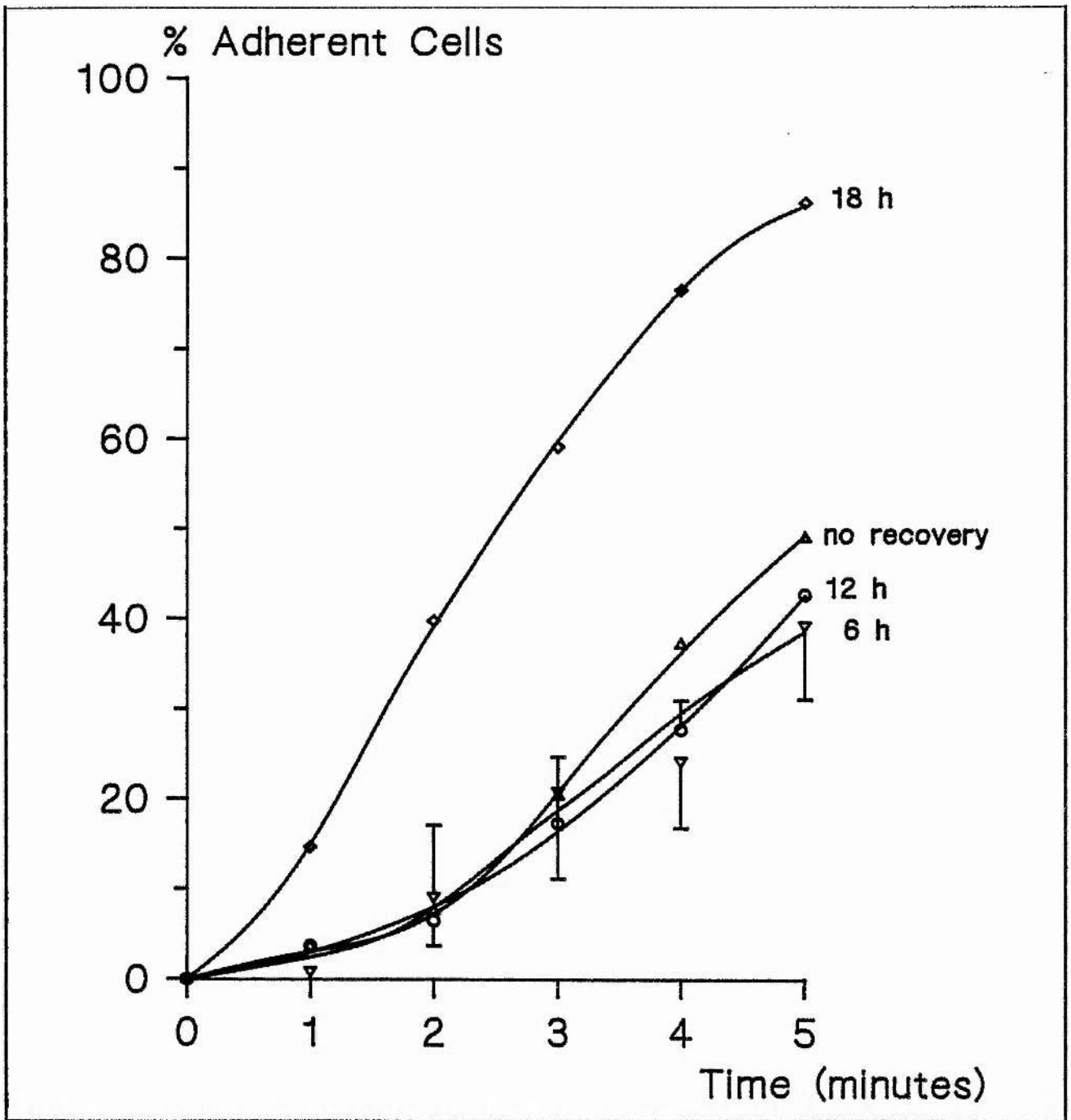


Figure 35: Adhesion of tumour cells to fibronectin: recovery after exposure to tunicamycin.

B16F10 cells were cultured in the presence of 0.45 ug ml^{-1} of tunicamycin for 36 hours. Tunicamycin was then removed and the cells were allowed to recover for various lengths of time before their adhesion to a plastic substratum coated with 5 ug cm^{-2} of fibronectin was tested.

△ = tumour cells exposed to tunicamycin for 36 hours (n=3).

▽ = tumour cells exposed to tunicamycin for 36 hours and allowed to recover for 6 hours (n=2).

○ = tumour cells exposed to tunicamycin for 36 hours and allowed to recover for 12 hours (n=3).

◇ = tumour cells exposed to tunicamycin for 36 hours and allowed to recover for 18 hours (n=3).

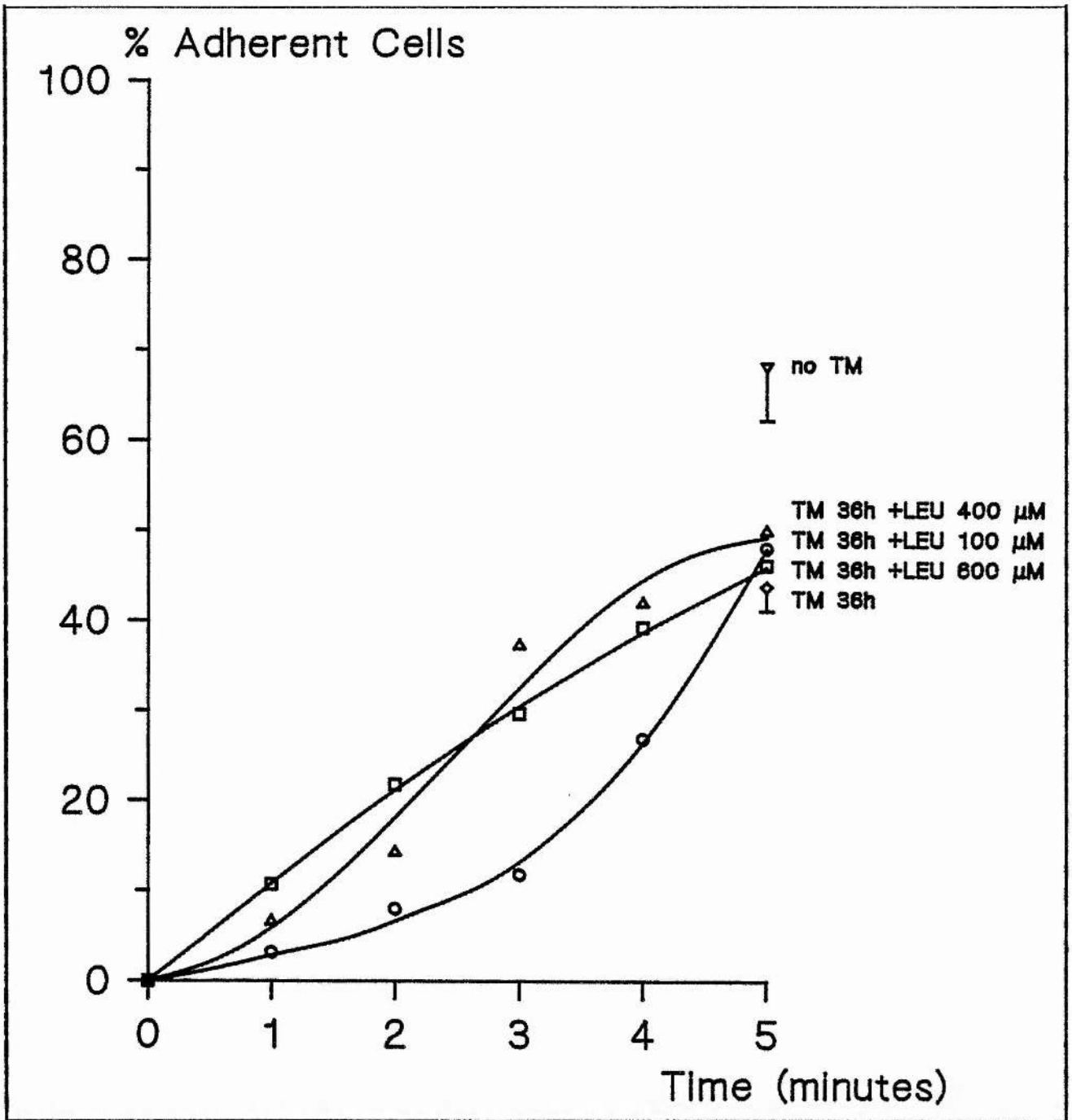


Figure 36: Adhesion of tumour cells to endothelium: protection from the effect of tunicamycin exposure by the protease inhibitor leupeptin.

B16F10 cells were cultured in the presence of 0.45 ug ml^{-1} of tunicamycin for 36 hours together with various concentrations of leupeptin. Their adhesion to a BAES monolayer was then tested.

▽ = tumour cells not exposed to tunicamycin (n=6).

◇ = tumour cells exposed to tunicamycin for 36 hours (n=6).

○ = tumour cells exposed to tunicamycin for 36 hours in the presence of leupeptin 100 uM (n=1; 5 min n=2).

△ = tumour cells exposed to tunicamycin for 36 hours in the presence of leupeptin 400 M (n=1; 5 min n=2).

□ = tumour cells exposed to tunicamycin for 36 hours in the presence of leupeptin 600 uM (n=1; 5 min n=2).

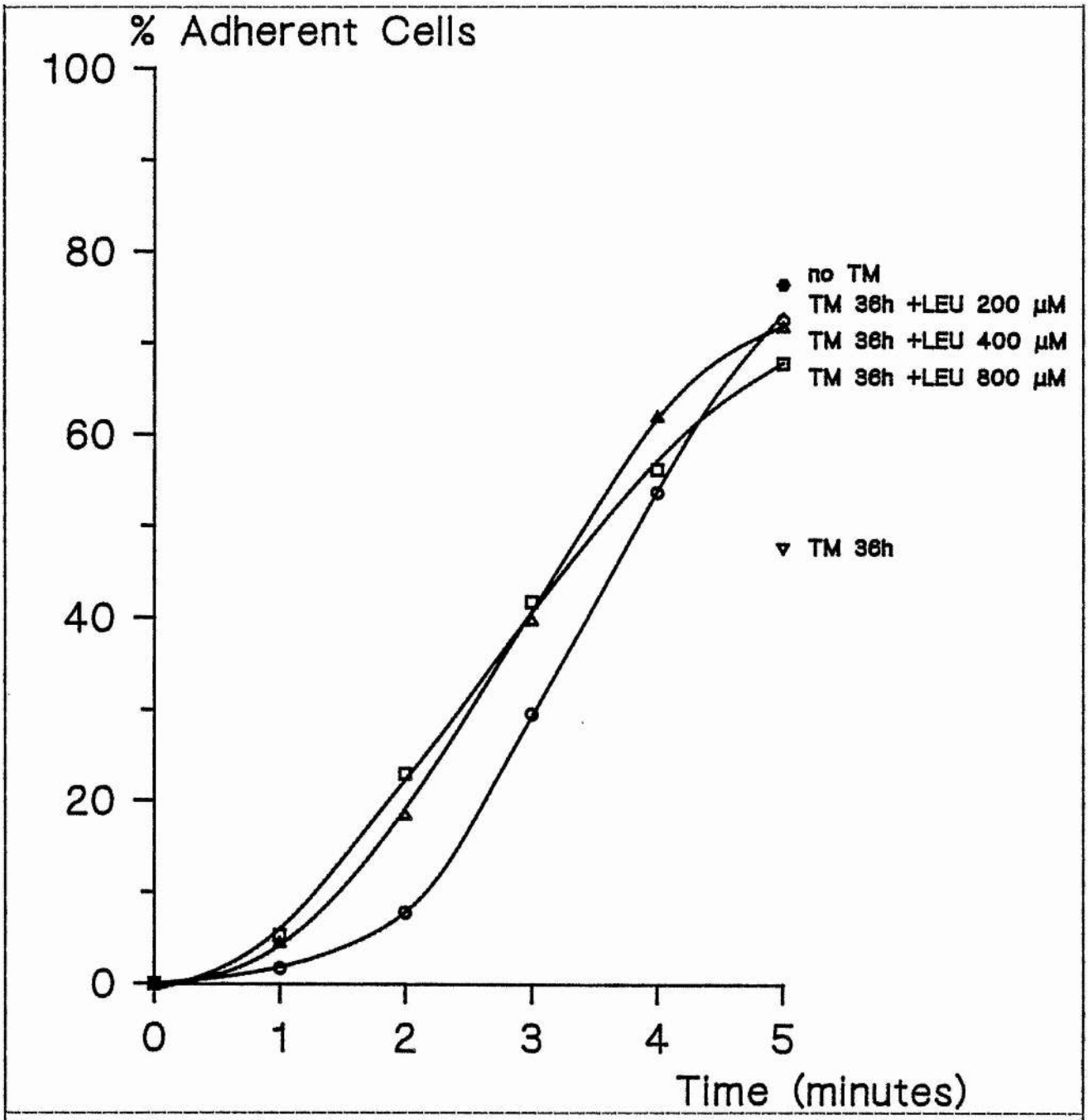


Figure 37: Adhesion of tumour cells to fibronectin: protection from the effect of tunicamycin exposure by the protease inhibitor leupeptin.

B16F10 cells were cultured in the presence of 0.45 ug ml^{-1} of tunicamycin for 36 hours together with different amounts of leupeptin. Their adhesion to a substratum coated with 5 ug cm^{-2} of fibronectin was then tested in adhesion assays.

● = tumour cells not exposed to tunicamycin (n=3).
▽ = tumour cells exposed to tunicamycin for 36 hours (n=3).
○ = tumour cells exposed to tunicamycin for 36 hours in the presence of leupeptin 200 uM (n=1; 5 min n=2).
△ = tumour cells exposed to tunicamycin for 36 hours in the presence of leupeptin 400 uM (n=1; 5 min n=2).
□ = tumour cells exposed to tunicamycin for 36 hours in the presence of leupeptin 600 uM (n=1; 5 min n=2).

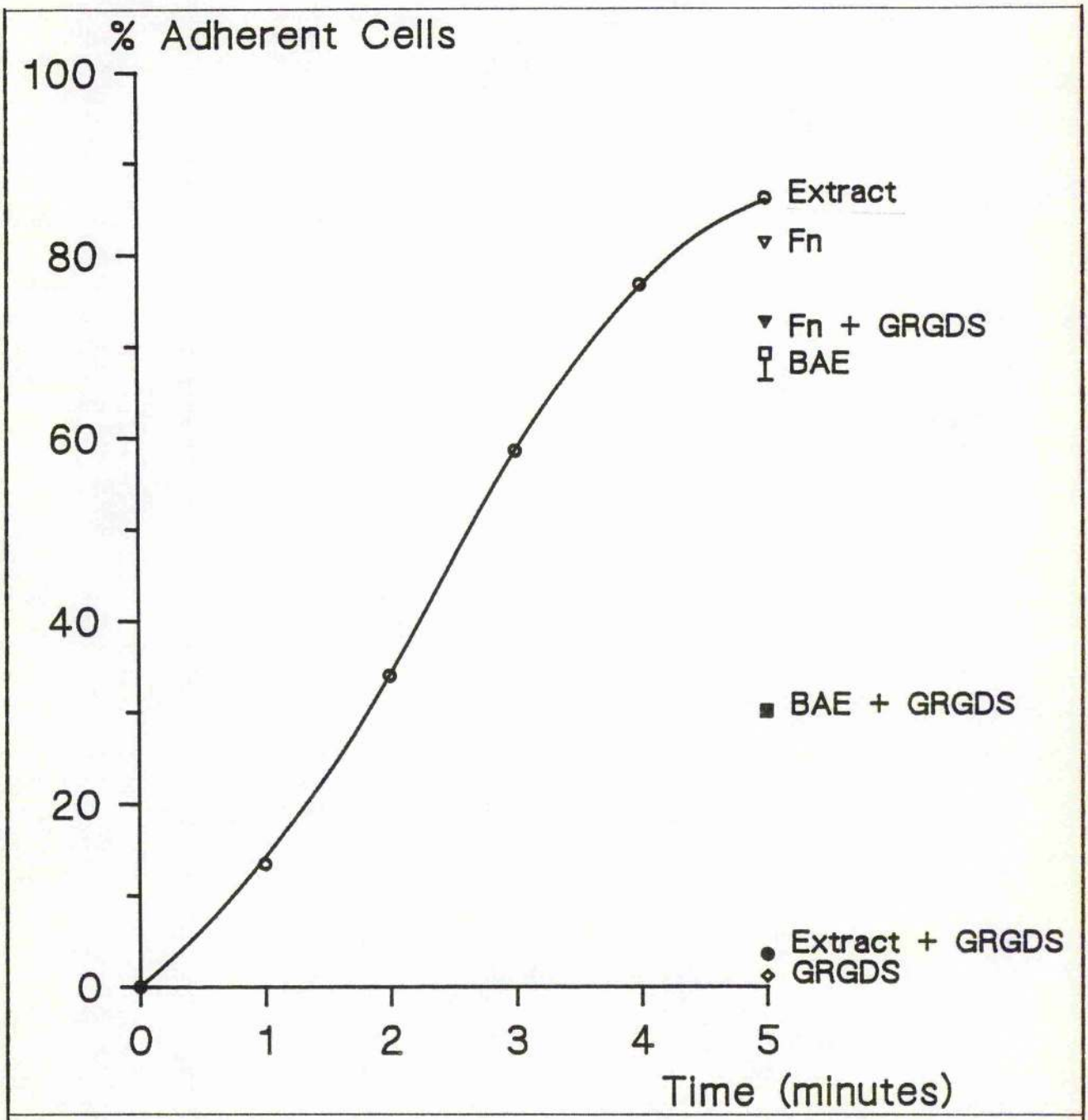


Figure 38: Adhesion of tumour cells to endothelial cell extract, fibronectin and endothelial monolayers: effect of the adhesive peptide GRGDS.

BAES monolayers and substrata containing 100 ug cm^{-2} of GRGDS, 5 ug cm^{-2} of fibronectin or 6 ug cm^{-2} of partially purified endothelial cell extract (1.0 M NACl elution of a Q-Sepharose column) were tested for their ability to support B16F10 cells adhesion. In some experiments the pentapeptide GRGDS was present during the assay.

○ = endothelial cell extract (n=3).
● = endothelial cell extract + 1 mg ml^{-1} GRGDS (n=3).
▽ = fibronectin (n=4).
▼ = fibronectin + 1 mg ml^{-1} GRGDS (n=2).
□ = BAES (n=7).
■ = BAES + 1 mg ml^{-1} GRGDS (n=2).
◇ = GRGDS (n=3).

Cell Spreading

Part 1: Background.

The adhesion assay was designed to assess the immediate responses of cells in suspension as they come into contact with the endothelium or various molecular substrata. The emphasis was placed on the very early phenomena occurring as the cell surface comes into close proximity with the substratum because it was assumed that in a dynamic environment such as the blood stream, cells would either rapidly engage in adhesive interactions with the vessel wall or they would be transported downstream by the flow, thereby missing the chance to extravasate.

During the first stages of adhesion cells retain their spherical shape. Although one of the prerequisites for adhesion is the alignment of the surface that confronts the substratum (probably so as to offer a larger area for interactions), the extent of such flattening is quite limited (Roos, 1984). Later events of cell-substratum interactions include the establishment of higher numbers of molecular points of adhesion, probably of varying nature and often this is accompanied by extensive re-organization of the cytoskeleton which causes the membrane of the cell to spread parallel to the substratum.

The spreading of cells onto a substratum is a slow event, taking several minutes to hours and not seconds. This timescale almost certainly exceeds the amount of time available to a circulating cell for initial adherence to the vessel wall in order to extravasate (cf. Bjerknes et al., 1986). Nevertheless, after adhering to the vessel wall, cells may need to

establish stronger interactions with it before being able to extravasate and this could be accompanied by changes in the cell shape so radical as to be detectable using low power light microscopy.

Although it may seem at first obvious that all molecular substrata that cause rapid adhesion will eventually support some degree of cell spreading, no systematic studies which correlate the two phenomena have so far been published. Furthermore, even if the supposition is proved right, the contrary may not hold true, namely that substrata unable to cause rapid adhesion will not support spreading, therefore it was felt that a spreading assay should be used to monitor this phenomenon.

Several spreading assays have been used in the past by many researchers, all differing in small details but using the same overall principles, and a considerable body of experience has been accumulated in the field (for a review see Silnutzer and Barnes, 1984 a). In order to carry out the proposed work it was necessary to have an assay that would allow the use of minimal quantities of the molecules to be tested. Together with this, it was thought to try and design the assay so that we could more confidently extract informations about the effect of the density of substratum-bound ligand on the entire cell population. At their best, most assays measure the number of cells spread per surface area rather than the percentage of spread cells. This is done by employing an excess of cells in relation to the surface area and, at the end of the incubation period, fixing and staining of the spread cells. This approach has the advantage of allowing the processing of a large number of samples in each experiment, the stained plates being counted at a later stage.

The disadvantages are obvious: firstly, spread cells may accidentally be detached from the substratum during the staining routine. Secondly, the presence of an excess of cells in suspension may increase the likelihood of cell-cell interactions and these could in turn interfere with the capacity to

interact with the substratum. Thirdly, cells may adhere and spread on already adhering cells or on areas of the substratum previously conditioned by other cells. Lastly, no information can be obtained on the effect of the substratum on the population as a whole.

To overcome these problems, quantification of the spreading at the end of the assay should be performed on live cells with the aid of phase contrast light microscopy rather than on fixed and stained preparations and the concentration of cells in the suspension should be kept as low as possible so that the chances of cell-cell collisions would be minimized.

A plastic substratum was preferred to glass in order to avoid derivatization of the glass which is quite time consuming and subject to day to day variability. In search of suitable tissue culture-grade plastic containers, 35 mm diameter Petri dishes and 24 well plates were discarded because of the large surface area that had to be adsorbed with molecules that were sometimes difficult to obtain in large quantities. Another disadvantage that comes with using large plates is that quantification of the assay needs the evaluation of a number of fields (usually between 5 and 10), since the relative percentage of spread and round cells vary quite considerably between different areas of the plate. In fact, ideally, the whole surface area should be counted, but this is in practice never done with large plates.

Plates with 96 wells, although possessing a much smaller surface area than Petri dishes or 24-well plates, have the disadvantage of a poor geometry for use with phase contrast light microscopy, so 72-well plates with a Terasaki configuration were chosen. The design of Terasaki plates, originally developed for immunological work (e.g. mixed lymphocyte reaction), has recently been improved by Gibco (Nunclon) with the introduction of tapering low profile walls that render the optical properties of the wells extremely good for use with phase contrasted light.

The wells have a very small surface area (0.01 cm^2) and a minimal volume (10 ul) and this makes them ideal for our assay. By using an appropriate magnification (between x 100 and x 160) the whole surface area of a well can be fitted into a microscopic field and all cells can be counted and scored for spreading with ease. Because of this we were in a position to inoculate into each well the minimum ammount of cells to obtain a significant count, reducing the possibility of cell-cell collisions. An added advantage of avoiding overcrowding is that the fewer the cells that were contained in the inoculum, the more single cells, either round or spread would be present on the substratum at the end of the assay, making the scoring fast and easy.

Part 2: Findings.

During the course of this study it was found that the spreading of B16F10 cells was dependent on the type of molecules adsorbed on the substratum and their concentration. Certain molecules, such as fibronectin, laminin and endothelial cell extract, induced more than 90% of cells to spread; these three substrata had similar values for the concentrations that induced half maximum (0.25, 0.25 and 0.2 ug cm^{-2} respectively) and maximum spreading (0.5, 0.6 and 0.4 ug cm^{-2} respectively) as shown in figures 39, 40 and 41. Other molecules, like gelatin, on the other hand, did not induce spreading at any of the concentrations used, as shown in figure 39. FCS did support the spreading of the tumour cells, although much higher concentrations were required (half max.= 0.5 ug cm^{-2}, max.= 3 ug cm^{-2}), as shown in figure 40. Spreading was not as successfull on haemoglobin, where the maximum spread (not more than 70 %) was achieved at concentrations above 3 ug

cm^{-2}, as shown in figure 41. Von Willebrand factor (0.001 to 1 ug cm^{-2}) did not support the spreading of tumour cells (results not shown).

The time dependent nature of spreading was investigated using substrata coated with supra- and sub-maximal concentrations of fibronectin (1.5 and 0.15 ug cm^{-2}), laminin (2.25 and 0.225 ug cm^{-2}) and endothelial cell extract (3 and 0.3 ug cm^{-2}). When supra-maximal cancentrations were used, the maximum amount of spreading was reached by 60 minutes. At submaximal concentrations the kinetics were slower and cell spreading continued even after 60 minutes of incubation. These results are summarized in figures 42, 43 and 44. High concentrations of gelatin did not elicit any spreading even after 90 minutes of incubation (see figure 42). In fact prolonging the assay on gelatin for up to 4 hours resulted in no spreading.

Since tumour cells produce and express ligands for gelatin (like fibronectin), the lack of spreading could be due to a defect in the adsorbtion of gelatin on the plastic substratum. To investigate this possibility, a "sandwich" experiment was performed in which 1 ug cm^{-2} of fibronectin was added to increasing concentrations of substratum adsorbed gelatin. As shown in figure 45, the tumour cells were now able to spread on the gelatin-fibronectin sandwich in a manner dependent on the concentration of gelatin adsorbed on the substratum. Because of these results, it was conceivable that tumour cell surface fibronectin was either poorly expressed or unable to mediate the spreading process. For this reason a further experiment was aimed at investigating the role of cell surface fibronectin during spreading: a substratum of anti-fibronectin antibodies was prepared and spreading was tested. Figure 46 shows that tumour cells spread well on these substrata in a concentration-dependent fashion (while a similar preparation of antibodies from non-immune sheep serum was unable to induce any spreading - results not shown). Adding

fibronectin to the antibodies (the soluble fibronectin binding to the adsorbed anti-fibronectin antibodies) resulted in a small but significant improvement in the amount of spreading ($p= 0.011$) as shown in figure 46.

The inhibitory effect of anti-fibronectin antibodies on the spreading of tumour cells were studied in a series of experiments where the antibodies were used to mask any molecules previously adsorbed on the plastic. The excess antibodies were then either washed away or left in the well and spreading assays were performed. Figure 47 shows two such experiments where a supra-maximal concentration of fibronectin was used to coat the wells and different amount of anti fibronectin antibodies were later added. Similarly to the results obtained with adhesion assays, the antibodies inhibited spreading of tumour cells on fibronectin and this inhibition was dose dependent. The presence of excess soluble antibodies during the assay resulted in further inhibition of spreading from 20-25 % to 0 %. The antibodies were tested on all the other molecular substrata that elicited adhesion and in all cases there was no effect on spreading when the excess soluble antibodies were removed, while there was a similar dose dependent effect when the excess antibodies were left in the wells (results not shown, but an example is given in figure 48). FCS-coated substrata constituted the only exception: as shown in figure 49, substrata with different concentrations of adsorbed FCS were incubated with 50 ug cm^{-2} of anti-fibronectin antibodies; when the soluble unbound antibodies were removed there was a dose independent increase of spreading to values of around 90 % or more. When, however, the unbound soluble antibodies were left into the wells, the usual dose-dependent inhibition was seen.

In other experiments, the effect of purification of the endothelial cell extract on the ability to induce spreading was studied. When spreading on the crude endothelial cell extract was compared to that on the extract purified with ion exchange (one step elution with 1.0 M NaCl), no

significant difference was found (multivariate analysis of variance, $p > 0.09$), although the purified extract gave more reproducible results (compare figures 39 and 50). Such partially purified cell extract was fractionated using ion exchange chromatography by means of a linear gradient of NaCl between 0.075 and 1.0 M, and each fraction was tested for its ability to support the spreading of B16F10 cells. Only two fractions (eluted with 0.3 M and 0.9 M NaCl respectively) showed any spreading inducing activity and their dose response curves are plotted in figure 50. Multivariate analysis of variance show no significant different between the the material eluted at 0.3 M NaCl and that eluted at 0.9 N NaCl ($p=0.99$), while both are significantly different from the (one step) 1.0 M NaCl elution and the unpurified extract ($p<0.001$).

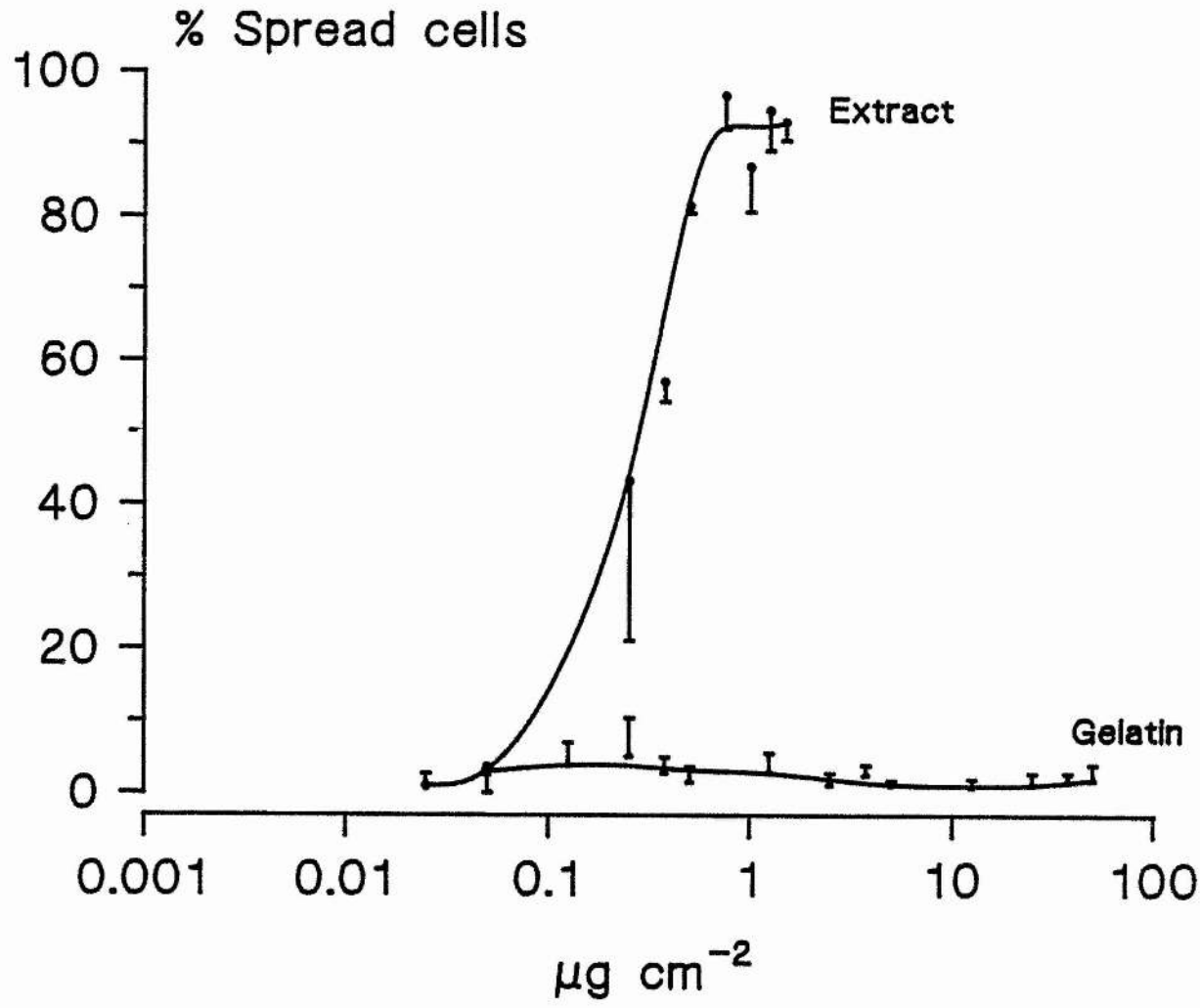


Figure 39: Spreading of tumour cells on substrata of gelatin and endothelial cell extract: dose dependent responses.

Substrata were coated with different concentrations of the molecules to be tested and their ability to support the spreading of B16F10 cells was tested.
● = endothelial cell extract (not purified, n=5).
▲ = gelatin (n=3).

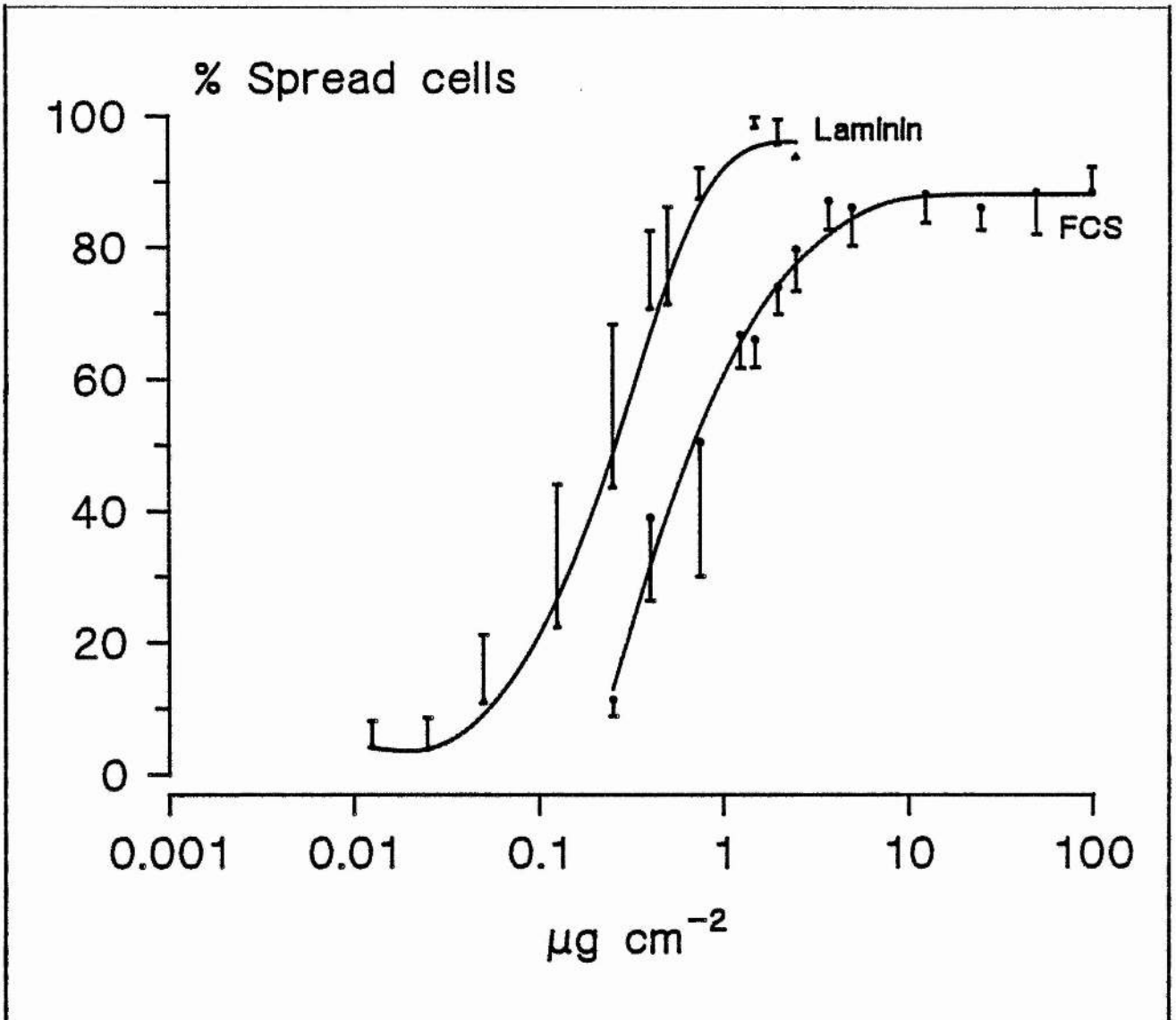


Figure 40: Spreading of tumour cells on substrata of laminin and FCS: dose dependent responses.

Substrata were coated with different concentrations of the molecules to be tested and their ability to support the spreading of B16F10 cells was tested.

▲ = laminin (n=6).

● = FCS (n=5).

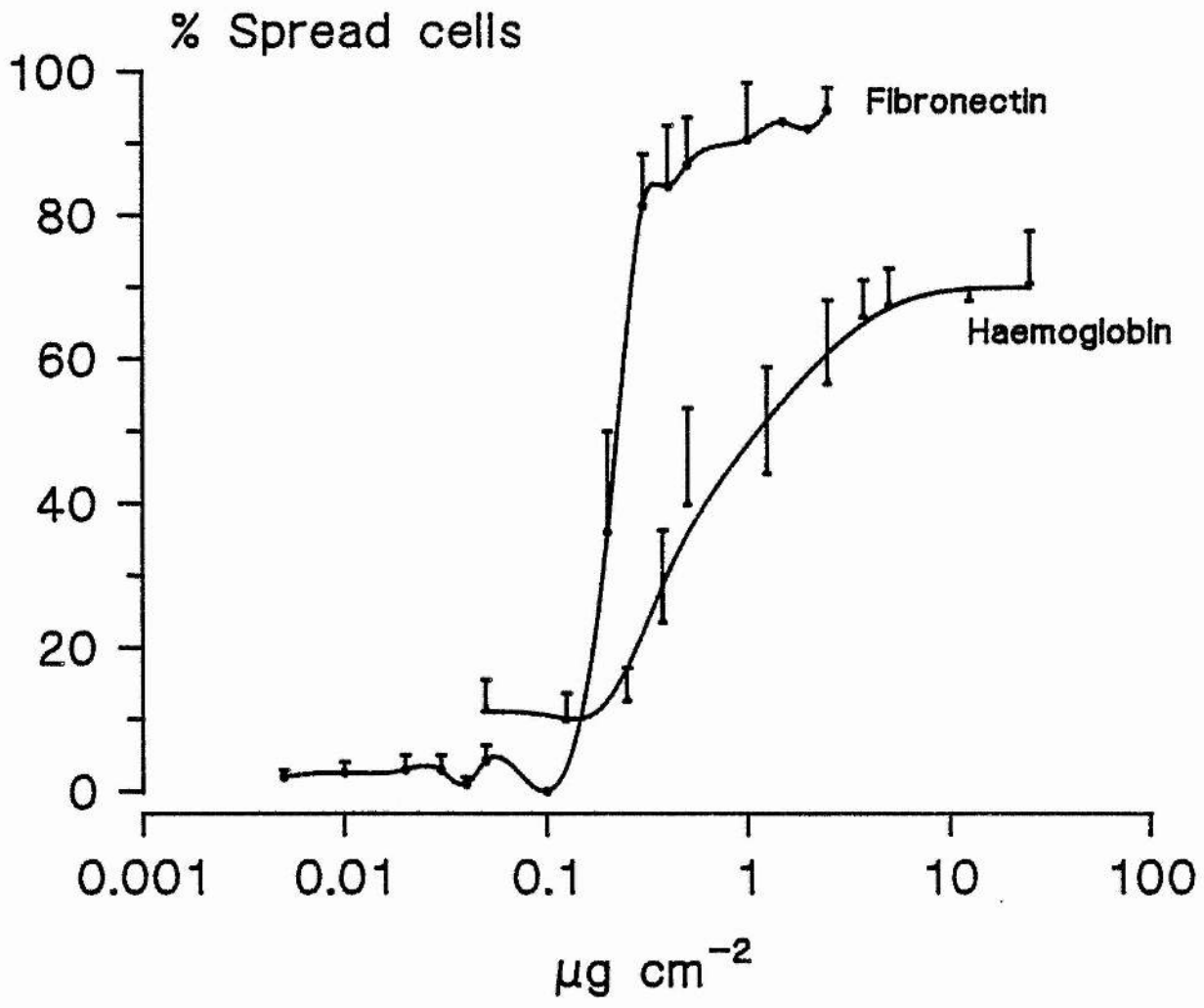


Figure 41: Spreading of tumour cells on substrata of fibronectin and haemoglobin: dose dependent responses.

Substrata were coated with different concentrations of the molecules to be tested and their ability to support the spreading of B16F10 cells was tested.
● = fibronectin (n=3).
▲ = haemoglobin (n=3).

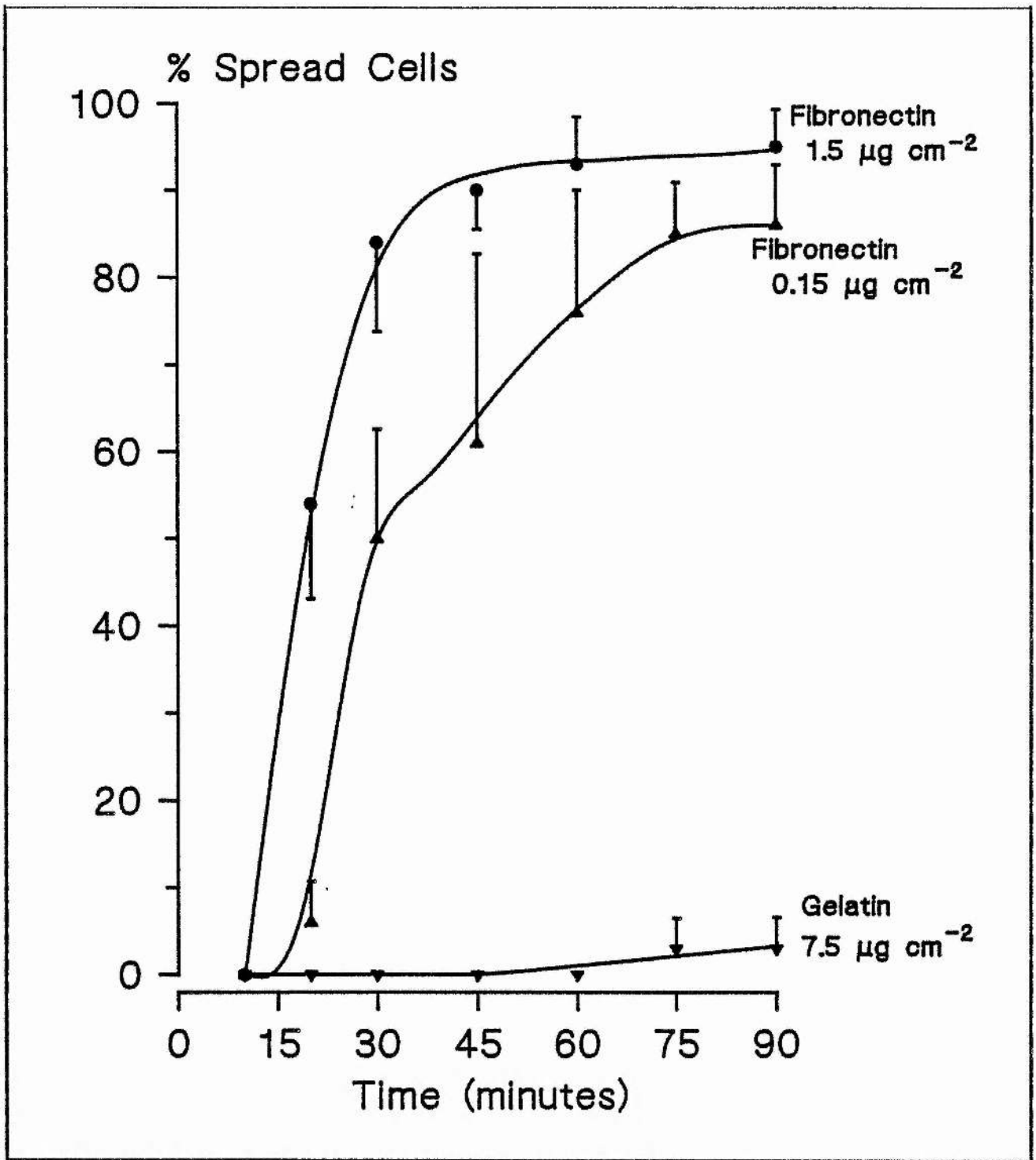


Figure 42: Spreading of tumour cells on fibronectin and gelatin: time dependent responses.

Substrata were coated with gelatin or with two different concentrations of fibronectin and their ability to support the spreading of B16F10 cells was tested.

● = fibronectin 1.5 ug cm^{-2} (n=6).
▲ = fibronectin 0.15 ug cm^{-2} (n=5).
▼ = gelatin 7.5 ug cm^{-2} (n=5).

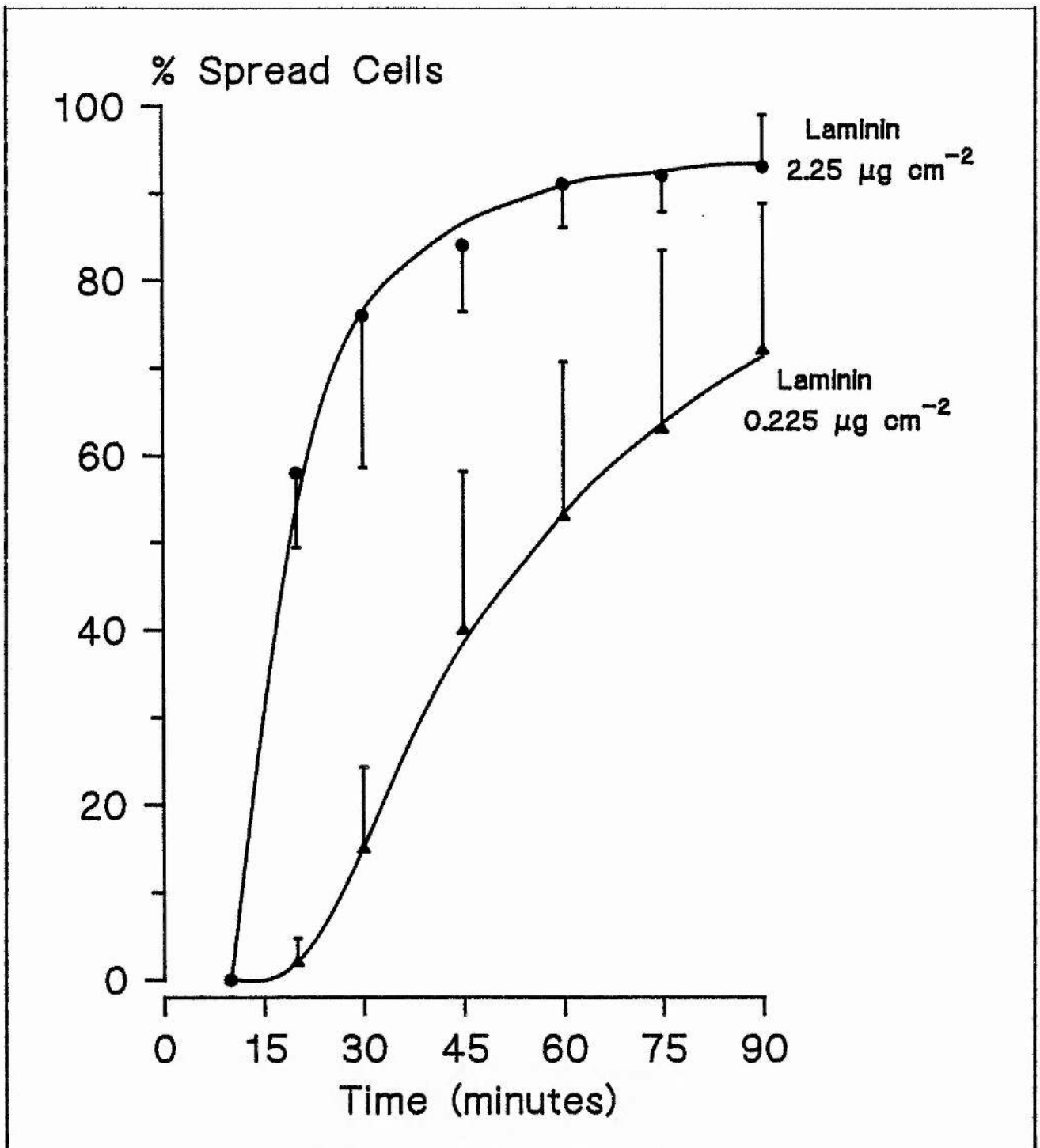


Figure 43: Spreading of tumour cells on laminin: time dependent responses.

Substrata were coated with two different concentrations of laminin and their ability to support the spreading of B16F10 cells was tested.
● = laminin 2.25 ug cm^{-2} (n=5).
▲ = laminin 0.225 ug cm^{-2} (n=5).

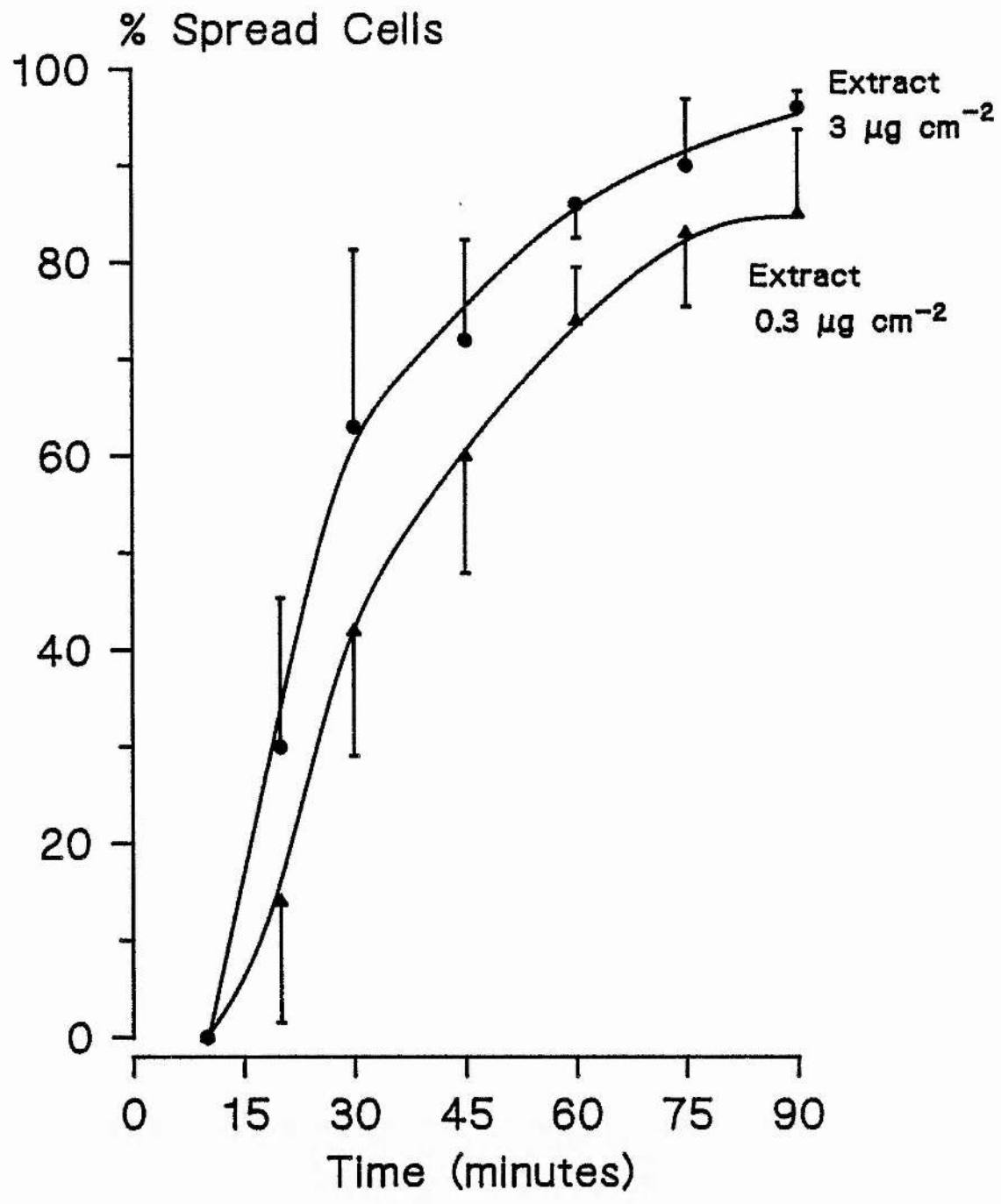


Figure 44: Spreading of tumour cell on endothelial cells extract: time dependent responses.

Substrata were coated with different concentrations of endothelial extract partially purified through a one step elution (1.0 M NaCl) from Q-Sepharose. Their ability to support the spreading of B16F10 cells was tested.
● = endothelial cell extract 3 ug cm^{-2} (n=3).
▲ = endothelial cell extract 0.3 ug cm^{-2} (n=3).

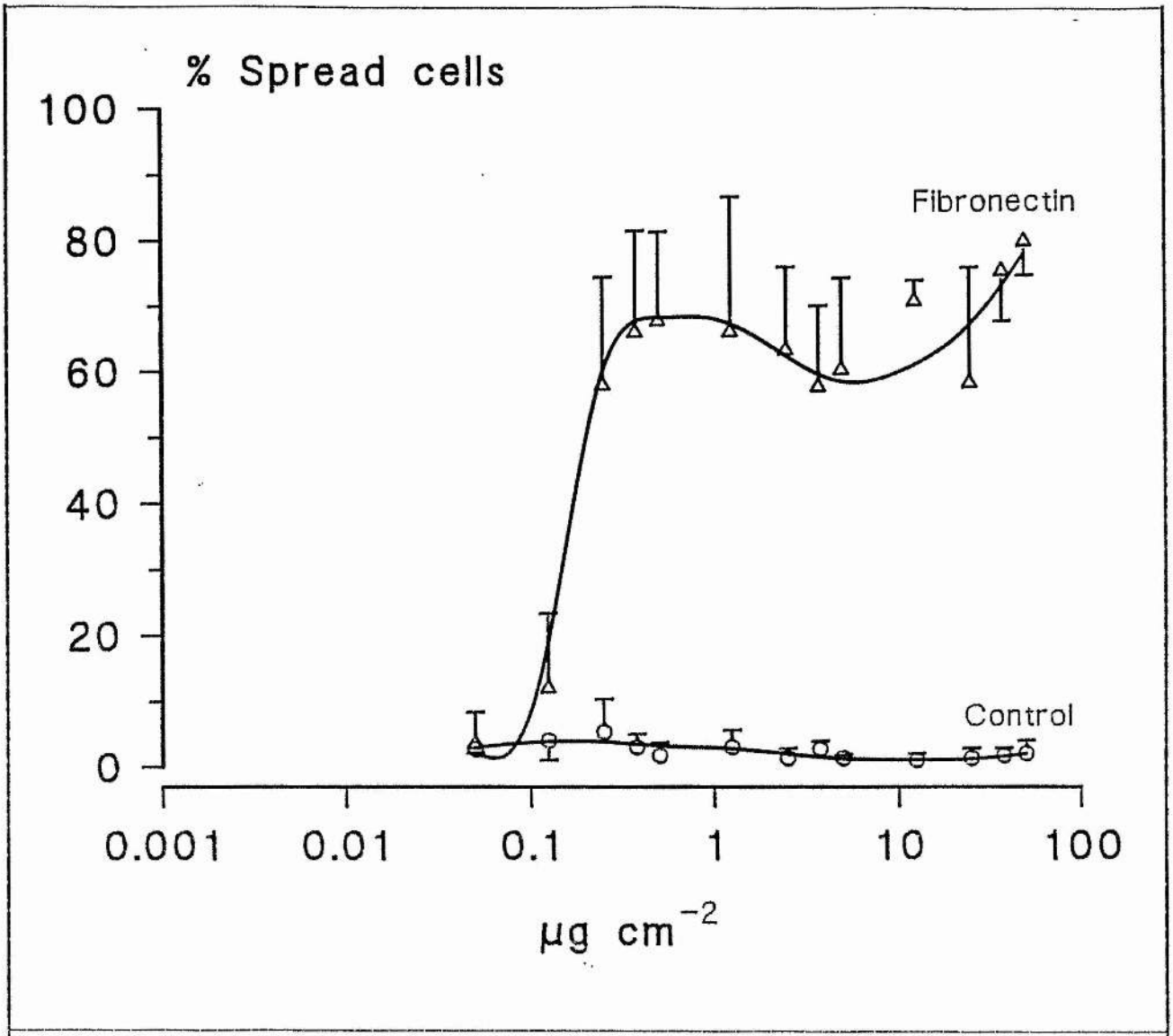


Figure 45: Spreading of tumour cells on gelatin: effect of fibronectin.

Substrata were coated with different concentrations of gelatin and, after blocking the remaining reactive sites on the plastic, the wells were incubated with fibronectin (1 ug cm ^{2}). The abilities of these substrata to support the spreading of B16F10 cells were then tested.
o = dose of gelatin, no fibronectin added (n=3).
Δ = dose of gelatin + fibronectin (n=3).

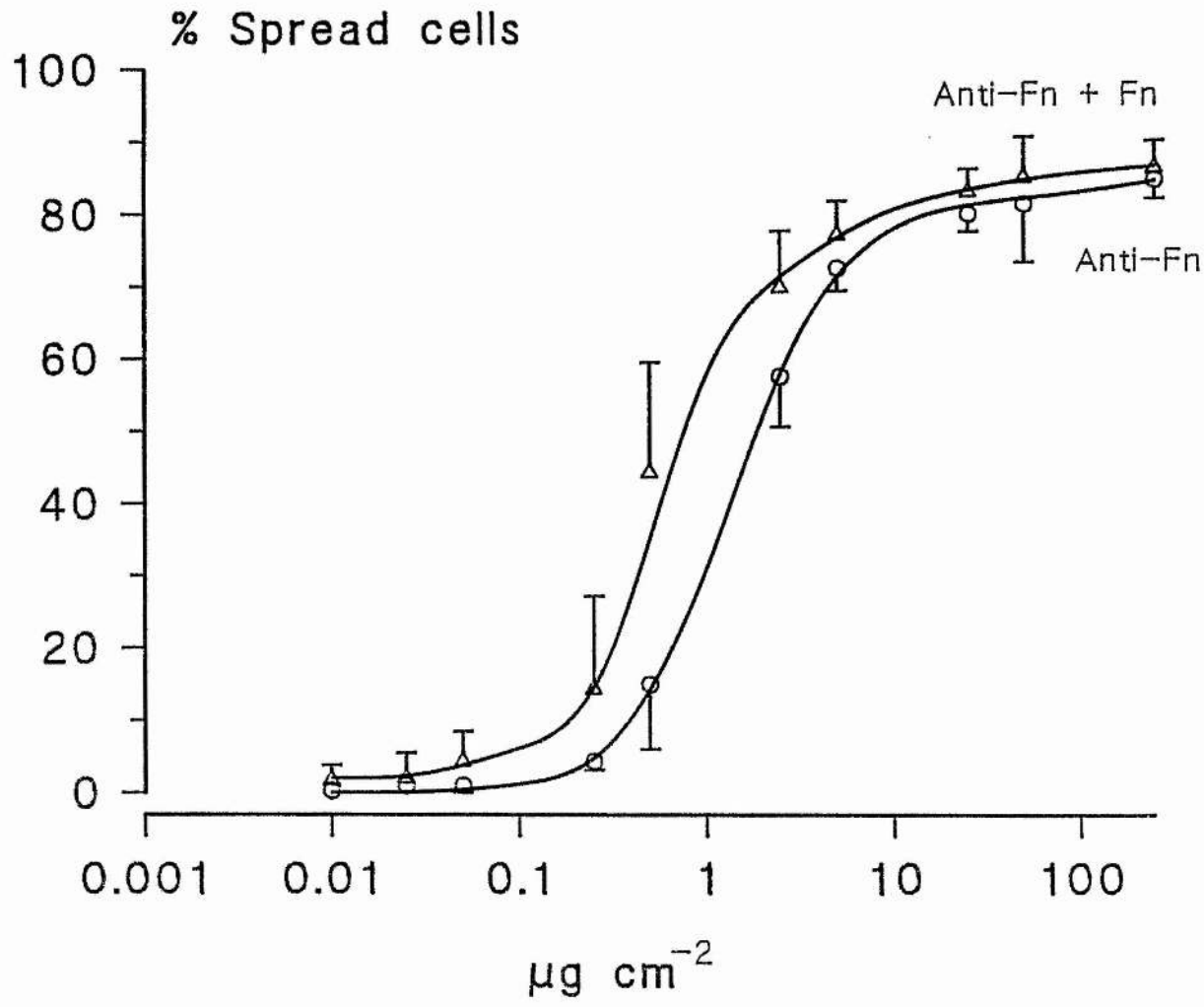


Figure 46: Spreading of tumour cells on anti-fibronectin antibodies.

Substrata were coated with different concentrations of anti-fibronectin antibodies. After blocking the remaining reactive sites on the plastic, the wells were incubated with 1 ug cm^{2} of fibronectin. The abilities of these substrata to support the spreading of B16F10 cells were then tested.
o = dose of anti-fibronectin antibodies (n=3).
Δ = dose of anti-fibronectin antibodies + fibronectin (n=3).

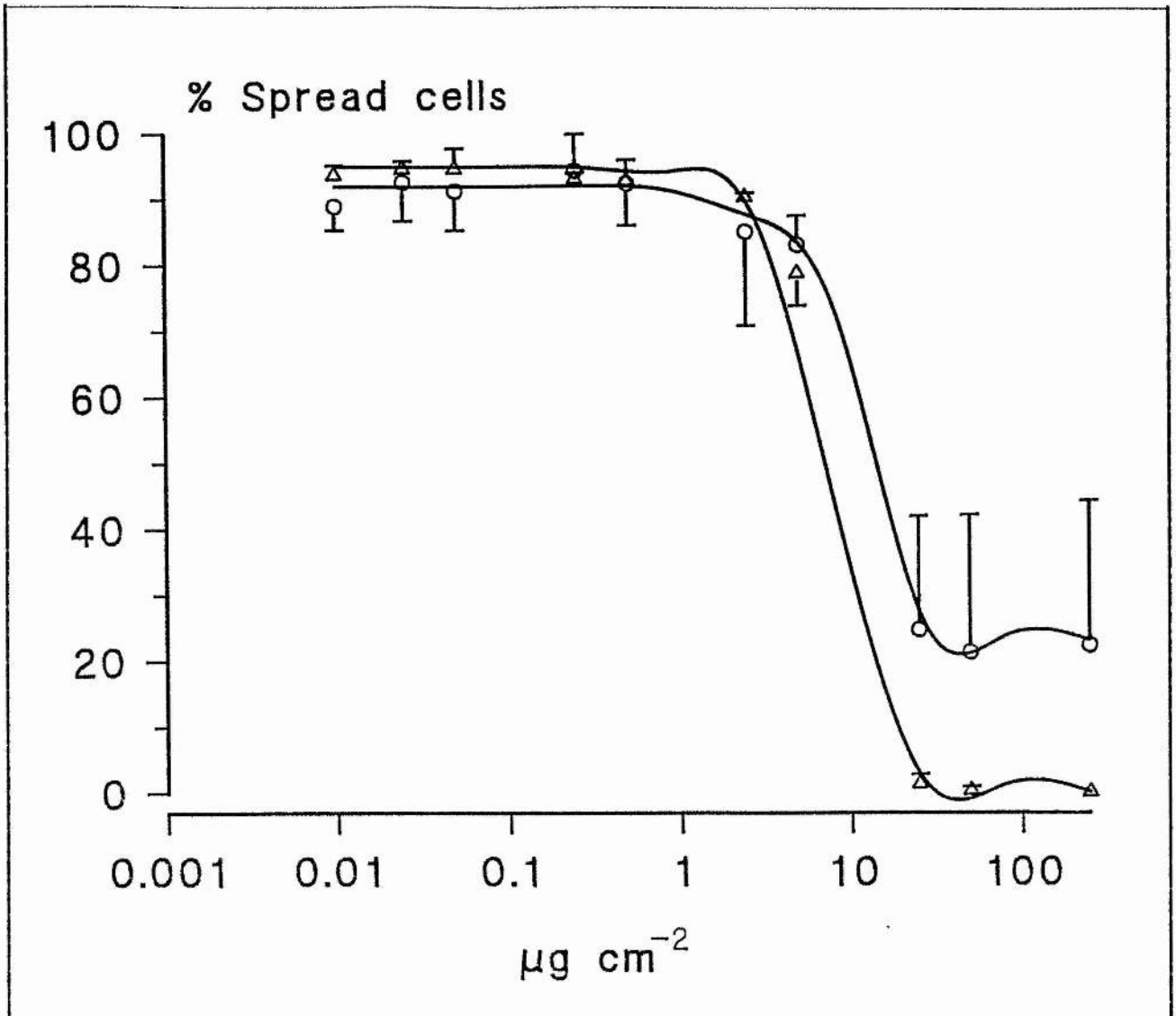


Figure 47: Spreading of tumour cells on fibronectin: effect of polyclonal anti-fibronectin antibodies.

Substrata were coated with 1 ug cm^{2} of fibronectin. After blocking the remaining reactive sites on the plastic, the wells were incubated with different concentrations of polyclonal anti-fibronectin antibodies. The abilities of these substrata to support the spreading of B16F10 cells were then tested.

O = fibronectin + dose of polyclonal anti-fibronectin. The unbound antibodies were removed before the assay (n=3).

Δ = fibronectin + dose of polyclonal anti-fibronectin. The unbound antibodies were not removed from the wells (n=2).

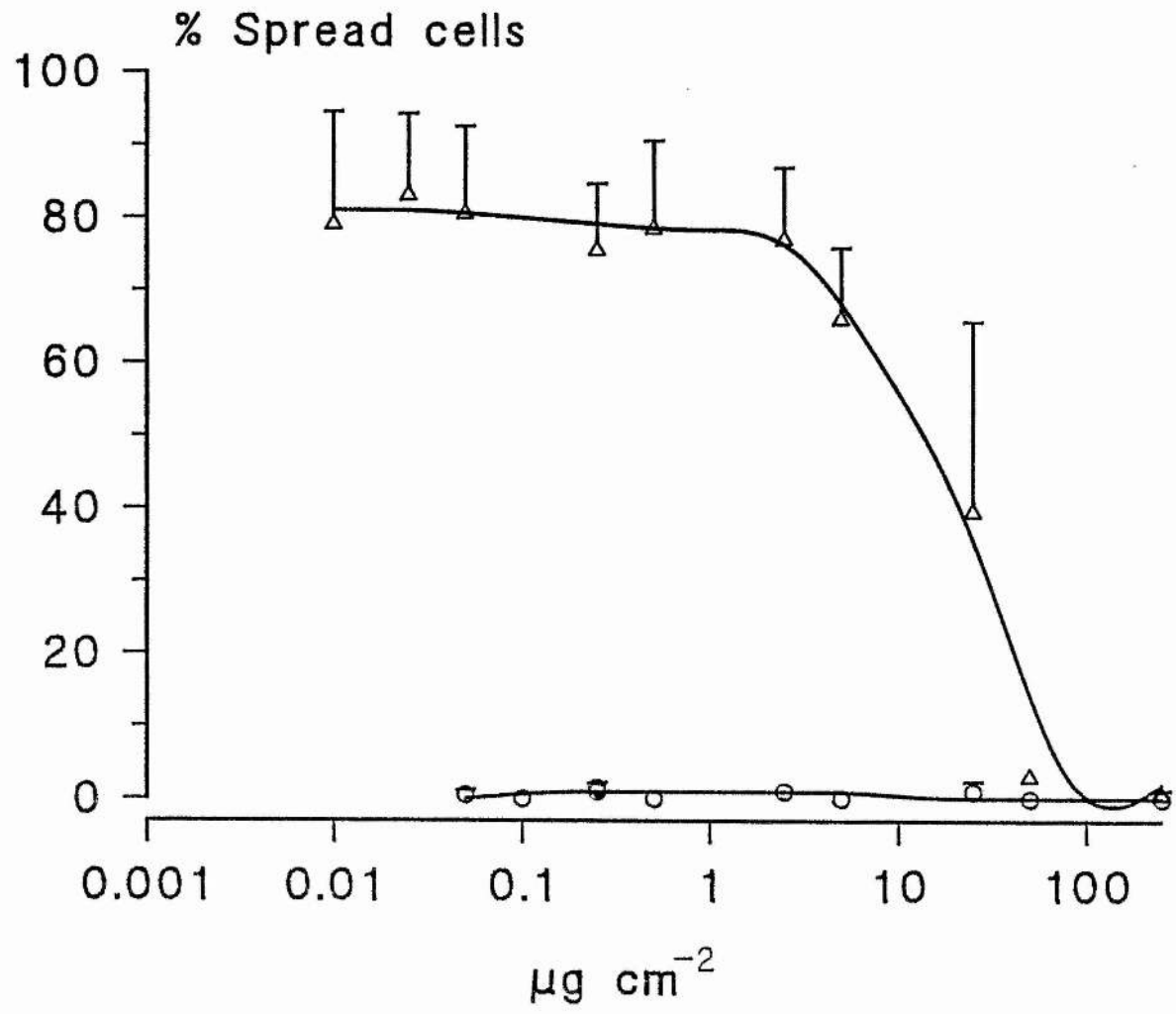


Figure 48: Spreading of tumour cells on anti-fibronectin antibodies: effect of anti-fibronectin antibodies.

Substrata were coated with different concentrations of anti fibronectin antibodies. After blocking the remaining reactive sites on the plastic, the wells were incubated with different concentrations of polyclonal anti-fibronectin antibodies. The ability of these substrata to support the spreading of B16F10 cells were then tested.

Δ = 50 ug cm^{-2} of anti-fibronectin antibodies + dose of anti-fibronectin antibodies. The unbound second antibodies were not removed from the wells (n=3).

o = dose of anti-fibronectin antibodies + 50 ug cm^{-2} of anti- fibronectin antibodies. The unbound second antibodies were not removed from the wells (n=3).

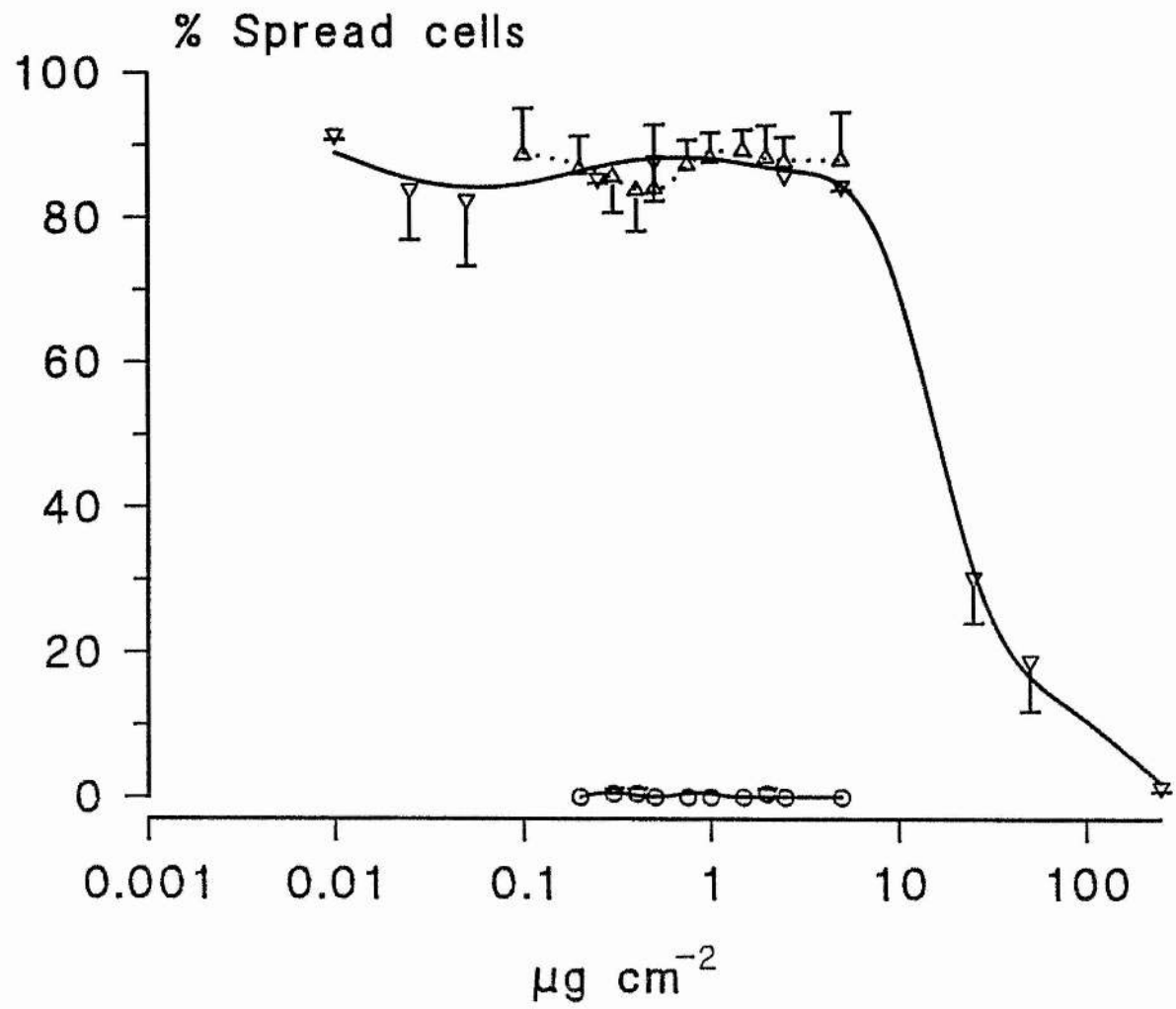


Figure 49: Spreading of tumour cells on FCS: effects of anti-fibronectin antibodies.

Substrata were coated with different concentrations of FCS and, after blocking the remaining reactive sites on the plastic, the wells were incubated with different concentrations of polyclonal anti-fibronectin antibodies. The abilities of these substrata to support the spreading of B16F10 cells were then tested.

▽ = FCS (5 ug cm^{-2}) + dose of anti-fibronectin antibodies. The unbound second antibodies were not removed from the wells (n=3).

○ = dose of FCS + anti-fibronectin antibodies (50 ug cm^{-2}). The unbound second antibodies were not removed from the wells (n=3).

Δ(---) = dose of FCS + anti-fibronectin antibodies (50 ug cm^{-2}). The unbound second antibodies were removed before the assay (n=3).

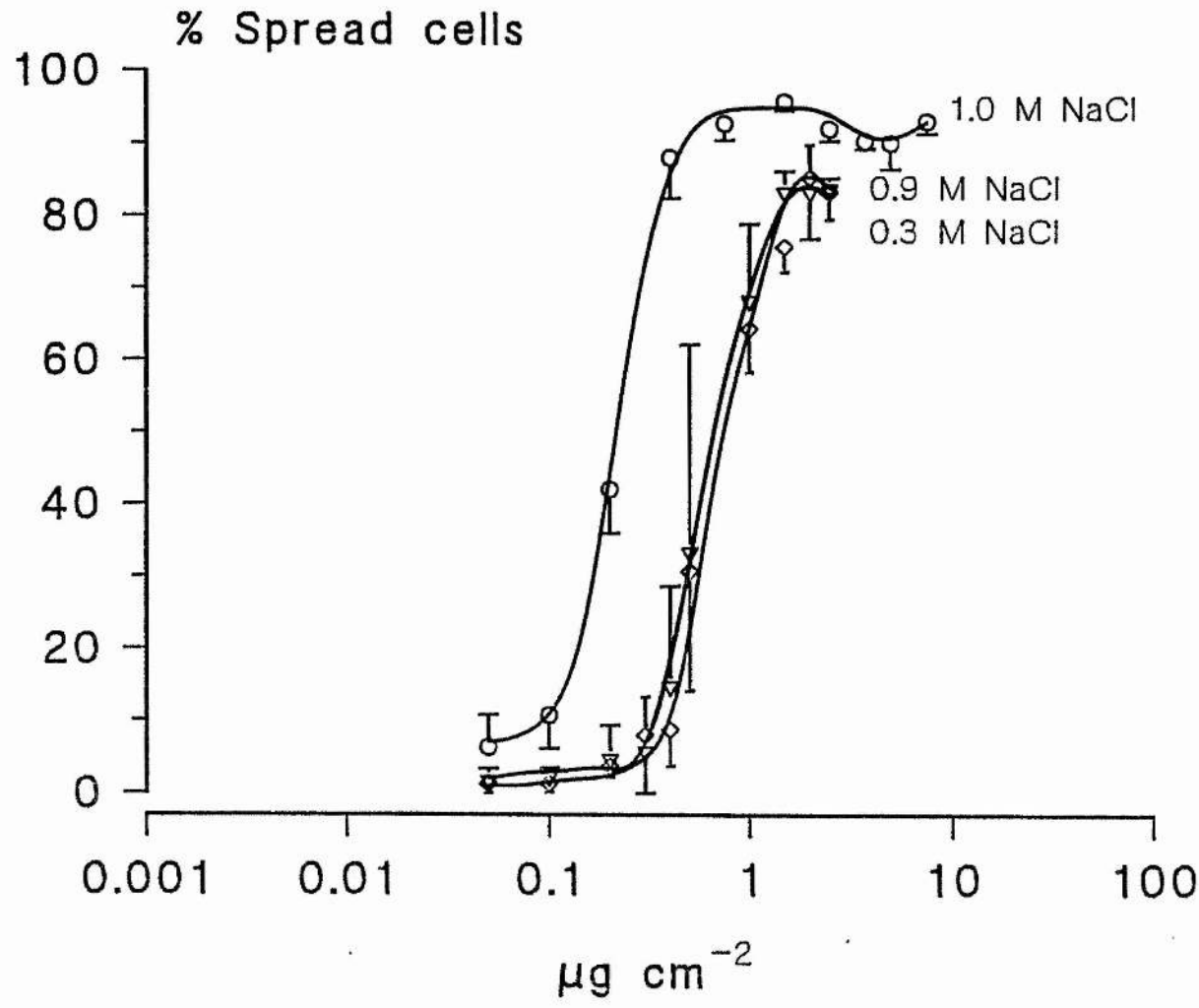


Figure 50: Spreading of tumour cells on endothelial cell extract: effect of extract purification.

Substrata were coated with different concentrations of the preparations to be tested and their abilities to support the spreading of B16F10 cells were tested.

○ = endothelial cell extract partially purified through a one step elution (1.0 M NaCl) from Q-Sepharose (n=3).

◇ = endothelial cell extract purified through Q-Sepharose and eluted with a linear gradient of 0.075-1.0 M NaCl; 0.3 M NaCl peak (n=3).

▽ = endothelial cell extract purified through Q-Sepharose and eluted with a linear gradient of 0.075-1.0 M NaCl; 0.9 M NaCl peak (n=3).

Production and purification of the endothelial extract

Extraction procedures varied slightly during the course of this study. Initially each roller bottle containing an endothelial monolayer was incubated with 25 ml of extraction buffer (EDTA 2 mM in T^{-}). The extraction volume was later reduced to 10 ml without any effects on either the biological activity or the amount of proteins extracted. Each roller bottle gave approximately 0.3 mg of protein as determined by the dye binding test of Bradford (1976).

Attempts to purify the material were made using ion exchange chromatography, first on ZetaPrep 15 ion exchange disks (LKB) and later on Sephadex- and Sepharose-based ion exchange media (Pharmacia). Two types of ZetaPrep disks were used, a cation exchanger with sulfopropyl (SP) groups and an ion exchanger containing diethylaminoethyl (DEAE) groups. The SP disk was used at pH 6.0 and no adhesion promoting activity was recovered in the bound fraction when this was eluted either at pH 7.5 or at pH 9.0; conversely, using the DEAE disk at pH 7.5, some adhesion promoting activity could be eluted from the column at pH 6.0 (results not shown).

From these preliminary results an attempt to purify and at the same time concentrate the activity contained in the extracted material was made using a 16 ml column (8 cm x 2 cm^2) of Q-Sepharose Fast Flow. The column was first loaded at a pH of 7.5 and the bound material was eluted with the addition of 1.0 M NaCl. Both the bound and unbound material contained adhesive promoting activity. The unbound material was re-applyed to the column at a pH increased by 0.25 units and the bound

material was eluted with 1.0 M NaCl in the same buffer. Bound and unbound fractions were tested in adhesion assays and the cycle of pH increase and subsequent testing was repeated until no activity was detected in the unbound fraction. This was found to be the case when the loading buffer pH was at 8.5 (results not shown). When freshly extracted material was applied to the Q-Sepharose column at pH 8.5 it was noted that some adhesion promoting activity was still present in the eluate and that this activity would disappear after repeated applications to the column in the same buffer (results not shown).

In an attempt to lower the loading buffer pH and improve the separation, extracted material was diluted with an equal volume of tris-HCl buffer pH 7.5 (this effectively reduced the NaCl concentration to 0.075 M), applied to the Q-Sepharose column at a pH of 8.0, 8.25 and 8.5 and eluted in the same buffer with the addition of 1.0 M NaCl. Following these experiments it was established that, with a NaCl concentration of 0.075 M, a pH of 8.25 was sufficient to determine the binding of all the adhesion-promoting activity of the extract to the column, while, when the pH was lowered to 8.0, some adhesion-promoting activity was detected in the unbound fraction. Figure 51 shows an example of purification. Extracted material from 8 roller bottles was diluted with an equal volume of tris-HCl buffer and the pH was raised with NaOH to 8.25. A two step elution was performed using 0.5 and 1.0 M NaCl in the same buffer to try and further isolate the active material. Both peaks contained spreading-inducing activity while only the 0.5 M NaCl peak had (rapid) adhesion-promoting activity.

The molecules present in the preparation purified using Q-Sepharose and a one step elution (1.0 M NaCl) were separated using gradient SDS-PAGE after the extracted material was concentrated. The only major band present in the gels had an estimated M_r of ~ 58,000 and, upon reduction,

it migrated slightly slower and had an estimated M_r of 62,000-65,000. Since no other bands were immediately evident, it was at first thought that this band contained the molecule responsible for eliciting adhesion. Althought this low molecular weight band was a prime candidate for the adhesive activity of the extract, it was possible that very low amounts of adhesive molecules present in the extracted material, such as laminin, thrombospondin or fibronectin, might be responsible for the adhesion observed, although at concentrations too low to be detectable with SDS-PAGE.

In an attempt to separate this M_r 58,000 molecule from higher molecular weight components of the extract, gel filtration was used. The extracted material, purified on Q-Sepharose by application at pH 8.25 and a one-step elution with 1.0 M NaCl, was then applied to short columns (~ 15 cm x 2 cm^2) of Sephadex G-25, G-50, G-75, G-100, G-150 and G-200. Typically 5 ml were applied to each column and two fractions recovered: one that did not enter the gel (or excluded fraction) and one that did. These fractions were subsequently tested for their adhesion promoting activity. In all these experiments the adhesion-promoting activity was always found in the excluded fraction and the separation between the two fractions was always well defined. Examples of these runs are shown in figures 52 and 53.

No high molecular weight material could be detected after SDS-PAGE. This could be due to the very low concentration of the putative adhesive molecule in the extracted material. Therefore a large quantity of extract was produced from 24 roller bottles, concentrated down to a small volume (0.4 ml) and SDS-PAGE was repeated in order to demonstrate the presence of bands with higher molecular weight. Unfortunately this exercise proved unsuccessful. Later experiments suggested that an explanation for this maybe due to the fact that upon concentration, especially at low temperature, the extracted material tended to form a precipitate that was

SDS, mercaptoethanol and urea insoluble.

In other experiments, in order to purify the active component(s) of the extract, the material was applied in tris-HCl buffer pH 8.25 + 0.075 M NaCl to the Q-Sepharose column and eluted with a linear gradient of 0.075-1.0 M NaCl in the same buffer. Each eluted fraction was then tested for its ability to support the adhesion and the spreading of B16F10 cells. Only one fraction eluting at approximately 0.3 M NaCl seemed to have adhesion-promoting activity. Two fractions were most active in supporting spreading, one being the same fraction that showed adhesion-promoting activity, while the other eluted at approximately 0.9 M NaCl (see figure 54).

Affinity chromatography was used in an attempt to characterize the adhesion- and spreading-promoting activity of the extracted material, and at the same time to remove extracellular matrix molecules that are known to possess such activity. As starting material an extract partially purified on Q-Sepharose with a 1.0 M NaCl elution was used. When such material was applied on a column of heparin-agarose (see figure 55) the activity was not bound to the column as was the case when gelatin-agarose and poly-L-lysine-agarose were used.

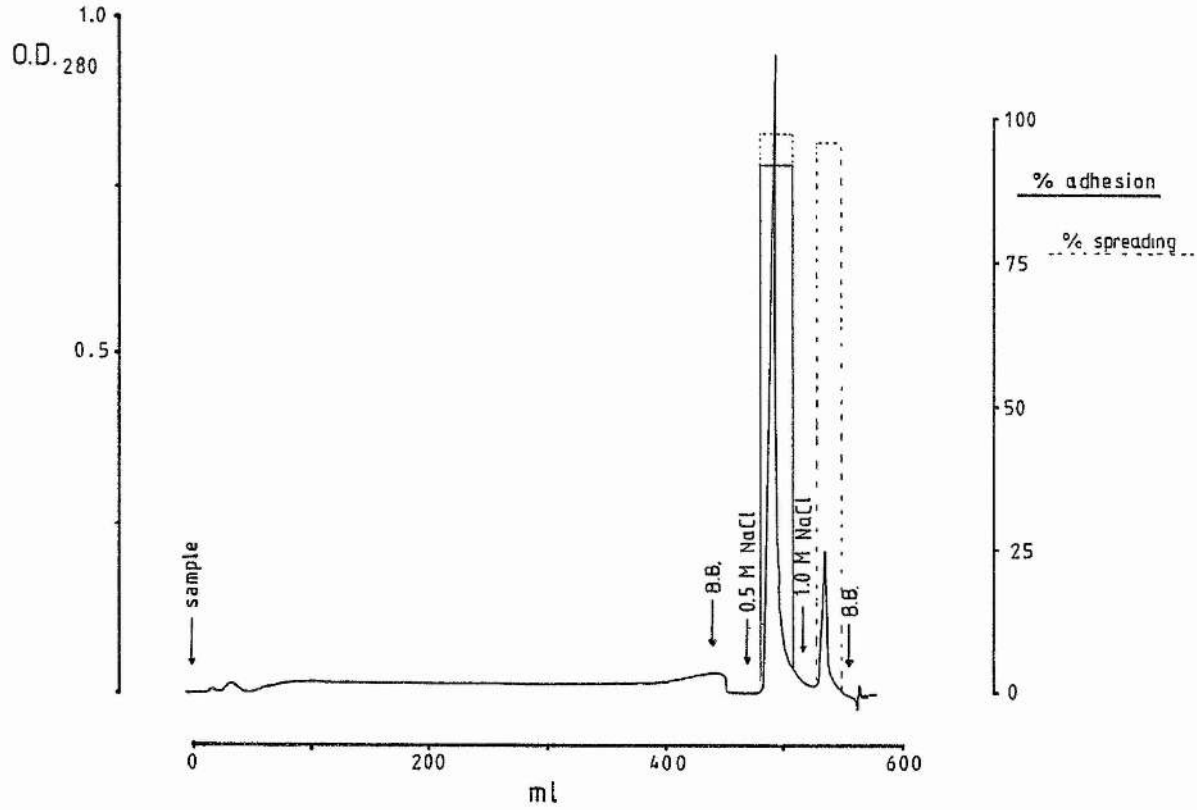


Figure 51: Purification of the endothelial extract on Q-Sepharose using a two step NaCl elution.

Extracted material from 8 roller bottles was diluted with an equal volume of tris-HCl, pH 8.25 and buffered to pH 8.25 with ~ 0.1 ml of NaOH 1 N. The sample was then applied to a 16 ml column of Q-Sepharose and eluted with 0.5 M and 1.0 M NaCl. Flow rate = 150 ml cm^{-2} h^{-1}. Histograms indicate the ability of the material to support the adhesion (5 min, continuous line) and spreading (60 min, discontinuous line) of B16F10 cells. Values below 10 % are not represented.

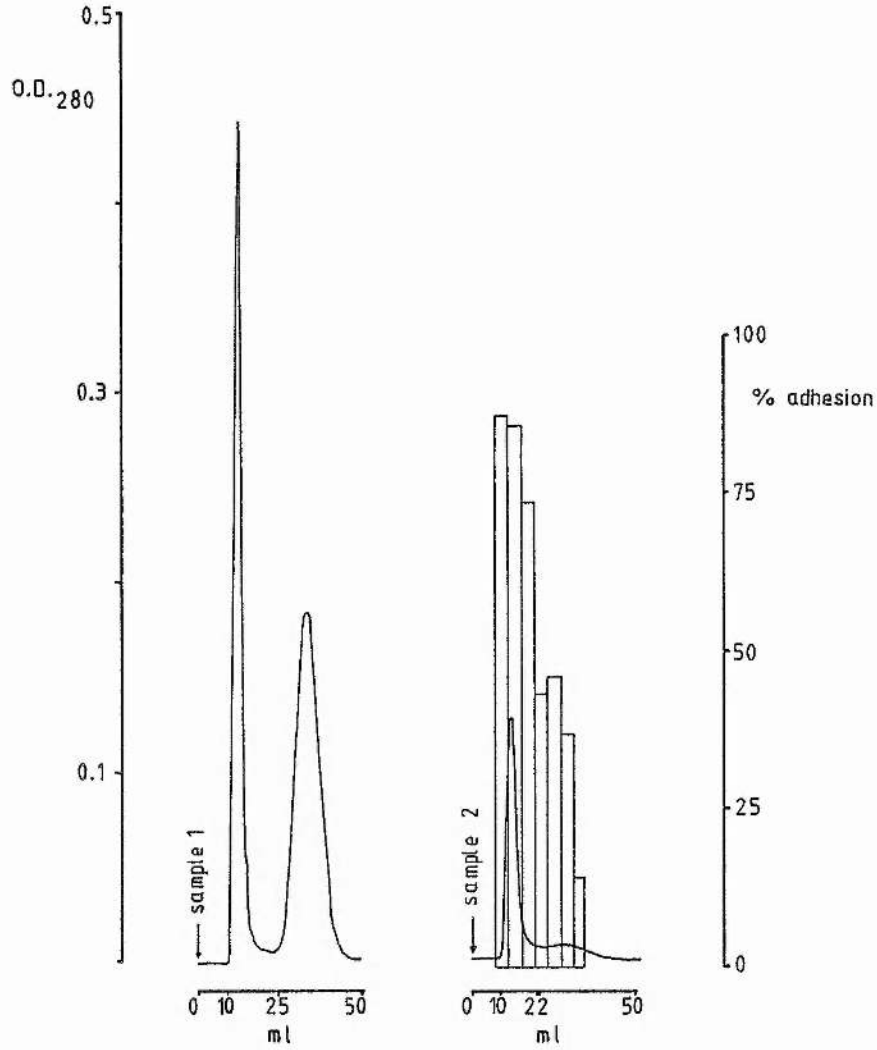


Figure 52: Characterization of the endothelial extract; exclusion chromatography on Sephadex G-50.

A 2.8 sample was applied to a short column (30 ml) of Sephadex G-50 F with a flow rate of 120 ml cm^{-2} h^{-1}. The sample is in this way separated into two fractions: the void volume fraction (first peak) and the fraction of the stationary volume (second peak). The cut-off point of this gel for globular proteins is ~ 30,000 D.

Sample 1: 0.5 mg of Blue Dextran + 0.5 mg of tyrosine.
Sample 2: Extracted material from endothelial monolayers, partially purified using Q-Sepharose and a 1 step elution with 1.0 M NaCl.

Histograms indicate the ability of the material in each fraction to support the adhesion (5 min) of B16F10 cells. Values below 10 % are not represented.

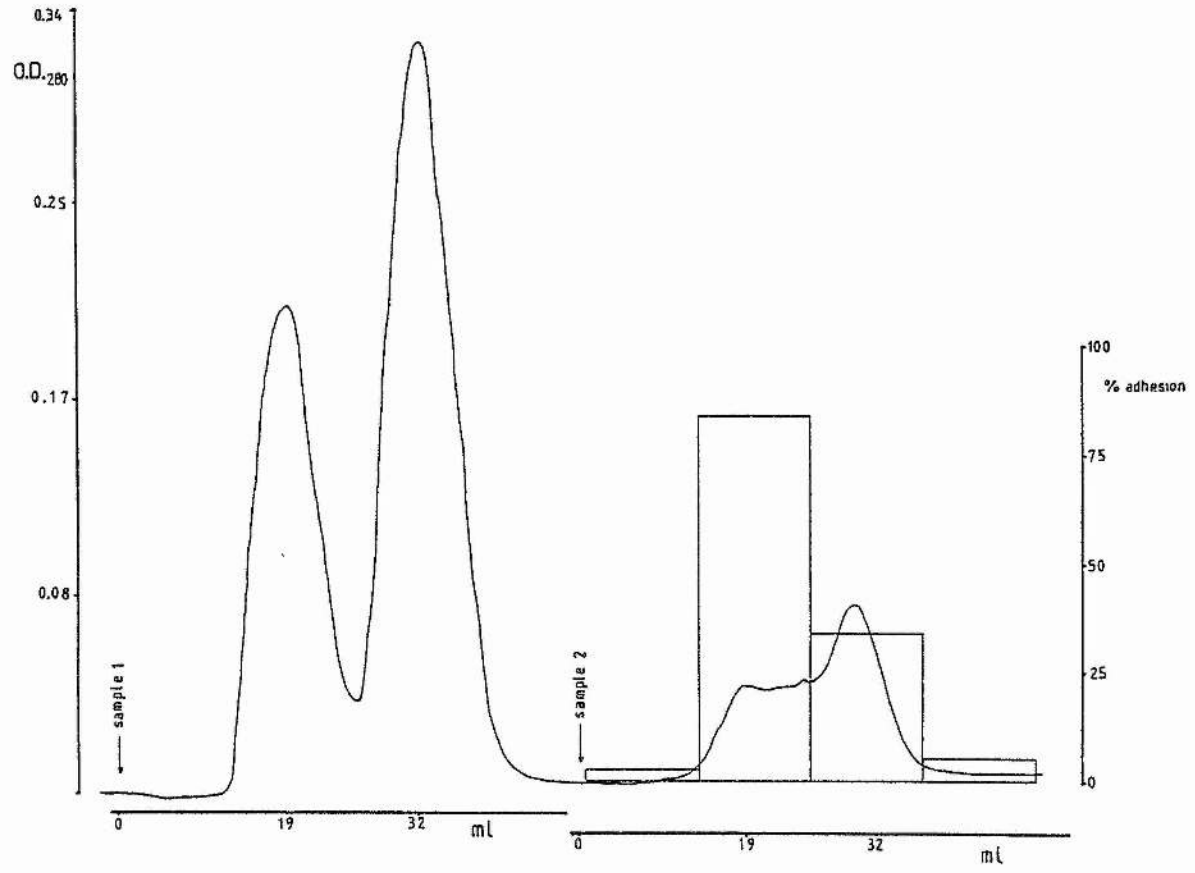


Figure 53: Characterization of the endothelial extract: exclusion chromatography on Sephadex G-200.

A sample was applied to a short column (33 ml) of Sephadex G-200 with a flow rate of 6.9 ml cm^{-2} h^{-1}. The sample is in this way separated into two fractions: the void volume fraction (first peak) and the fraction of the stationary volume (second peak). The cut-off point of this gel for globular proteins is ~ 600,000 D.

Sample 1: 1.5 mg of Blue Dextran + 1.5 mg of tyrosine in 8 ml of buffer.
Sample 2: 5 ml of extracted material from endothelial monolayers, partially purified using Q-Sepharose and a 1 step elution with 1.0 M NaCl.

Histograms indicate the ability of the material in each fraction to support the adhesion (5 min) of B16F10 cells.

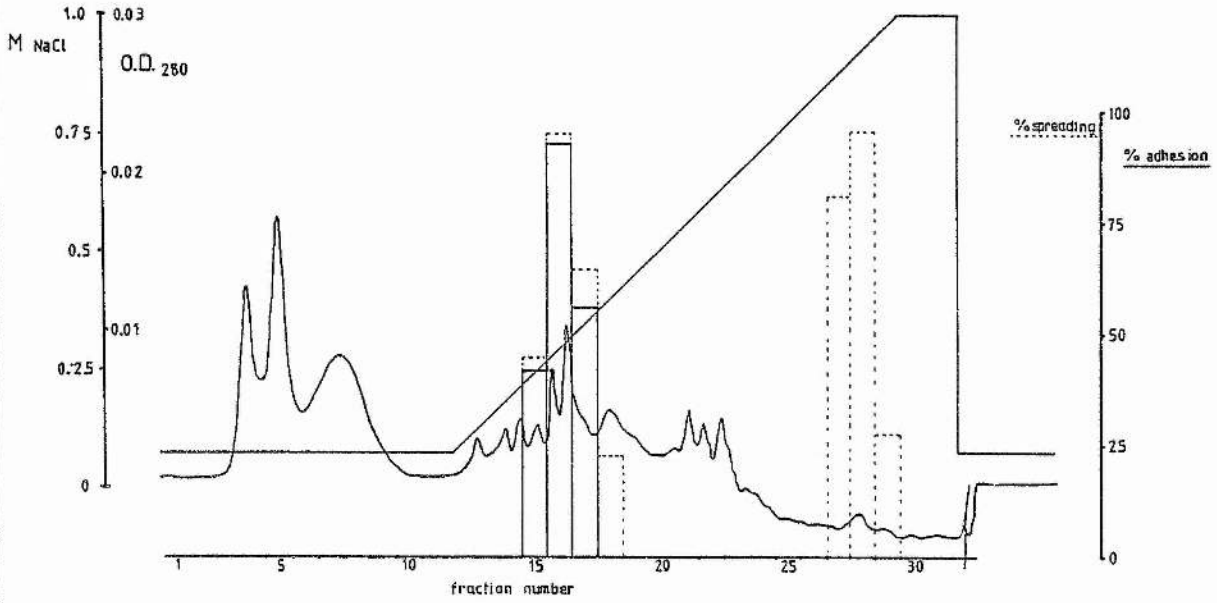


Figure 54: Purification of the endothelial extract on Q-Sepharose using a continuous NaCl gradient.

Two ml of extracted material were diluted with an equal volume of tris-HCl, pH 8.25 and buffered to pH 8.25 with ~ 0.1 ml of NaOH 1 N. Such sample was applied to a 16 ml column of Q-Sepharose and eluted with a 0.075-1.0 M linear gradient of NaCl. Fraction size = 4 ml, flow rate = 75 ml cm^{-2} h^{-1}. Histograms indicate the ability of the material in each fraction to support the adhesion (5 min, continuous line) and spreading (60 min, discontinuous line) of B16F10 cells. Values below 10 % are not represented.

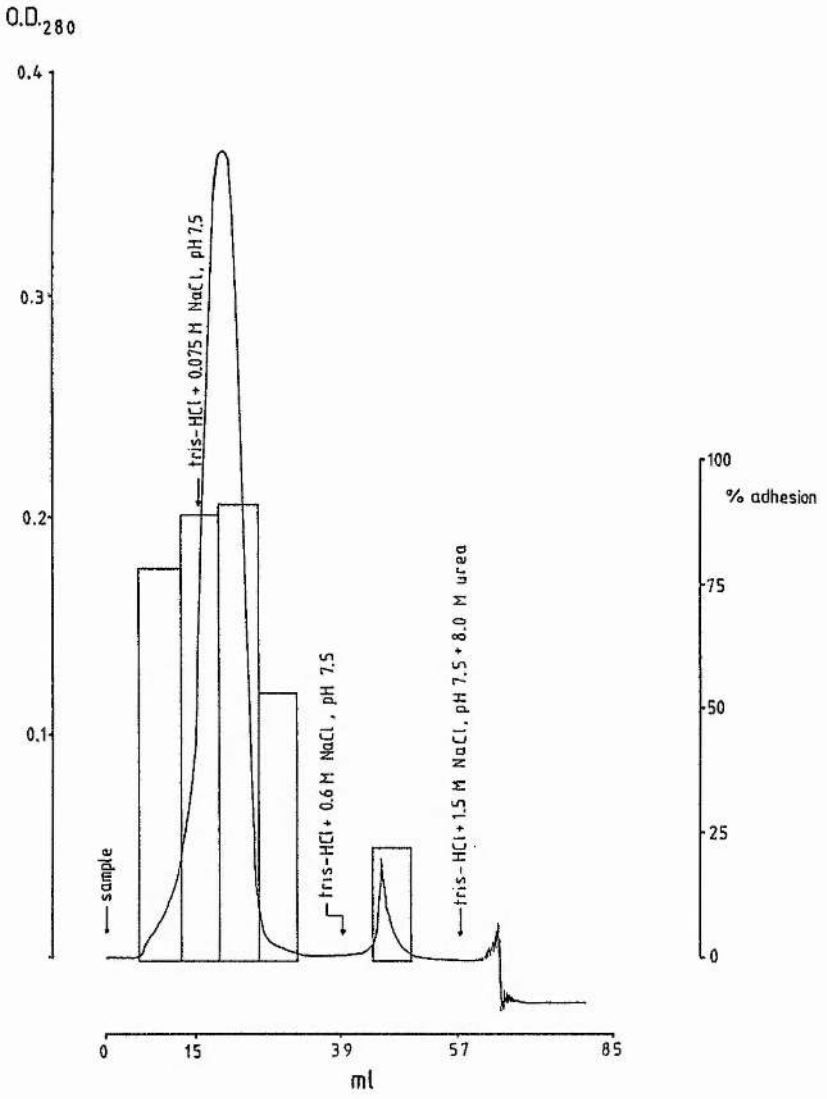


Figure 55: Characterization of the endothelial extract: affinity chromatography on heparin-agarose CL-4 B.

Extracted material from 6 roller bottles was diluted with an equal volume of tris-HCl buffer. A sample (15 ml) was applied to a column of heparin-agarose CL-4 B and eluted first with 0.6 M NaCl and then with 1.5 M NaCl + 8 M urea. Flow rate = 75 ml cm^{-2} h^{-1}. Histograms indicate the ability of the material in each fraction to support the adhesion (5 min) of B16F10 cells. Values below 10 % are not represented.

Discussion

1: Designing the assay.

The successful development of an easy, rapid and reliable adhesion assay was of prime importance to this study and this therefore constituted a major part of the early work. The approach was centred on two ideas:

1 Adhesion of circulating cells to the vessel wall is an extremely rapid event (Bjerknes et al., 1986). The assay should therefore be able to measure cell adhesion after the shortest possible period of incubation.

2 Given such short incubation, the assay must easily discriminate between the amount of adhesion elicited when using a known adhesive substratum and that measured using a substratum that does not induce adhesion.

Since the original work of Walther and colleagues (1973), various studies have been based on the collecting lawn assay principle: single cells in suspension are confronted with a monolayer or lawn of cells and the rate of their adhesion to the monolayer is measured. Although this approach can yield valuable information, the original assay as described by Walther and colleagues (1973) does have some significant disadvantages, as discussed below.

Static vs. dynamic assays.

Two basic variations of the collecting lawn assay exist: a static one where the suspension is left to settle and adhere to the monolayer undisturbed (Nicolson, 1982 b; Varani et al., 1980; Tao and Johnson, 1982; Pohlman et al., 1986; DiCorleto and de la Motte, 1985; Kramer and Nicolson, 1979; Murray et al., 1980; Terranova et al., 1982; Vlodavsky and Gospodarowicz, 1981;), and a dynamic one, where adhesion occurs under flow. In the latter version the flow is usually generated by shaking the plate in an orbital or a linear shaker (Walters et al., 1973; Winkelhake and Nicolson, 1976; Nicolson et al., 1981; Alby and Auerbach, 1984) and this is done in an attempt to simulate the in vivo environment tumour cells would encounter when adhering to the endothelium. The use of orbital or reciprocating shakers is quite a crude way to generate flow and some attempts have been made to try and achieve a more controlled experimental environment. Most of these generally use a flat flow chamber that is optically transparent and suitable for microscopy and time lapse photography studies (Doroszewski, 1980; Forrester and Lackie, 1984; Owens et al., 1987).

Flow undoubtedly represents a very important factor that influences in vivo cell adhesion to the endothelium. Nevertheless it is extremely difficult, if not impossible, to make a precise estimate of the forces that circulating tumour cells may encounter when extravasating. A discussion about the reasons behind this is outwith the scope of this dissertation but the main points are:

1 There is no consensus regarding the anatomical location of tumour cell arrest in the circulation. This might happen in the precapillary arterioles, in the capillary bed, in the postcapillary venule or in larger diameter veins. In each of these locations blood flow has markedly different characteristics.

2 Even if we could be sure about the anatomical location of the arrest, the geometry of vascular beds varies between organs. The capillaries in the liver, for example, present a totally different environment to those in the lungs. In the lungs the capillaries spread over several adjacent alveoli and branch and anastomose to form intricate networks over the surface of the alveoli. Lung capillaries can be compared more to a continuous thin sheath rather than a number of individual small tubes connected in parallel. In fact there is so much organ individuality that the in vitro mimicking of a vascular environment would probably approximate only to one particular organ. Besides, our current knowledge of the functional anatomy of the microcirculation of many organs is somewhat deficient.

3 Blood is a very complex fluid. Its behaviour under flow seems to obey physical rules governing Newtonian fluids (constant relationship between shear rate and shear stress), but at times it can behave like a non-Newtonian fluid (shear stress does not increase linearly with shear rate).

4 The mechanical properties of the blood are not uniform but change dynamically. There are several phenomena that contribute to this, such as plasma skimming towards the vessel walls, particle sorting and dynamic haematocrit. Additionally, the flow rate decreases and increases as the blood vessel curves, the pattern of flow changes as the blood goes through restrictions, bifurcations or junctions and the pressure fluctuates as districts are open or shut. All these contribute to changing the basic pattern of flow from laminar to turbulent, and indeed there may be different types of flow within the same area of the vessel. In view of all the necessary assumptions it is not possible to make a reliable estimate as to the

likely flow in any given part of the microcirculation.

5 Even if we were to accept these approximations, there are very substantial differences between the environment in model systems and that in vivo due to the very nature of the biological material. Particles suspended in the blood are not rigid spherical bodies but asymmetric and elastic, they are able to modulate their shape and the smoothness of their surface. Furthermore the blood vessels are irregular and elastic and their shape is always changing.

(For a comprehensive discussion on the mechanics of blood flow see the book by Caro, Pedley, Schroter and Seed, 1978). Because of the very complicated nature of blood flow, all the different dynamic adhesion assays developed in the past reproduce environments so far removed from the in vivo situation that they have no real advantages over static systems. In fact, contrary to initial impressions, these dynamic assays often complicate the picture, introducing variables and constraints which can make the results more difficult to evaluate. For all these reasons it was thought that a static adhesion assay, although not without limitations, would nevertheless give some insight into the adhesive phenomenon.

Cell density and volume.

A common pitfall of many methods for measuring adhesion is that the results are often influenced by many parameters, some of which are irrelevant to the biological phenomenon under study. For instance, in a static system (like the one used here), two factors are likely to have a very significant effect on the results obtained: the amount of cells in the suspension and the amount of fluid in which the assay is performed.

The effects of cell number is related to the area of substratum available to the cells as well as to the influence of cell-cell interactions on their adhesion to the substratum. When there is an excess of cells in

relation to the surface area of the substratum, only a proportion of these cells would be able to come into direct contact with it, the rest piling up on each other. This could be a source of bias because cells may prefer to adhere to each other rather than to the substratum. They may therefore adhere less rapidly or establish fewer molecular interactions with the substratum, which would weaken the strength of cell to substratum adhesion. There may also be differences among individual cells of the suspension, either because of the presence of different phenotypes (that are known to arise even within cell lines of monoclonal origin), or because of the presence of cells at different stages of the cell cycle. Such differences are known to alter the adhesive properties of a cell population (Elvin and Evans, 1982; 1983). Also, if cells are in excess, phenotypic differences within the cell population can give further bias. For example, if the density (mass/volume) of individual cells varies within the population and the adherent phenotype is preferentially associated with an increased or decreased density of the cell, the heavier cells would "sink" to the bottom of the settled suspension and into contact with the substratum. The assay would therefore record an increased or decreased number of adherent cells only because of the presence of characteristics associated with the adherent phenotype, not because of an overall difference in the numerical expression of such adherent phenotype among the cell population.

When the volume of medium used in the assay is taken into consideration, one consequence is immediately obvious: the higher the column of fluid, the longer the cells will take to reach the substratum while settling through the medium. This has a tangible effect when adhesion kinetics are studied. It can be assumed that, within the suspension, cells are uniformly distributed. If this is the case, some will be in direct contact with the substratum and begin to establish adhesive

interactions with it almost as soon as the assay starts, while others will first need to settle through the fluid. When the geometry of the system is such that the column of liquid is tall in relation to its base, a high proportion of cells will not be in the immediate vicinity of the substratum. Additionally, if we were to measure the distance of each individual cell from the substratum at the beginning of the assay, it would become apparent that the higher the column of fluid in relation to its base, the wider the spatial distribution of the cells would be, which would in turn result in a large variation of the time of first contact of each cell with the substratum. Some adhesion assays have tried to minimize this variable, as in the case of Nicolson and colleagues (1981), who let the cell suspension settle onto the substratum before starting the assay by raising the temperature to 37° C. This is a rather inelegant solution since the system will require a considerable and unknown amount of time to reach a stable temperature and during this time adhesion will occur and will be enhanced in areas that warm up at a faster rate. If very rapid adhesion has to be measured, settling of the suspension would significantly bias the results and centrifugation onto the substratum could be a way of minimizing such bias by reducing the settling time. Although this would improve the assay in theory, it also poses practical problems with the shaking of the system during the deceleration of the rotor and the transport of the plate to an incubator, as well as introducing changing g forces during adhesion and shape changes of the cells as additional variables.

Further considerations arise from the fact that cells, while settling, collide with each other. An increased frequency of such collisions can be induced by raising the concentration of the cell suspension and the distance cells have to travel in order to reach the substratum. Cell-cell adhesion occurring within a cell suspension (aggregation) is a phenomenon

which is among other things dependent on the number of collisions and on the relative speed of the colliding cells (for a discussion on cell-cell aggregation see Evans and Proctor, 1978). If aggregates do form, then the results could be extremely biased. Each aggregate, in fact, could have as little as one cell adhering to the substratum, resulting in a marked over-estimation of substratum adhesion.

For these reasons, when developing the assay, efforts were made in the attempt to minimize the number of cells relative to the surface area of the substratum. Furtermore, the incubation chamber was designed so that it would allow the height of the medium to be kept to a minimum (2 mm).

Determining the number of adherent cells.

Another important aspect of adhesion assays is the method employed to quantify the outcome of the experiment. Ideally, after removing the non-adherent cells, a count of all the adherent cells should be performed. This can be done by visual count using a microscope (Fligiel et al., 1985; Scott et al., 1986) but it is a tedious and labour intensive task. Other strategies are available: the adherent cells can be harvested and counted with an electronic device, alternatively a radioactive label can be incorporated into the cells which can be later solubilized and counted. This latter method has been widely used in the past (Walther et al., 1973; Winkelhake and Nicolson, 1976; Alby and Auerbach, 1984; Pohlman et al., 1986).

Labelling of the cells makes quantification easier, a very high number of assays can be performed and the amount of radioactivity later determined. There are several disadvantages to this technique however. Firstly, radiolabelling involves quite a significant amount of manipulation of the cells, especially if labels such as ^{51}Cr are used. Secondly, there is the possibility that the label (whether or not metabolic) introduced into

the cells would accumulate preferentially into certain phenotypes rather than others. Thirdly, even during a short term adhesion assay such as that used in this study, the label may leak out of the cells, may adsorb onto the plastic or otherwise be internalized by other cells. Fourthly, a long incubation with a metabolic label or the incorporation of radioactive sources within a cell poses questions about the effects of radiation on cell behaviour. Although this last point is often regarded by the mainstream researchers as being insignificant, there is no ground for adopting such a dismissive attitude. On the contrary, there are increasing numbers of reports suggesting that very low doses of radiation can have a profound effect on selective cell activities (Kimler et al., 1981; Olivieri et al., 1984; Shadley and Wolff, 1987; Sankaranarayanan et al., 1989). Cell adhesion, like any other cellular activity, is likely to rely on the coordinated functioning of many different components such as surface receptors, cytoskeletal elements, intracellular organelles, degradation pathways and so on; the exposure to radiation obviously increases the risk of interfering with any of these components. It is probably for these reasons that some authors have tried, more recently, to overcome the possible effects of such manipulations by labelling the cells after the assay (Mentzer et al., 1986).

Other techniques have been employed in the past, as in the case of the use of a vertical pathway photometer after fixation and staining of the adherent cells (Piershbacher and Ruoslahti, 1984) or the measuring of the turbidity before and after removal of non-adhering cells (Grinnell and Minter, 1978; Grinnell and Feld, 1979). The first approach offers advantages over other techniques especially because of its rapidity, low cost and the fact that it allows the processing of large numbers of samples. Personal experience casts doubts as to the reliability of such measures and no published indications were given by the authors

regarding variations between duplicates nor has any statistical analysis, graphical or numerical, been provided. The second (turbidimetric) approach is relatively fast and again it offers low cost and reduced manipulation of the cells, but, on the other hand, turbidimetry is not a reliable estimator of the number of cells in solution and the presence of cellular aggregates reduces the accuracy further. In fact, a relatively high number of cells in suspension are required (0.5-1 x 10^6 cell ml^{-1}) for an accurate reading, with an increased probability of aggregate formation.

As discussed earlier, the assay would benefit from the use of a small number of cells in relation to the available substratum. If reliable results are to be obtained by visual counting, this has to be performed over the entire surface of the substratum. Cells do not distribute on the bottom of the dish uniformly, due to, amongst other things, the geometry of the system and, in particular, the presence of a meniscus. The visual counting should be done as soon as the assay is terminated because adherent cells may detach and float, they may divide or they may lyse. Although fixing and staining of the adherent cells allows counting to be performed at a later date, the quantification of the assay is very labour intensive and, therefore, the use of such a technique for investigative and screening purposes becomes impractical.

Another way of determining the number of adherent cells would be to harvest and resuspend them and count a sample. For some cell lines (like the B16 cells) it is possible to use chelating agents for routine detachement but the molecular composition of the substratum of an adhesion assay is often very different from that of a dish that had cells growing to confluency. It is therefore possible that some cells may not detach following such gently treatment and, for this reason, it is best to harvest the adherent cells using enzymes. Although this method would have the advantage of counting only a small proportion of the adherent

cells either with an haemocytometer or, even better, an electronic particle counter (Murray et al., 1980; Terranova et al., 1982), bias could arise from the exposure to enzymes, which may cause lysis among a population of cells that have already been harvested, centrifuged, resuspended and allowed to adhere again within the last 10-15 minutes.

By counting the number of non-adhering cells, the number of adherent cells can be determined with a good approximation. Since at the end of an adhesion assay non-adherent cells have to be removed anyway, their collection does not greatly complicate the procedure. Although haemocytometers can be used for the counting (Varani et al., 1980), these non-adherent cells would be in suspension and would therefore be ideally suited for electronic counting. Counting cells with the help of a Coulter counter is inexpensive and cell manipulation is kept to a minimum since no labelling is required and large numbers of samples can be processed easily and rapidly. Furthermore, results are available immediately. In our experience, when the Coulter counter is coupled to a channelyzer, the counts obtained are quite reproducible, especially if the cell concentration is kept well below the threshold for coincidence (which, using a 100 um orifice, is ~ 5.5×10^6 cells ml^{-1}).

2: Evaluation of results.

From the results of the study four points can be made:

1 Rapid adhesion is temperature and divalent cation dependent.

2 The kinetics of adhesion to a particular molecule depend on the concentration of the given molecule on the substratum.

3 The adhesion kinetics to endothelial monolayers compare favourably with those of the most adhesive molecular substrata.

4 Some molecules are unable to support rapid adhesion regardless of

their concentration on the substratum.

Divalent cations and temperature.

Not surprisingly, rapid adhesion is dependent on the presence of divalent cations in the incubation medium and on the temperature of the system (see figures 18, 24 and 27). This is in agreement with the general consensus that cell adhesion is Ca^{2} and Mg^{2} -dependent and decreases as the temperature decreases.

There are several possible explanations for this:

1 The folding and unfolding of the long arms of some adhesion molecules is Ca^{2+}-dependent. Thrombospondin, for instance, has several binding sites for Ca^{2+} which modulate its ternary structure (reviewed in Frazier, 1987) and, as a result, binding to other ligands may be impaired.

2 The affinity of some ligands for their receptors are Ca^{2+} and Mg^{2+}-dependent (for more details see the pages 25-66 of the introduction about the subendothelial matrix).

3 Calcium is necessary for cells to degranulate and chelating agents can inhibit degranulation. In platelet adhesion to the endothelium for example, Weber-Palade bodies are brought into contact with the cell membrane allowing the generation on the endothelium of patches containing very high densities of membrane bound von Willebrand factor (Sporn et al., 1986; Sporn et al., 1987). If tumour cell adhesion to endothelium relies on exocytosis of selective endothelial granules, the presence of chelating agents may reduce exocytosis and/or the binding of the granule contents to the membrane acceptor sites and therefore impair adhesion.

4 Chelating agents may affect the cytoskeleton. If adhesion depends on a rearrengement of cell surface adhesion receptors (like patching

or capping) and if such redistribution is mediated by the cytoskeleton, adhesion may be inhibited.

5 The divalent cation-free medium could affect the general metabolism of the cells, rather than disrupting specific ligand-receptor interactions.

Some experiments to evaluate the action of EDTA on the system gave results that are in contrast to those published by Kramer and co-workers (1980). These authors exploited the action of EDTA on endothelial monolayers to compare the adhesion of tumour cells to confluent endothelium and to retracted endothelial cell monolayers. In their hands, exposure to EDTA for 30 min caused the rounding up and retraction of the endothelial cells, leaving the underlying extracellular matrix exposed. These authors reported that EDTA pre-treatment of the endothelial monolayer resulted in a ~ 8 fold increase in the number of adhering cells after 5 minutes of incubation. They observed that in these conditions tumour cells are mostly bound to the exposed extracellular matrix and then they proceeded to use these results to support the interpretation, at the time in vogue, that tumour cell arrest relied on patches of exposed subendothelial matrix. In our hands exposure to EDTA prior to testing of either the endothelium (for 30 min as described in the work of Kramer et al., 1980), the tumour cells or both, did not alter the kinetics of adhesion. Although for these experiments we used B16F10 and not B16F1 cells, in our experience these two cell lines behave quite similarly as regards adhesion to the endothelium and other substrata (see figures 22 and 25). Finally, according to Kramer and his colleagues (1980), adhesion to the endothelial monolayer was not as fast as we have reported, with less than 10 % tumour cells adhering after 5 minutes. These authors used bovine aortic endothelial cells similar to those used in our laboratory. There may

be several reason for the marked difference in the results we obtained, mainly related to the handling of the endothelial cells. Their endothelium was, in fact, maintained with a different routine and cultured in a different medium. We routinely kept the cells on a strict 3 days cycle and no aged monolayer was used for adhesion assays, while there is no mention of these details in their work; their cells were grown in medium containing 10 % FCS but at confluency this was replaced with a medium containing only 5 % FCS; fibroblast growth factor was added every other day but no insulin nor other supplements were used. Other differences relate to the tumour cell suspension, which was ^{51}Cr-labelled (with the inherent artefacts of exposure to radioactive sources and manipulation) and inoculated into the wells in a volume 2.5 times larger than that used in our study (which resulted in a much increased settling time).

The effects of low temperatures on adhesion (which are reversible - as shown in figure 18) can be due to an overall slowing down of the metabolism of the cells. Additionally, at low temperatures, membrane fluidity is reduced and this could impair the movement of receptors towards the patches of contact with the substratum. In this scenario, the speed with which ligand receptor interactions are undertaken would be much reduced. Low temperatures could also have a significant influence on the K_d of the ligands and receptors involved in adhesion. It is known that fibrinogen, for example, is not a ligand for fibronectin at 37° C but its affinity for it increases drammatically at 4° C (Grinnell, 1981, Mosesson and Amrani, 1980). If at low temperature the K_d of laminin for its receptor increases, this could in turn result in a much slower rate of formation of molecular bonds between the cell surface and the substratum-bound laminin, which would result in a de facto inhibition of adhesion as measured with this assay system (see figures 24 and 27).

If the results of the fixation experiments (see figure 19) are considered

within this context, it would seem that tumour cell-endothelium adhesion may require an active and fully functional membrane-cytoskeleton assembly, at least on the tumour cells. Generally the cytoskeleton plays a central role in cell adhesion, and this is confirmed by the work of Chong and his colleagues (1987). The absence of divalent cations would affect the cytoskeleton (Roos, 1984) as well as the fusion of granules to the cell membrane, while fixation would have the effect of stiffening the cell membrane denying the cell the possibility of modifying its shape, redistributing its surface molecules or expressing new ligands on its surface.

That adhesion of cells is a more complex phenomenon than simply bringing two particles together and letting their surface molecules create a bond is not a new concept. A fundamental step for adhesion to occur is, for instance, the alignment of the two confronting surfaces (see Roos, 1984 for a review). This is probably accomplished by the cytoskeleton. Cells which are unable to align their confronting surfaces will not adhere. Using anchorage independent cells and normally adherent cells, it has been shown how this alignment can be induced by just one of the cell pair (see Roos, 1984 and references within). It is therefore very interesting to note that fixing of the tumour cells completely abolishes adhesion while fixing of the endothelium only reduces it. Since adhesion is dependent on the ability of the confronting cells to align their surfaces, in tumour cell adhesion to the endothelium this alignment must rely predominantly on an active role of the tumour cells rather than the endothelium.

Concentration on the substratum.

Small differences in the concentration of adhesive molecules on the substratum have a great influence on the rate at which cells adhere to it

(see figures 23 and 26).

Although this in itself seems quite obvious and has been stated in the past (for a review see Grinnell, 1981), it is surprising how this effect has been overlooked by many researchers. Grinnell and Minter (1978), Pierschbacher and his colleagues (1984) and Nicolson and his colleagues (1981), for example, do not give any indications as to the amount of fibronectin used to coat the dishes. Giancotti and colleagues (1985), on the other hand, state the concentration of the protein solution used to coat the dishes, but not the volume used. Vlodavsky and Gospodarowicz (1981) reported that tumour cells adhered quite well to endothelial extracellular matrix (more than 80 % after 30 minutes) but poorly to fibronectin coated substrata (less than 10 % after up to 1 h). The fibronectin substrata used, were coated with 10 ug of protein per dish, but there is no mention of the type of dishes used. If these were 24-well plates, the substrata would have been exposed to 5 ug cm^{-2} of fibronectin, but if they were 35 mm Petri dishes (as seems more likely from the volume of medium used) the substrata had a concentration of fibronectin which, in our experience, is too low (approximately 1 ug cm^{-2}) to promote adhesion. This lack of reference to the density of adhesion molecules on the substratum prevents comparison of data from different work and at times cast doubts on the validity of the conclusions.

Throughout this study no attempts were made to quantify the amount of molecules bound to the substratum, although, in retrospect, an effort to try and determine the concentration at the substratum should have been made. A major problem was the radiolabelling of the adhesive factors used: it is impossible to determine whether this procedure would alter the affinity of the molecule for the substratum. In some respects, any manipulation could results in substantial changes, as exemplified by antithrombin III, where iodination exposes a cryptic site for the binding of

vitronectin (Ill and Ruoslahti, 1985). Therefore all the figures quoted represent an estimate of the concentration, assuming that all the molecules present in the solution at the time of incubation with a clean plastic dish would bind to the plastic and that there would be an even distribution across the exposed surface.

There is no real understanding of the mechanisms regulating the adsorption of molecules onto plastic substrata but it is useful to summarize some phenomenological observations related to it:

1 Adsorption of proteins depends on the characteristics of their charged groups that can interact with the plastic (Butler, 1981). Some proteins may therefore exhibit unique behaviour in this respect.

2 The binding of molecules to plastic seems to have an initial phase where molecules are adsorbed very rapidly to the plastic reaching a plateau within the first 45-60 minutes. Beyond that the concentration of substratum-bound molecules increases significantly only if incubation times are extended to 18 hours or more (Herrmann, 1981).

3 Below a certain threshold, the amount of bound molecules is independent of the amount of molecules present in solution. This threshold is inversely related to the molecular weight, and for small molecules it is around 0.15 ug cm^{-2} (Butler, 1981).

4 Above this limit for independent binding there is an apparent saturation limit which is above 2 and below 20 ug of protein per cm^2 of plastic. Below this limit the amount of protein adsorbed is proportional to its concentration in solution and for those molecules tested is ~ 50 % (Herrmann, 1981).

5 It is possible to bind more molecules to the plastic above the saturation limit by significantly increasing the incubation time and

the concentration of the solution. The efficiency of the process decreases dramatically and most of the additional bound molecules will be easily solubilized once the coating solution is removed (Herrmann, 1981).

6 Below the independent threshold most of the molecules are tenaciously bound to the substratum: salts, chaotrophic agents, acid treatment, detergents, organic solvents, oxidizing or reducing agents all fail to remove the adsorbed proteins. Only boiling in aqueous solution for at least 15 minutes may solubilize all the immobilized molecules (Parsons, 1981).

7 Some molecules seems to be able to displace other molecules already bound to the plastic. Fibrinogen is one of these proteins that displaces previously adsorbed ones (Parsons, 1981).

8 Given a complex molecular mixture, below the threshold for independent binding there is no competition for the binding sites on the plastic. Above the threshold for independent binding, the quantity of each of the types of molecules adsorbed to the plastic may not be representative of the composition of the solution (Butler, 1981).

9 Below the saturation point the binding seems relatively independent of pH, temperature and ionic strength, although more and faster binding occurs at higher temperatures (Parsons, 1981; Herrmann, 1981).

10 The bound molecules retain some of their biological activities and in some cases acquire others not displayed when in solution (Ill and Ruoslahti, 1985).

Since most of these data refer to studies on the binding of selected molecules to plastic, it cannot be assumed that all molecules will behave in

the same way. It is therefore difficult, for instance, to claim conclusively that B16F10 cells do not adhere to collagen or gelatin since it cannot be ruled out that these molecules, once adsorbed to the plastic, lose their adhesive activity.

The endothelium as an adhesive substratum.

One of the central aims of this work was to compare the kinetics of adhesion of tumour cells to the endothelium and to isolated subendothelial matrix components. Previous published work suggested that endothelial cells were not a suitable substratum for tumour cell adhesion and that, in fact, tumour cells arrested in vivo by adhering to patches of the vessel wall denuded of endothelium. In their paper, Kramer and colleagues (1980) reported that endothelial monolayers were poorly adhesive for tumour cells and that other substrata, such as immobilized fibronectin or endothelial extracellular matrix, were much more effective in eliciting tumour cell adhesion. Although their basic adhesion assay ran for 1 h, from the published figures we can extrapolate some data of adhesion after 5 min incubation: tumour cell adhesion to the endothelium was ~ 7 % while adhesion to the subendothelial matrix was around 60 % and adhesion to fibronectin alone was between 80 and 90 %. The same group (Nicolson et al., 1981) later confirmed these results. Around this time there were reports from related research groups supporting the hypothesis that the endothelium was a most unlikely target for tumour cell arrest, as shown by the work of Poste and Fidler (1980) where tumour cells were allowed to adhere to the intact vessel wall or to one denuded of vascular endothelium. Reports on the adhesive role of isolated subendothelial matrix components like laminin and fibronectin, such as those by Vlodavsky and Gospodarowicz (1981) and Terranova et al. (1982), indicated that a consensus of researchers gave the subendothelial matrix a prime

role in the arrest of tumour cell. Indeed in a review paper Nicolson (1982) stated that tumour cells would be expected to bind preferentially to regions of injured endothelium.

This supposition must be met with reserve for two basic reasons:

1 The exposed subendothelial matrix is a very potent stimulus for platelet adhesion. Platelets are extremely numerous (values fluctuate, but in most vertebrates there are 2-8 x 10^8 platelets per ml of blood) and, although no numbers can be reliably quoted for circulating metastasizing tumour cells, it is safe to say that platelets are present in the blood in overwhelming numbers in comparison to cells of non-haematological tumours. With this in mind, it seems unlikely that tumour cells ever come into contact with exposed subendothelial matrix. Indeed, platelets would rapidly and effectively coat the denuded area before any tumour cell could have a chance to adhere to it. It can be argued, of course, that tumour cells adhere to platelet plaques (although the finding of tumour cells adhering to platelets adhering to the vessel wall is very rare in electron microscopy studies), but this is not pertinent to the endothelium/subendothelial matrix debate.

2 The other point to consider is the fact that the arrest and extravasation of circulating cells is of common occurrence throughout the lifetime of every individual. Apart from colonization of various organs by circulating cells during fetal life (liver, spleen, thymus, and so on), leukocyte and lymphocyte traffic across the endothelium is a physiological phenomenon for which no exposed subendothelial matrix is required. Indeed, it would be strange if a physiological function were to rely on what is considered to be a pathological condition (exposure of subendothelial matrix). Since tumor cells grow, locomote and function mainly by utilizing

mechanisms commonly used by normal cells, it is possible that tumour cells use the same mechanisms as other circulating cells to arrest in the circulation.

For these reasons it was felt that the endothelium itself, rather than the subendothelium, might be more important in tumour cell arrest. The data collected during this study disagree with the previously published work discussed above and show that the endothelium is indeed an adhesive substratum for tumour cells, certainly comparable to some isolated subendothelial matrix components such as fibronectin and laminin (see figure 29). Although the extracellular matrix of the endothelial cells (obtained by detergent extraction with 0.1 % Triton X 100) was not systematically tested in our adhesion assay, pilot experiments had shown that adhesion to such a matrix was comparable to that reported in previously published work (Kramer et al., 1980; Vlodavsky and Gospodarowicz, 1981; Nicolson et al., 1981), and the kinetics of adhesion were similar to those obtained using intact endothelium.

The reason for the differences in tumour cell-endothelial monolayer adhesion in our assay as compared with the work of Kramer et al., (1980), Nicolson et al., (1981), Vlodavsky and Gospodarowicz (1981), Tao and Johnson (1982) and Nicolson (1982 b) is not at first evident, but it is probable that the endothelium is a substratum which can modulate its adhesiveness according to external stimuli. This is supported by reports that indicate how the endothelium can change from an anti-thrombogenic to a pro-coagulant surface (see page 20). Indeed, many factors may influence the final status of the endothelium as an adhesive or non-adhesive surface. This is shown, for instance, by the work of Nakache and colleagues (1985) who found that different substrata onto which the endothelial cells were cultured induced changes in the mobility of

membrane lipids, although no gross shape and size differences were noticed. The different fluidity of the lipid bilayer, although not necessarily important in adhesion, is only an example of how the environment can subtly change the status of an endothelial monolayer. The density of the monolayer may also drastically alter adhesion, as shown by Di Corleto and de la Motte (1984), who tested adhesion of U937 monocyte cells to a number of endothelial cell lines of various derivations.

Further support for the notion that the adhesive status of the endothelial monolayer has the potential for modulation arises from the results of the experiments using trypsin (see figure 21) which showed that trypsin can induce the endothelium to become much more adhesive for the tumour cells.

With this in mind, we can attempt to explain the discrepancies between our findings and the published work by pointing out the differences in culture conditions, cell handling and assay techniques. We had recognized the need to culture the endothelial cells using a strict routine and a 3-day cycle (although endothelial monolayers could be kept in culture for months) in order to avoid the introduction of the effects of culture ageing and different cell densities. In their work, Tao and Johnson (1982) performed their assays 7 days after seeding of the endothelial cells, while in both the work of Kramer et al. (1980) and Nicolson et al. (1981) there is no reference to the age of the monolayers used. The culture medium is also very important, and in our experience even short exposure to slightly more acidic pH can cause disruption of the endothelial monolayer after a delay of several hours. For this reason we decided to maintain as stable a pH of the medium as possible during the adhesion assay, since a shift of the pH could cause modulation of the adhesiveness of the endothelium. Culture media usually rely on bicarbonate buffering with CO_2 in the incubator and unless CO_2 levels are controlled during the assay, the

buffering system fails to be effective. Hepes was therefore added to the medium and cells were allowed to adapt to it for 24 h. Even at the time of the assay due care was taken to ensure that the endothelium was not injured as the medium was aspirated and the tumour cell suspension inoculated. Results have shown that wounding modulates adhesion (Di Corleto and de la Motte, 1985), but it is likely that differences can occur even if less obvious injuries are inflicted on the endothelium. This could happen when, for instance, the medium is aspirated from the wells and the monolayers are temporary exposed to the dry heat of the thermostatic cabinet before tumour cells are added.

With respect to the comparison between the adhesion to endothelium and to fibronectin and laminin, it would be of great interest to establish what the concentration of these two ligands is in their functional states on the plastic substrata and on the endothelium. Endothelial cells produce and incorporate many types of adhesive molecules into the extracellular matrix as well as on their membrane, and it is possible that tumour cell adhesion to the endothelium is indeed mediated by one or more of these molecules. In this case their concentration on the surface of the endothelium should correlate with the data obtained using purified molecules. If in the experiments shown in figures 23 and 26 the adsorption of laminin and fibronectin occurred below the saturation limit, it is reasonable to assume that the real concentration of these ligands on the substratum is approximately 50 % of the quoted value. Therefore, if the binding of tumour cells to the endothelium occurred via laminin or fibronectin, the endothelium should possess at least ~ 4 ug cm^{-2} of fibronectin or laminin (confront figures 18, 23 and 26).

On the other hand there may be basic differences between an artificially constructed substratum and a monolayer of cells, the most obvious one being that a fibronectin substratum of a given density is

probably composed of randomly spaced molecules, while on the cell membrane fibronectin may form patches of tightly spaced molecules interspersed by large areas which are depleted of fibronectin. In this case, although the fibronectin density on the cell surface may be well below that of the fibronectin substratum, there will be localized areas of the cell membrane where the concentration of this molecule could be as high as that on the synthetic substratum (or higher). Rapid adhesion may rely on the formation of small adhesion patches with a high density of ligand-receptor interactions, therefore the possibility of such diversity should be taken into consideration when comparing the results of adhesion to endothelial monolayers with those to molecular substrata.

The experiments performed using formaldehyde (see figure 19) allow us to formulate some comments on the adhesive process. Because of the total inhibition of adhesion when B16F10 cells were exposed to the fixative compared with only a partial inhibition when the endothelium was fixed, it seems that rapid adhesion depends on the ability of the cell in suspension to modify itself as it comes into contact with the substratum. Rearrangements of surface molecules (capping?), exocytosis and localized shape changes (alignment of confronting surfaces?) would be almost certainly impaired in a cell exposed to fixative. In this light it is interesting to note that fixing of the endothelium resulted only in partial inhibition of adhesion. If an overtly active role of the endothelium during tumour cell adhesion is envisaged, as it would be in the case of adhesion and engulfing of circulating foreign bodies, we should have recorded a marked reduction of adhesion.

It is important to point out though, that fixation can have far more profound effects on the system than merely to cross-link and stiffen the cell membranes. It can, for instance, modify the physico-chemical properties of the surfaces to such an extent that the subsequent adhesion

observed could be due to totally different kinds of interactions, as decades of experiments with fixed platelets have shown.

As for the absence of adhesion elicited by von Willebrand factor, the preparation tested contained only the basic dimer plus perhaps small amounts of the low molecular weight multimers. Although it is known that von Willebrand factor is an extremely adhesive substratum for cells such as platelets, this property seems to reside exclusively in the very high molecular weight multimers (Sporn et al., 1986; Sporn et al., 1987) and therefore the absence of any adhesion (and spreading - see below) inducing activity does not rule out an involvement of von Willebrand factor in extravasation of tumour cells. It is not clear why high molecular weight multimers are highly adhesive molecules while the low molecular weight ones are not, since they are all assemblies of the same basic dimer and no post-polymerization modifications have so far been reported. We can only speculate that on the basic dimer there are low affinity binding sites for membrane acceptors. The high molecular weight multimers are highly coiled polymers (Slayter et al., 1985) and would therefore possess a high density of such low affinity binding sites and this would raise the efficacy of the molecule to bind to the cell membrane. A similar property has been demonstrated for the aggregation factor in the marine sponge Microciona prolifera where a very high molecular weight polymer containing a high density of low affinity cell binding sites promotes the specific re-aggregation of single cells, with the association constant increasing linearly with the molecular weight of the reconstituted polymers (Misevic and Burger, 1986).

An interesting observation in the present study was that von Willebrand factor coated substrata were completely non-adhesive (no cells were found on visual inspection, while we always noticed a small (2-7 %) amount of cells adhering to BSA-coated plastic). The non-adhesive

property of this molecule could well be worth further investigations in view of the possible industrial applications for substrata that are non-adhesive for blood, bacteria or algae.

Modulation by antibodies.

Attempts have been made to inhibit adhesion using anti-fibronectin and anti-laminin antibodies which were polyclonal in origin. Polyclonal antibodies contain a mixture of tens of thousands of different antibody sub-populations. Each of these sub-populations is directed against a different determinant of the target molecule and each individual antibody has a fairly high K_d for its determinant. Monoclonal antibodies are instead directed against a single determinant of the target molecule. In the case of monoclonal antibodies, only those with low K_d for the target molecule would successfully interfere with adhesion by competing with the ligand for the target molecule. This makes monoclonal antibodies more likely to react also with similar determinants present in different molecules.

Among the various polyclonal preparations against fibronectin tested, the one obtained through the Scottish Antibody Production Unit (SAPU) was found to exert an inhibitory influence on adhesion and it was used for further investigations. Although results showed that it causes an almost complete inhibition of adhesion when incubated with fibronectin substrata (87 % inhibition after 5 minutes), its inhibitory effects on adhesion to endothelial monolayers was much less (33 % of the total adhesion in 5 minutes). This could indicate that fibronectin is indeed not responsible for mediating adhesion between tumour cells and endothelium but some alternative interpretations should be considered. While in the first instance (fibronectin substrata) there is a well defined and comparatively simple situation where it can be envisaged that the

antibodies coat the fibronectin molecules and in doing so mask either directly or indirectly the sites that are involved in adhesion, in the case of the endothelium many alternative explanations may apply.

Since the substratum is composed of live cells, it can be hypothesized that the antibodies coat the cell bound fibronectin and that, once removed from the solution, the endothelium internalizes the bound molecules and replenishes its surface with more fibronectin which is then available for adhesion. If soluble antibodies are left in the medium during the assay so that they are still available to coat fibronectin molecules that are recycled to the membrane, adhesion is abolished. Unfortunately this inhibition is totally independent of the type of molecules bound to the substratum, being observed also when laminin and endothelial cell extract substrata were used. This is likely to be due to the action of the antibodies on the fibronectin present on the tumour cell surface. This could be either by functional blockage of the fibronectin molecule or by less specific means such as perturbation of the pattern of electric charges on the cell surface or because of the injury that can be associated with formation of immune-complexes on the cell surface.

The inhibition of tumour cell adhesion caused by the anti-fibronectin antibodies on fibronectin substrata is specific since they do not have any effect on other molecular substrata. The moderate inhibition exerted by such antibodies on the tumour cell adhesion to endothelial monolayer may be explained as having been caused by the formation of immune complexes on the surface of the endothelium. It must be remembered that the endothelial cells may be considered part of the immune system by virtue of their phagocytic and antigen presenting-cells activities. It has also been shown that the anti-thrombogenic status of the endothelial surface can be subverted very rapidly using a number of stimuli, challenge with immune complexes being one of them. For these reasons it is not

surprising that, although fibronectin may not be directly involved in the adhesion between endothelium and tumour cells, exposure of the endothelial cell surface to anti-fibronectin antibodies causes a slight inhibition of such adhesion.

Modulation by enzymes.

Another way of modulating adhesion is by means of exposure of cells to trypsin. Such an effect is in itself not surprising, since the enzymatic digestion of surface molecules directly or indirectly involved in adhesion would markedly influence this process.

Some research groups working with B16 cell lines use trypsin for the detachment of these cells during routine passaging. From discussion with Dr. Vollmers it emerged that B16 cells subjected to such treatment show a substantial decrease in the number of lung colonies formed after i.v. injection after only 3-5 in vitro passages. Generally our investigations pointed towards a reduction of adhesion following trypsin treatment, which could explain the methodological difficulties experienced by Vollmers and colleagues.

The action of trypsin may be influenced by the presence in the incubation medium of divalent cations within the normal physiological range, or of chelating agents. In the presence of chelating agents some molecules unfold part of their tertiary structure revealing sites of the polypeptide chain more susceptible to proteolytic cleavage. Usually, the enzymatic activity of trypsin in the absence of divalent cations is therefore enhanced both in term of speed of reaction and extent of the damage caused.

Exposure of tumour cells to trypsin (either in the presence or absence of divalent cations) caused an inhibition of adhesion to the endothelium. This inhibition was dependent on the time of exposure to the enzyme and

appeared to require longer exposure to the enzyme when divalent cations were present (confront figures 20 and 21). These data suggest that the structures responsible for the adhesion are proteinaceous. It is also possible that the carbohydrate moiety on the cell surface is active in some stages of the adhesive process since an effect of the use of trypsin (and other proteolytic enzymes) upon the cell surface is the loss of some oligosacharides side chains from the polypeptides cores. The differences recorded when digestion occured in the presence or absence of divalent cations cannot be due the action of the chelating agent per se on the tumour cells. Control experiments have, in fact, shown that incubation of tumour cells in a divalent cation-free buffer and in the presence of EDTA for up to 15 minutes did not alter their normal adhesiveness when this was later tested in normal complete medium.

Trypsin can also exert a stimulatory influence on the system. In the presence of inactivated enzyme, adhesion of tumour cells (not previously exposed to proteolysis) to the endothelium was enhanced (35 % increase of adherent cells after 5 minutes, as shown in figure 21). Since this enhancement was not detected when adhesion was tested on fibronectin substrata, these results would point to a possible action of the trypsin molecule on the endothelium. This action should not be due to the enzymatic activity of the molecule, which was blocked, although this may not be a correct assumption. Alternatively, contaminants present in the trypsin preparation and not trypsin itself may be responsible for this enhancement of adhesion.

Carbohydrate involvement.

Cell adhesion may be mediated by carbohydrate chains of surface molecules and indeed it has been proposed that lectins are involved in the process of metastasis (Raz et al., 1986). Lectins (carbohydrates-binding

proteins) have been implicated in many aspects of tumour biology, from "contact inhibition" of growth to involvement in tumour cell-platelet aggregation during metastasis (Raz et al., 1984 and references within). There are reports that carbohydrates are involved in cell-cell adhesion (for a review see Feizi, 1985) and lectin-resistant variants of B16 melanoma cells have been selected to show how the presence of lectin binding sites may correlate with the ability to metastasize (Tao and Burger 1982; Tao et al., 1983).

The adhesion to fibronectin, laminin and the endothelium may be mediated by carbohydrate chains present on tumour cell surface molecules, as indeed some reports have suggested (Nicolson, 1982 b). Since by using our adhesion assay we had already obtained data quite dissimilar from the published dogma, we decided to investigate the effects of tunicamycin treatment on adhesion. For this, tumour cells were treated with tunicamycin, which is an antibiotic that blocks the N-linked glycosylation by inhibiting the transfer of the first N-acetylglucosamine-phosphate to the dolichyl-phosphate molecule.

Results obtained after exposure of cells to tunicamycin (and other glycosylation inhibitors) are to be carefully evaluated in that, although the action of the antibiotic is thought to be very specific upon brief exposures, it may ultimately inhibit protein syntesis selectively and unpredictably. An explanation for this relates to the role of proteins themselves within the cellular machinery for protein synthesis: some unglycosylated proteins may have a shorter life or not function properly and, if the correct components of this machinery are not supplied, a long term effect could be disruption of protein synthesis. Proteins are also reqired for modulating DNA synthesis, mRNA production and transport, so that long term effects of impaired glycosylation may involve the perturbation of some genes coding for certain proteins. A different

explanation put forward is that the antibiotic itself may selectively interact at the nucleic acid level, thereby impairing the synthesis of some proteins. For a review on the inhibitors of glycosylation see Elbein (1987).

Adhesion of tumour cells to fibronectin or endothelial cells is affected only after long exposure (36 hours) to the antibiotic, the inhibition was partial and did not further increase after even longer exposure (up to 48 hours, see figures 31 and 32). Therefore adhesion of tumour cells to the endothelium and fibronectin does not seem to rely on the presence of N-linked sugar chains. The partial inhibition observed only after prolonged incubation with the antibiotic could be accounted for by its effects on protein, mRNA and DNA synthesis. An alternative explanation is that the molecules responsible for adhesion have an extremely slow turnover or that there is a substantial stored pool of such molecules that can be used before requiring de novo synthesis. This, it must be said, seems an unlikely scenario.

The inhibition recorded might be due to the fact that carbohydrate groups, although not directly involved in adhesion, could have the function of protecting adhesion molecules from proteolytic attack. If this was the case, the addition of inhibitors of proteolytic enzymes would reduce such inhibition. By using leupeptin it is possible to show that, in the case of the adhesion to the endothelium, the partial inhibition after 36 hours of treatment with tunicamycin is not dependent on increased enzymatic digestion of unglycosylated proteins (see figure 36). In the case of adhesion to fibronectin the use of leupeptin seems to partially overcome the inhibition caused by the antibiotic (figure 37). Interestingly, when tunicamycin was used to assess the adhesion to laminin (figure 33), the pattern of inhibition was found to be different from that observed for fibronectin and endothelium since there was a

quite marked inhibition after only 24 hours of exposure to the antibiotic.

This gives a stronger indication that adhesion to laminin, unlike that to the endothelium or to fibronectin, may depend on the presence of carbohydrate side chains. Certainly these experiments with tunicamycin must be interpreted with caution since it is possible that the effects seen on laminin may again be due to selective inhibition of the synthesis of some proteins rather than to the lack of glycosylation.

A number of cell surface receptors for fibronectin and laminin belong to the integrin family (Buck and Horwitz, 1987; Gehlsen et al., 1988). Integrins are composed of a beta subunit which is common to numerous receptors in the integrin family, while the affinity for fibronectin or laminin is probably given by the ligand-specific alpha-subunit. Different integrins seem to possess similar molecular mechanisms for the recognition and binding of the various ligands. These receptors bind to a discrete amino acid sequence (usually RGD) on the target molecules, with differences in structures adjacent to this sequence probably responsible for ligand specificity. If B16 cells adhered to both fibronectin and laminin via an integrin receptor, we would have expected similar results when glycosylation was blocked, but this was not the case. We can therefore postulate that different types of receptors are used during the rapid adhesion to fibronectin and laminin by B16F10 cells. Cells possess at least one non-integrin receptor for laminin (that binds a quite dissimilar sequence - YIGSR - see page 43) and have many surface-associated molecules that can bind both laminin or fibronectin; these results suggest that one of these could indeed be used during rapid adhesion. The polypeptide sequence YIGSR was not available and it could not be tested for inhibition of adhesion to laminin, but the lack of inhibition by the pentapeptide GRGDS suggested that B16F10 cell adhesion to fibronectin is not mediated by the RGD sequence. These results are in agreement with

those of other coleagues (Aplin, personal comunication) but do not imply that this adhesion is integrin independent; there are, in fact, two other sequences on fibronectin (LDV and RGVD, see pages 38 and 62-63) recognized by cell receptors of the integrin family. The lack of inhibition by GRGDS has also been described by McCarthy and colleagues (1986) who found that the peptide actively inhibited adhesion to low density fibronectin substrata (80 % inhibition) but not when the fibronectin concentration on the substrata was 5 times as much (20 % inhibition). Unfortunately the authors do not give exact indications of the volume used to coat the substrata but, by extrapolation from other work by the same group, we believe the fibronectin concetration was 0.25 ug cm^{-2} and 1.25 ug cm^{-2}, which would be in accordance with our findings.

Although the main emphasis of this work was the study of rapid adhesion, events subsequent to adhesion may be important in the metastatic cascade. Of particular interest is the fate of these rapid adhesive interactions and three possible outcomes can be envisaged:

1 Cells could continue to maintain only those molecular bonds that were established at the time of adhesion.

2 After a certain length of time, cells may decrease their number of adhesive bonds with the substratum and eventually even release their grip and detach.

3 Alternatively, cells will remain adherent and establish progresively more molecular interactions with the substratum.

The outcome of cell-cell or cell-substratum adhesion is important when migration and invasion are considered. Although the importance of the long term interactions during the metastatic process is undisputed, their possible interpretations are more controversial. Consider for example the possibility that, after rapidly adhering to the endothelium, the tumour cell later detaches from it. It can be argued that such a cell will be impaired from metastasizing since, by losing its adhesion to the endothelium, it will be washed away by the blood flow. Conversely it can be speculated that it is indeed such a character that will allow metastasis formation since the tumour cell will need to migrate through the endothelium and into the tissue: if a strong adhesion to the endothelium is maintained the tumour cell will be "glued" to the lumen and unable to extravasate.

The concept that cell adhesion is a dynamic process and that the

amount and strength of adhesion depends on the time elapsed after first contact, can be deduced from the work of Fligiel and her colleagues (1985) who monitored the adhesion of fibrosarcoma cells over a period of 48 hours and showed that adhesion increased and then decreased with time. Their work also exemplifies the importance of the substratum in determining the long term fate of adhesion, since laminin substrata gave a very different pattern of long term adhesion when compared with untreated substrata.

After rapid adhesion has been established, cells may develop some shape changes, sometimes very obvious, and cells may assume morphologies that range from extensively flattened to almost spherical. Cell flattening (spreading) is believed by some authors to be an extreme attempt by the cell to phagocytose a particle of infinite radius (the substratum), but this is not by any means demonstrated and there may be other reasons for such behaviour (for a review see Roos, 1984). In all cases it must be remembered that, although cell spreading occurs in vivo, the spreading of cells on the flat surface of a dish may be an activity that occurs only in vitro, and may have no biological importance in vivo.

Whatever the significance, cell spreading is a phenomenon deriving from a complex series of events which occur over a much longer time scale compared to rapid adhesion (at least to the type of rapid adhesion described in this study). During rapid adhesion cells still maintain an overall round shape, as perceived through light microscopy observation, while during spreading, cells lose their round shape and tend to flatten onto the substratum and at the same time extend their membrane to cover several orders of magnitude more surface area than was originally involved during rapid adhesion. The nuclei of spread cells become more evident, nuclear components like nucleoli and chromatin can be appreciated, and the cytoskeleton can be seen in association with the

formation of pseudo- and lamello-podia and the appearance of ruffled edges.

The assay.

Despite the fact that the phenomenon is clearly complex and probably relies on a multitude of different events and interactions, it is relatively easy to devise an assay that assesses the ability of a substratum to cause cells to spread, regardless of the complexity and interrelationship of all the events that give rise to the spreading. The spreading assay used in this study gives information only on the overall phenomenon and although in some cases it was obvious that some substrata induced cells to occupy more surface area than others, there has been no attempt to quantify such a parameter.

Spreading or, as some authors prefer, attachment assays are widely used in tumour cell biology research and many different variations have been employed in the past. They are all based on the same principle: a substratum (usually plastic or glass) is coated with a molecular solution (very often by passive adsorption but sometimes using cross linking molecules or derivatization of the substratum), a cell suspension is then inoculated onto the substratum and incubated for a considerable length of time (mostly between 1 and 4 hours), after which the number of spread cells are determined by microscopy (either with or without removal of non-attached cells, and on fresh or fixed and stained preparations). Typical of this technique is the work of Ruoslahti and his colleagues (1982).

The assay we developed used very small surface area polystyrene plates treated for tissue culture. During the years there has been a shift from the use of large Petri dishes to small-well plates, mainly because of convenience as well as the need to economize on expensive attachment

factors. Recently, microtiter (96-well) plates have been the standard for this work, but their use has not been completely satisfactory because of the poor optical quality in phase contrast microscopy. Because of this many authors have included fixing and staining of the attached cells in their protocols (Giancotti et al., 1985; Piershbacher and Ruoslahti, 1984; Ruoslahti et al., 1982). Evaluation of the assay by phase contrast microscopy is desirable because it avoids possible artefacts introduced by the fixing and staining procedures and is prefered by many research groups such as the one of Grinnell (for a review see Grinnell, 1981). Because of this, we experimented with Terasaki plates and found their phase contrast optics satisfactory, providing the wells were completely filled with medium. The Terasaki plates also give the added bonus of having a much smaller surface area to coat (~ 1/10 of the 96-well plates, depending on the volumes used).

Some authors prefer to use polystyrene plates not treated for tissue culture (i.e. bacteriological-grade), and one of the main reasons for this is that many cell lines do not spread on such a surface, unless it has been previously conditioned by a suitable molecule (Ruoslahti et al., 1982; Grinnell, 1981). This gives the great advantage that the substratum does not need blocking with a non-adhesive molecule after its conditioning.

Despite this, we chose the tissue culture-grade plastic for two reasons: firstly we wanted to compare the same substratum for its ability to induce rapid adhesion and spreading. Since adsorption of molecules may be different on differently treated surfaces and since tissue culture grade plastic had already been used for adhesion assays, we chose not to introduce a possible variable that could prevent useful comparisons. Secondly, in our hands, the B16F10 cells would spread on untreated bacteriological-grade plastic (although not as well as on tissue culture-grade dishes), therefore the blocking of the plastic would have been

required in any case.

BSA is a common blocking agent (Grinnell, 1981; Giancotti et al., 1985) although rarely used at concentrations higher than 1 %. In our assay this treatment allowed a small number of cells to spread on the plastic. We experimented with many different BSA preparations at various concentrations and found that high percentages of BSA (15-20 %) were required to inhibit such spreading. Although longer incubations (perhaps overnight) with less concentrated BSA solutions would have had the same effect, the benefits of streamlining the assay were preferred.

As for the medium used for the experiments, two variations of the assay exist: one that employs FCS containing medium and one which has no FCS. By allowing cells to spread in the presence of a complex and undefined mixture of molecules (FCS), there is always the risk that some of these molecules interact with the substratum, changing its original quality. Although arguably, because of the very presence of the cells, it is difficult to be sure that the substratum has not been in any way manipulated, it was thought better to avoid this if at all possible.

Evaluation of results.

Despite the fact that B16 cell adhesion has been the subject of many investigations, there are very few reports on the spreading abilities of these cells. McCarthy and his colleagues (1986) used B16F10 cell and fibronectin fragments in an attachment assay and concluded that, in addition to the RGDS sequence, there may be other attachment sites on the fibronectin molecule. They also found that the inhibition of attachment by soluble RGDS is inversely related to the concentration of fibronectin or fibronectin fragments coating the substratum. Terranova and his colleagues (1982) investigated the role of laminin and collagen in attachment of B16BL6 cells and showed that these cells spread more

readily on collagen type IV rather than type I. B16BL6 cells spread very readily to substratum adsorbed laminin, and soluble laminin improved the kinetics of attachment to collagen type IV. Anti-fibronectin antibodies did not interfere with their spreading to collagen type IV while anti-laminin antibodies inhibited BL6 attachment to collagen type IV. When cells were preincubated for 4 hours with cyclohexamide, an inhibitor of protein synthesis, there was no inhibition of cell attachment to laminin substrata or to substrata of collagen type IV + soluble laminin.

Other interesting information about the spreading phenomenon derive from studies with other cell lines. Fliegel and her colleagues (1985) noted that the spreading of mouse fibrosarcoma cells on laminin was dependent on the concentration of the ligand and that higher concentrations produced faster responses. Piershbacher and Ruoslahti (1984) tested the ability of various synthetic peptides adsorbed to the substratum to support the attachment of rat kidney cells and found that only RGD containing peptides were effective. Gehlsen and colleagues (1988) showed that A375M melanoma and RuGli glioblastoma cells attachment to various substrata including laminin, vitronectin, fibronectin, collagen type I and type IV can be selectively modulated by different RGD containing peptides. Following on the work of Piershbacher and Ruoslahti (1987), these authors showed how esapeptides based on GRGDxP with different amino acids in position x could inhibit the binding of fibronectin, collagen type I and vitronectin with their specific receptors. When these peptides were tested in an amniotic membrane invasion assay, it emerged that fibronectin was a crucial ligand involved in invasion.

To date no systematic studies of the spreading of B16 cells on different substrata has been done and, by using our spreading assay, some useful data regarding this phenomenon emerged. These are briefly:

1 B16F10 cells spread readily on a number of substrata ranging from

fibronectin and laminin to FCS, but do not spread on other substrata such as collagen, BSA or gelatin.

2 The kinetics and percentage of cell spreading depends on the concentration of the spreading factors on the substratum.

3 Within a cell population there is a considerable heterogeneity with regard to readiness to initiate spreading.

4 Some molecules, although unable to cause rapid adhesion, can trigger cell spreading.

The time-dependent experiments (figures 42-44) showed that the first cells begin to spread after 10 and before 20 minutes of incubation and most cells were spread within 30 to 45 minutes. These observations are interesting since, although most of the cells have the ability to spread, there are indications that within the population there are cells that are responding to the spreading stimuli much more readily than others. This delay in spreading shown by some cells could be due to many diverse factors. The substratum could be a mosaic of patches with different supramolecular organization of the spreading molecules so that in places spreading can be initiated almost immediately, while in others some processing of the substratum by the cells may be needed before spreading can be observed. This is, of course, quite difficult to disprove but there are data that point towards an even and regular adsorption of molecules on the substratum, as shown in the work of Grinnell (1981). A more plausible hypothesis is that within the population there are a series of phenotypes for the character "spreading", some of which can be distinguished by the rapidity of their response. Such diversity may only be the effect of differences related to cell cycle (for the influence of the cell cycle on cell adhesion see the work of Elvin and Evans, 1983) but alternatively they could be caused by the presence of a more stable type of phenotypic variation. If this was the case, it would be of interest to

select from the population the fast responders and assess this character for its association with a higher or lower metastatic potential. Selected populations could be easily obtained by removing either the fast or the slow responders with the help of a micropipette mounted on a micromanipulator.

Like adhesion, spreading is dependent on the density of molecules adsorbed on the substratum (see figures 39-41), with the difference that the concentration of a particular molecule needed to elicit maximum spreading is lower (around one order of magnitude) than that needed to obtain fast adhesion (confront with figures 23 and 26). In general, the concentrations of purified molecules needed to induce spreading was ~ 0.1 ug cm^{-2} and the maximum effect was noted around 1 ug cm^{-2}.

It has also emerged from this study that there are molecular substrata that, although unable to support adhesion, do allow cells to spread. FCS, for instance, stimulated a good proportion of cells to spread, as did the polyclonal antibody anti-fibronectin (figures 40 and 46). Both these substrata, and especially the anti-fibronectin preparation, were unable to support rapid adhesion.

Because cells do not spread on an immunoglobulin fraction of normal sheep serum, it is quite interesting that the anti-fibronectin preparation stimulates cells to spread because such spreading must be dependent on cell surface fibronectin interactions with the substratum-bound anti-fibronectin antibodies. Since no rapid adhesion can be detected, the kinetics of this interaction is probably far too slow. Nevertheless, the binding of cell surface fibronectin to an acceptor on the substratum induces the cell to carry out a spreading response which, therefore, does not rely on a fast reaction.

The role of cell surface fibronectin on cell spreading is intriguing since soluble anti-fibronectin antibodies block spreading regardless of the

type of molecular substratum used. These findings could lead to the assumption that cell surface fibronectin plays a crucial part in the cascade of events that lead to the spreading of the cell. On the other hand, as discussed before, the mere binding of antibodies to the cell surface may be a perturbing event: there may be a non-specific coating effect, there may be the masking of important ligands present on the cell membrane near the fibronectin molecules, there may be an injurious stimulus to the target cell and, lastly, the binding of these antibodies to the cell surface fibronectin may act as a signal to inhibit spreading.

Alternatively, it could be envisaged that, in order to spread, cells must deposit extracellular material on the substratum. If fibronectin is a crucial component of such material, the presence of the antibodies in the medium could prevent fibronectin interacting with other molecules, ultimately disrupting the deposition of such extracellular material.

Although the inhibition of spreading caused by the anti-fibronectin antibodies was quite specific for fibronectin, when tested on FCS substrata they had the effect of enhancing spreading (see figure 49). This is quite puzzling since either no effect or an inhibition was originally expected and no definite reason can be given for this phenomenon. One way of explaining such enhancement is to envisage that within FCS there are molecules which are capable of binding immunoglobulins (components of the complement system are good candidates), possibly but not necessarily, via their Fc tails. In this way the substratum would present a number of anti-fibronectin antibodies with their antigen-binding site free to interact with the cell surface fibronectin, thereby increasing the density of molecules that can mediate cell spreading.

Another interesting observation came from the experiments in which gelatin was used (figure 45). Denatured collagen has quite a good affinity for fibronectin and in view of the fact that cells do spread on a lawn of

anti-fibronectin antibodies presumably via cell surface fibronectin (since these antibodies induce spreading only when they have their fibronectin binding site available, and not if they are heads down with their Fc tails sticking up as when they coat a fibronectin substratum), it was expected that cells would spread on denatured collagen too. Nevertheless denatured collagen was not able to mediate either adhesion or spreading, although it was able to bind soluble fibronectin and, by creating such a sandwich, cause cells to spread. A reason for this is that cell surface fibronectin could be different from plasma fibronectin in its binding to gelatin and, in fact, purification protocols for cell surface fibronectin use a very high pH (11.0) to ensure its binding to immobilized gelatin. Whether this indicates a real difference between these two forms of fibronectin or merely derives from methodological convenience (cellular fibronectin is said to be poorly soluble at physiological pH) is not clear. It could also be the case that the fibronectin of the cell surface may not have the gelatin binding site available, due to the way the two arms of the molecule are folded. Alternatively it could be postulated that cell surface fibronectin is not a ligand involved in spreading and that the anti-fibronectin antibodies cause spreading by some other mechanism. However, this last possibility seems unlikely.

In general, and following comparison of these data with those derived from the adhesion assays, it was found that substrata that allow rapid adhesion also had the correct stimuli for cells to spread. Non-adhesive substrata could also induce spreading although in some cases (e.g. gelatin) no spreading was observed. It is interesting to report here that in similar experiments performed in our laboratory with different cell types (endothelial cells) some substrata that induced rapid adhesion (e.g. BSA) were not effective in eliciting spreading, at least not during the time course of the assay (60 min).

During the course of this study it was apparent that the endothelial monolayer provided a good substratum for tumour cell adhesion and evidence suggested that this adhesion was not due to fibronectin or laminin molecules present on the endothelium. Since the earlier strategy to try and identify the moiety present on the endothelial surface responsible for such adhesion by raising monoclonal antibodies were abandoned, attempts were made to extract material from the endothelium that would support the adhesion of tumour cells when it was adsorbed onto a plastic substratum.

Many extraction approaches have been used in the past to recover activity from a cell monolayer, some are severe and cause cell disruption and yield a complex mixture that has to be extensively purified. Some rely on the use of various enzymatic treatments which lead to the solubilization of far fewer molecules from the cells but carry a higher risk of inactivating the molecules under investigation. This would therefore render functional identification quite difficult. Some milder treatments include the use of chelating agents that release material from the cell layer in two ways: either by disrupting interactions within molecules of the supramembranous coat, or by stimulating the cells to excrete or release some molecules. In either case it is conceivable that only comparatively few types of molecules would be recovered in the supernatant. This has obvious advantages for the identification and purification of active compounds. Furthermore, since the extracting procedure does not rely on enzymatic digestion or denaturing but simply

on changes in ternary and quaternary molecular structure and changes in affinity of reciprocal binding sites (all usually reversible upon reinstating of a physiological environment) it would be less likely that, if the molecules responsible for the adhesion were present in the extract, the adhesive activity would not be detected because of inactivation. Early experiments showed that such an approach was promising since the extracted material adsorbed on plastic would support tumour cell adhesion in a way comparable to that obtained with the intact monolayer.

Exclusion chromatography indicated that the active material had a molecular weight of several hundred kD or more (see figures 52 and 53). Molecular weight determination of very large molecules by gel filtration is not very precise and can sometimes be unreliable since the molecules in solution may interact with some reactive groups present in the gel. Molecules like thrombospondin, laminin, fibronectin and von Willebrand factor as well as GAGs had to be taken into consideration as possible candidates for the unidentified factor, because of their molecular weight. All these molecules are produced by the endothelial cells and immuno dot-blot analysis of the extract revealed, albeit with a certain degree of variability, the presence of both fibronectin and laminin. Anti-fibronectin antibodies did not block the adhesion or spreading activity of the extract, but this alone did not rule out the presence of fibronectin. In fact, if the activity was due to the presence of many different adhesive molecules, blocking one would still leave all the others to elicit adhesion. To exclude this, it was decided to remove any such adhesive molecules, if present, by affinity chromatography. Fibronectin binds to gelatin and heparin; laminin has a heparin binding site and so has thrombospondin; heparan sulfate and other GAG's bind to poly-L-lysine; vitronectin (which can self associate to form higher molecular weight aggregates) binds to heparin. Additionally each one of these molecules has a strong affinity for most of

the others when these are immobilized.

All the affinity chromatography ligands used failed to remove the adhesion activity from the extract, and this gives strong, although not conclusive, indications that the high molecular weight adhesive factor extracted from the endothelium is not one of the above mentioned adhesive molecules. Alternatively if such adhesive molecules were engaged in interactions with each other to form large molecular weight complexes, they could have reduced affinity for the immobilized ligands used in these experiments. The existence of soluble complexes of proteins and glycosaminoglycans (adherons) has been confirmed by the work of Schubert and his group (Schubert et al., 1986 and references within; Tsui et al., 1988 and references within).

Attempts to filter sterilize the extract resulted in rapid clogging of the filter and the loss of some activity. The type of filter used was a low protein binding membrane with a mean pore size of 0.2 um. If a solution of laminin is passed through such a filter, a very marked loss of activity of the preparation will result, while a solution of fibronectin can be filtered with no detectable loss of activity. This indicates that, in the case of the extract and of laminin, these large size molecules are retained by the filter because of physical trapping.

Adhesion to a plastic substratum coated with purified endothelial cell extract was almost totally inhibited by the peptide GRGDS (which failed to inhibit the adhesion to fibronectin). It has been shown that many interactions between adhesive ligands and their receptors at or near the cell surface involve the binding of RGD or closely related sequences to specific RGD receptors, implying that these are specialized sequences for adhesive phenomena. In the course of this work adhesion to fibronectin was not modulated by the peptide, nor was the adhesion to laminin. This would restrict the search among known adhesive molecules to

thrombospondin and von Willebrand factor. Other RGD containing molecules like fibrin and fibrinogen did not support adhesion of tumour cells when tested in the adhesion assay. Thrombospondin, which was present in the crude extract albeit at very low concentration (results courtesy of Dr. Nick Hunter, MRC Blood Components Assay Group, Edinburgh), should have been removed during purification against heparin-agarose.

Of the known RGD containing adhesive proteins, von Willebrand factor fit many of the observations characterizing the extract. Stored by the endothelium, it is released upon stimulation and forms the bases of platelet adhesion to the vessel wall (Weiss et al., 1986). Injurious stimuli can cause the release of stored von Willebrand factor by the endothelium. The released factor will be immobilized on the surface of the endothelium and this is thought to happen via the binding to the complex GPIIb/IIIa, a receptor for RGD (Parker et al., 1986). If exposure to chelating agents is an injurious stimulus for the endothelium it is conceivable that its cells would react by degranulating. The released von Willebrand factor would not be able to bind to the endothelial membrane because of the absence of divalent cations in the medium and would therefore be recovered in the supernatant.

Alternatively, during extraction, some components of the subendothelial matrix could be solubilized. Chelating agents are used for instance to extract laminin from the basement membrane of EHS tumours (this is in fact part of the standard protocol for the purification of commercially available laminin) and could have similar effects in the case of the subendothelial matrix. Regardless of the localization of the extracted adhesive factor, whether it is associated to the cell membrane, released from an intracellular pool or incorporated in the subendothelial matrix, there are indications that it is an endothelial cell product. Although no

metabolic labelling has been done, the adhesive factor doesn't seem to be derived from the FCS of the culturing medium since there is no trace of it in extracts of glass adsorbed medium. There is of course the possibility that such a factor is indeed present in the fresh medium but is not incorporated to the glass-adsorbed molecular layer in the absence of endothelial cells, at least not in an extractable/active form. Preliminary experiments have shown that endothelial cells cultured in defined medium with an FCS substitute yield a similar extractable factor.

Summary

In summary, the endothelium is a very adhesive substratum for B16 malignant melanoma cells. Results indicate that this is as adhesive as the subendothelial matrix or as isolated adhesive proteins such as laminin and fibronectin. The adhesiveness of the endothelium for the tumour cells can be modulated and rapidly increased (by trypsin, for instance). The role of the endothelium during the arrest of metastasizing tumour cells should be reviewed in the light of these findings.

Rapid adhesion is dependent on the concentration of adhesive factors on the substratum and is inhibited at low temperatures and in a cation-free medium. Spreading is a slower phenomenon and requires a smaller concentration of adhesive factors on the substratum. Collagen and gelatin do not elicit either adhesion or spreading of B16F10 tumour cells.

Carbohydrate moieties on the tumour cell surface do not seem to be involved in adhesion of these cells to the endothelium. Trypsin, on the other hand can digest molecules on the tumour cell surface which are important in adhesion to the endothelium.

An attempt to extract from the endothelium the moiety responsible for the rapid adhesion of the B16 cells was successful, and partial characterization of this factor suggests that it is a high molecular mass, RGD-containing or RGD-binding molecule.

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