

Adhesion molecules in cancer invasion and metastasis

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For more than one hundred years the question “what defines which organs and when will be affected in case of a disseminated cancer” expressed by Paget remains basically unanswered, despite the bulk of published studies regarding the cancer behaviour. In 1944, Coman developed the theory of adhesion forces regarding the cancer problem. Nowadays, it is known that the interactions either among cells (cell-cell) or among cells and substratum components (cell-matrix) are mediated by some molecules, named adhesion molecules. Until now, more than a hundred of adhesion molecules have been identified. Research on physiology, biochemistry, genetics, and biology so in basic as in clinical level, attracts with intense interest the attention of many scientists. In the majority of the human cancers it has been clearly demonstrated that alterations in the adhesion forces are implicated in the invasion and metastasis process. Furthermore, an effort to establish a role of adhesion molecules as potential biomarkers in various human malignancies is attempted. Involvement of adhesion molecules in diagnosis, prognosis and treatment possibly has been implicated in many cases of human cancer. Adhesion molecules comprise of five known families; cadherins, integrins, immunoglobulin gene superfamily (IgSF), selectins, and CD44. In the present article a global approach of the whole subject is attempted. In addition, the role of some adhesion molecules in carcinogenesis is discussed in some detail. *Hippokratia* 2005; 9 (3): 106-114

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In multicellular organisms, the development of adhesion bonds, either among cells or among cells and components of the extracellular matrix, is a crucial process. These interactions are mediated by some molecules which are named adhesion molecules. That is why these molecules play a main role both at the early stages of tissue consistency and later on. This happens because this adhesive process is directly related to the differentiation, architecture and normal development of the tissue. The development of cancer represents a disturbance of normal tissue homeostasis. The interaction of neoplastic cells with surrounding cells and underlying extracellular matrix (ECM) is a central component of this pathological process. It is not surprising, therefore, that the adhesion receptors, those molecules responsible for maintaining normal tissue architecture and regulating cellular interactions, should play such an important part in modulating cancer development and evolution¹. It is, however, unlikely that the major contribution of these adhesion molecules occurs during the initial phases of tumour development. Rather it is the late stage of cancer spread, a process known as metastasis, which involves the migration of neoplastic cells from one anatomical site to another, that is likely to be most affected by dearrangement or alterations in adhesion receptor levels or function^{2,3}. At present, adhesion molecules embrace five categories: cadherins, integrins, immunoglobulin gene superfamily members, selectins and CD44.

Involvement of ECM components in cellular proliferation and apoptosis

The ways that cells dissociate, migrate, arrest and reassociate with other cells are basic knowledge in biology and physiology. According to this approach, the formation and function of specific cell-cell or cell-matrix junctions appears very interesting. While the ECM has long been known to play a part in regulating cell growth and differentiation, only recently it has become apparent that it also plays a major role in controlling cell growth via the regulation of apoptosis^{4,5}. Apoptosis, which is also known as programmed cell death, is a means of controlling, via specific biochemical pathways, the self-destruction of cells. It is the dynamic balance between cellular proliferation and apoptosis in particular tissues which determines the overall cellular equilibrium of that tissue. Obviously, imbalances such as increased cellular proliferation or decreased apoptosis could be involved in the non-regulated growth observed in tumour development. It has been shown that both in vivo and in vitro, the loss of cellular adhesion to specific components can be initiated by interfering with the way in which specific members of the integrin superfamily of adhesion molecules interact with the ECM⁶⁻⁸. How the integrins manage to mediate these apoptotic survival functions in normal cells is unclear at present, but probably relates to the induction of signaling molecules as a consequence of integrin occupancy. In cancer development and

growth, cellular contact with the ECM is often lost. How, then, does the cancer cell manage to avoid the induced apoptosis that would result in normal cells if they were subjected to a similar loss of contact with the underlying ECM? It could be that the formation of a nidus of transformed cells generates signals from cell-cell communication which might to some extent offset this apoptosis. However, neoplastic cells have the capacity, especially during the process of tumour dissemination, to survive as individual single cells where there is little likelihood that such compensatory cell-cell signals would be generated to offset the lack of ECM-derived signals⁹. An alternative way that tumour cells might preserve the capacity to avoid programmed cell death would be via the constitutive activation of their integrin subunits, are continually generated within the cell to suppress the apoptotic response.

The precise nature of the intracellular signals generated may be dependent on the specific integrin-ligand interaction^{9,10}. Thus it was recently shown that up-regulation of Bcl-2 expression, a means of suppressing apoptotic death, occurred when attachment to a fibronectin substrate was mediated through one ($\alpha 5\beta 1$) but not the other of two fibronectin receptors (table1). Table 1 presents a classification of integrins receptors regarding their binding-specificity to ECM components¹¹. There is, then, the possibility that cells which find themselves in the incorrect tissue compartment (i.e. attaching through certain integrins which would not normally be involved in mediating this attachment) may be eliminated as a result of the physiological induction of apoptosis. The converse of this prospect is that metastatic cells have, through mechanisms which require determination, lost this capacity and can survive in these inappropriate sites or even generate growth-promoting signals upon arrival in these sites.

Table1. Functional classification of integrin receptors according to their ligand specificity.

Integrins ligands specificity to matrix components

Collagen receptors	$\beta 1-\alpha 1, \alpha 2, \alpha 3, \alpha v$
Fibronectin receptors	$\beta 1-\alpha 3, \alpha 4, \alpha 5$ $\beta 3-\alpha v, \alpha IIb$ $\beta 6-\alpha v$ $\beta 7-\alpha 4$
Laminin receptors	$\beta 1-\alpha 1, \alpha 2, \alpha 3, \alpha 6, \alpha 7$ $\beta 3-\alpha v$ $\beta 4-\alpha 6$
Vitronectin receptors	$\beta 1-\alpha v$ $\beta 3-\alpha v, \alpha IIb$ $\beta 5-\alpha v$
Non-defined	$\beta 1-\alpha 8, \alpha 9$ $\beta 8-\alpha v$

Tumor neovascularisation depends on adhesion molecules stimulation

It is frequently stated that in order for a solid tumour to grow beyond 2 mm in size, the size at which passive diffusion of nutrients is growth-limiting, new blood supply must be induced by the growth of vessels from surrounding normal tissues. Changes that are observed in invasion and metastasis not only do they concern the whole cell collections, but also they refer to various molecules in different subcellular anatomic sites. Hence, adhesive cell-cell or cell-matrix contacts may be created at the invading or frontal edge of the cell, while the posterior edge of the cell may disengage from either cell neighbours or surrounding extracellular matrix proteins. One must point out here that a great number of investigators occupied by the cancer problem, from a therapeutical point of view, are directed to the neovascularisation field, since angiogenesis is of vital importance for tumor growth and development. By in vitro and in vivo experiments it has been shown that soluble members of adhesion molecules are able to stimulate angiogenesis and tumor development¹². It occurs with members of the selectins and the immunoglobulin superfamily (e.g. PECAM, platelet endothelial cell adhesion molecule)¹¹. This process of neovascularisation, or angiogenesis, has long been thought to depend mainly on the release of angiogenic peptides whether from infiltrating normal host cells, such as macrophages, or from the neoplastic cells themselves. Cell adhesion and cell adhesion receptors have not, until now, been thought to make major contributions to the angiogenesis phenomenon. Thus, the role of adhesion molecules must be compared to the role of other angiogenetic factors such as angiogenetic cytokines, e.g. IL-8 (interleukin-8), TNF- α (tumor necrosis factor-alpha), and VEGF (vascular endothelial growth factor). Relatively recently, however, certain soluble adhesion molecules have been shown to be capable of inducing angiogenic responses both in vitro and in vivo experimental test systems¹²⁻¹⁴. Such molecules should be liberated at the site of cancer development, possibly by the cleavage of cell-associated receptors through the activity of cancer-derived proteases, and then the contribution of adhesion molecules to this means of regulating gross cancer growth could be highly significant. Where cell adhesion has been realised to play a vital part in modifying angiogenesis, is in the control of endothelial cell adhesion. Cords of endothelial cells, arising from pre-existing capillaries or venules, form new capillary sprouts which grow in towards the angiogenic stimulus. The interaction of these endothelial cells with the underlying extacellular matrix is necessary for the formation of new capillary loops. It is of interest, therefore, that expression of the integrin $\alpha v\beta 3$, the vitronectin receptor, at the surface of endothelial cells is a key event in angiogenesis¹⁴. Thus, antibodies or soluble ligands capable of inhibiting $\alpha v\beta 3$ activity can abrogate the angiogenic response. This abrogation of neovascu-

larisation has led either to an inhibition of further tumour growth or to the diminution of the size of already established tumours (possibly as a consequence of nutrient deprivation). In general, the continuous growth of a tumour beyond a critical size is dependent on the development of new vessels, which not only supply nutrition for further growth of the tumour, but also serve as the route for systemic metastasis. In the metastatic cancerous process, a cancer cell must gain access to the target organ, escaping the vascular system into the target organ and inducing angiogenesis into the target organ to further growth and additional metastasis.

Finally, adhesion molecules may be involved in the regulation of tumour growth at least in two levels: either by regulating the balance between cell growth and cell death on an individual cell basis, or by controlling angiogenesis which is responsible for regulating growth of the multicellular tumour at a gross level. As already discussed, though, it is the process of metastasis which is most likely to be affected by adhesion molecules activity.

Tumor spread and adhesion molecules

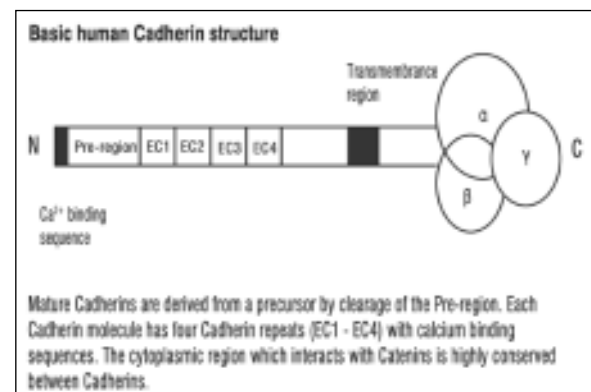
Cancer cells have been shown to express most of the adhesion receptors found on the surface of normal cells. Complex and coordinated reductions and increases in adhesion have been proposed to be necessary in order of tumour invasion and metastasis to take place^{2,15}. This hypothesis has fuelled the interest of cancer research teams in evaluating the expression of various adhesion molecules in a wide range of human malignancies in the hope of pinpointing some of the cell adhesion alterations underlying tumour behavior^{2,15}. Today, the use of monoclonal antibodies directed relatively against certain adhesion molecules provides the possibility of studying the majority of human cancer cases. Both basic and applied research are attracting the increasing concern of many scientists. It is entirely probable that the cancer cell utilizes many of the mechanisms used by normal cells to migrate around the body. The invasive and migratory capacities of cells like leukocytes, fibroblasts and trophoblasts are controlled by changes in cell-cell and cell-matrix interactions.

Adhesion molecules of the five known families are biochemically and genetically unique although in some instances they have related roles. Qualitative and quantitative changes in the expression and function of adhesion molecules have now been identified in the majority of human tumors using *in vitro* experimental model systems and *in vivo* immunolocalization studies. The hallmarks of malignancy e.g. invasion and metastasis, can now be explained at least partly, by alterations in the adhesive properties of the neoplastic cells. Additionally, interest is found in the role of adhesion molecules as potential biomarkers in malignancy for use in screening, in early and differential diagnosis, and in the assessment of prognosis as well as for the monitor of disease progression.

Cadherins

One class of cell-cell adhesion molecules implicated in tissue formation is the Ca^{++} -dependent, developmentally regulated transmembrane cadherin proteins¹⁶. Cadherins are the most interesting among all adhesion molecules since when they are present all adhesion interactions may be continued even in the absence of all the other rest adhesion molecules. Cadherins also help in tissue architecture maintenance in adult animals and humans. Cadherins participate in homophilically interactions in a subclass-specific manner on the extracellular side^{17,18}. On the cytoplasmic side, cadherin molecules interact with catenins (α , β and γ) which link cadherin molecules to cytoskeleton¹⁸⁻²¹. Figure 1 shows schematically the basic structure of cadherins. Mature cadherins are derived from a precursor by cleavage of the pre-region. Glycosylation and phosphorylation represent other post-translational modifications. The mature protein is between 723 and 728 aminoacids long. The most important epithelial cadherin (E-cadherin) molecules is a 120kda peptide mapped on chromosome 16q.22.1. Each cadherin molecule has four cadherin repeats (EC1-EC4) with calcium binding sequences. The cytoplasmic region which interacts with catenins is highly conserved between cadherins. For many years it has been known that malignant cells have a reduced capacity to adhere to one another. This, it is thought, facilitates the malignant cell's ability to detach from a primary tumour and to initiate the process of invasion into surrounding tissue²². Cadherins are cell surface membrane glycoproteins capable of mediating cell-cell adhesion in a calcium-dependent fashion. Since the major ligands of cadherins are the same molecules expressed on adjacent cells, the adhesion mediated by these receptors is termed homophilic or homotypic binding. These interactions serve to hold together and maintain tissue architecture¹⁷. A number of cadherin molecules have been identified and characterised, but there are three major members of this family: E-cadherin (epithelial), N-cadherin (neural) and P-cadherin (placental)^{17,23,24}. Of these, it is E-cadherin which has the most obvious involvement in cancer development since more than 90% of human cancers are carcinomas arising

Figure 1.



from epithelial tissues. Cadherins are the most important of all adhesion molecules and when they are expressed the inactivation of the other cell-cell adhesion molecules has little effect. Loss of E-cadherin expression is associated with dissociation and dispersal of epithelial cells. Thus, when monoclonal antibodies to E-cadherin have been used on E-cadherin-expressing epithelial cell types in tissue culture, loss of epithelial morphology has been observed and the dispersed cells have acquired a fibroblast-like morphology. Conversely, the introduction of cDNAs encoding for E-cadherin into non-E-cadherin-expressing cells has resulted in *de novo* expression of E-cadherin at the cell surface and the acquisition of a more epithelial morphotype. When tumour cells have been recipients of these cDNAs they have exhibited a reduced capacity to invade or migrate out of invasion assay systems. Cadherins are rapidly degraded by protease action in the absence of the calcium ions. Their function is regulated via the -COOH terminal intracellular domain by α , β and γ catenins which interact with cytoskeletal elements of the cells with which they bind^{10, 18, 19}. A recently identified protein p120 has a role similar to that of catenins and therefore is regarded as a new catenin molecule. Apart from their essential role in modulating cadherin adhesivity, catenins have been implicated in cell and developmental signaling pathways²⁶. For example, β -catenin is associated directly with at least two receptor tyrosine kinases and transduces developmental signals within the Wnt pathway. Catenins also combine with the tumor suppressor protein adenomatous polyposis coli (APC), which appears to have a role in regulating cell proliferation^{27, 28}. Mutations of the adenomatous polyposis coli tumor suppressor gene have been linked to familial polyposis, an inherited predisposition to colon cancer and high percentage of sporadic colon adenomas. Clinical analysis of human tumour specimens has shown an inverse correlation between expression of E-cadherin and the stage of cancer progression. Until now, a bulk of reported studies have shown that E-cadherin downregulation is a general phenomenon observed in many malignancies in humans including colorectal cancer²⁹, prostate cancer³⁰, bladder cancer^{24, 31}, pancreatic cancer³², esophageal cancer³³ and Barrett's esophagus³⁴, gastric cancer^{35, 36}, head and neck cancer³⁸, primary liver cancer³⁷ and cervical intraepithelial neoplasia³⁹. This downregulation has been shown to occur both at the messenger RNA and at the protein level. However, lack of normal protein is not the only way in which tumours may be released from the constraints of cadherin-mediated cell-cell adhesion. Thus, both mutations which disrupt the binding site of the cadherin molecules and mutations in the catenins, those proteins which serve to link the cadherins to the internal cytoskeleton of the cell can result in loss of functional cadherin activity. Though little information is available regarding the incidence of these changes in clinical material, it is apparent that both such mutations can occur not only in human cancer cell lines but also in fresh human tumor specimens. It

appears, therefore, that the release of neoplastic cells within the primary tumor mass from cadherin-mediated cell-cell cohesion is an important prelude to the initial events of tumor invasion.

Integrins

Integrins are a superfamily of transmembrane glycoproteins found predominantly on the surface of leukocytes that mediate cell-cell and cell-substratum interactions⁴⁰. They are heterodimers consisting of α and β subunits. Beta subunits of all integrins are remarkably similar. Their amino-sequences present a 40-48% homology with specific structural features. Integrin expression appears to be universal; at least one member of the integrin family has been found on every cell/tissue studied⁴¹. Integrins are heterodimers consisting of noncovalently associated α and β subunits. Nowadays, there are at least nine β subunits and fifteen α subunits, but new integrins and new ligands continue to be discovered. Integrins form a bond with a variety of proteins including fibronectin, fibrinogen, laminin, collagen, thrombospondin, vitronectin, and von Willenbrand factor as well as with C3b_i complement protein⁴⁰. They also bind to members of the immunoglobulin gene superfamily such as ICAM-1 (intercellular cell adhesion molecule-1 or CD54), ICAM-2 (intercellular adhesion molecule-2 or CD102), and VCAM-1 (vascular cell adhesion molecule-1 or CD106). There are different specificities of the integrins depending on the cell type in which they are expressed⁴². The RGD (arginine, glycine, aspartic acid) sequence is a recognition site for some integrins being their epitope^{42, 43}. It is of great interest that peptides with RGD sequence when given intravenously in cancer bearing wistar rats abrogate lung metastasis⁴⁴. Another interesting role of integrins is their participation in signal transduction pathways. The largest number of integrins are members of the β_1 or VLA (Very Late Antigen) subfamily, expressed by a variety of cell types⁴¹. The β_2 subfamily of integrins consists of leukocyte receptors LFA-1 (Leukocyte Function Associated Molecule-1), Mac-1 and p150^{41, 45, 46}. The β_3 subfamily, also named cytoadhesins, consists of the platelet glycoprotein gpII_b/III_a and the vitronectin receptor⁴¹. Other β subunits such as β_4 , β_5 , β_6 , β_7 and β_8 have been also characterized⁴⁷.

Once the neoplastic cells have been released from the primary tumour mass they must then migrate through and interact with the underlying ECM. This local invasive spread is a prerequisite for infiltration of the tumour cells into small lymphatics or blood vessels. The interaction between the ECM and disseminating cells involves both adhesion to and destruction of this structure. Attachment to the adhesive glycoproteins of the ECM is a consequence mainly of expression of the integrin heterodimers. However, the destruction of ECM which is mediated by proteolytic enzymes may also involve members of the integrin family. An association between enzymes of such important protease groups as

the metalloproteinases or the plasminogen activators and various members of the integrin family has been documented^{48,49}. Although the integrins are constitutively expressed, they do not bind to their receptors unless the molecule is activated. The conformation of both the integrin and its ligand is crucial to interactions among integrins and their ligands. Some possible mechanisms exist to explain the possible mechanisms of integrins activation. A region of 190 amino acids located towards the N-terminus from the metal ion binding sites, termed I domain, is present in most leukocyte integrin alpha subunits and offers functions of ligand recognition⁴⁵. This domain is capable of conformational changes important in ligand binding⁴⁵. Much work has focused on simple documentation of the absence or presence of specific integrin subunits in a wide range of tumour types⁵⁰. These immunocytochemical analyses have produced conflicting results with no clear correlation. Thus in some instances it has been recorded that progression of tumours from the more benign to the more malignant phase is associated with loss of expression of specific integrin subunits. This is a clinical finding which appears to substantiate those experiments where the introduction of cDNAs for particular integrin subunits into recipient cells has resulted in the reduced tumorigenic capacity of such transfectants. However, in other tumour types, like malignant melanoma, there appears to be a gradual up-regulation in the number of integrin subunits expressed by the more advanced or malignant tumours⁵¹. Whether this documentation of the mere existence of expression of different integrins tells us a lot about their possible involvement in affecting tumour cell behavior is questionable. Presence of the protein tells us little about the functional capacity of the molecule. It is known from experimental analysis that integrin activation status may vary considerably not only between cell types but also according to the influence of exogenous factors. It therefore becomes of crucial significance to know what the activation state is of those integrins which are expressed by the neoplastic cells. Thus in much the same way that cadherin function may be down-regulated, even when no detectable change is apparent in protein levels, so the simple presence or absence of immunoreactive integrin is no guide to likely effects on biological behavior.

As discussed, the integrins may play a part in regulating the enzymes which cause disruption of the ECM. Thus the binding of integrins to specific ligands has been shown to result in up-regulation in gene expression of particular types of metalloproteinases^{48,52}. These neutral-pH-acting enzymes are able to digest the major constituent proteins of the ECM. Equally, the urokinase-type plasminogen activator (uPA), which activates plasminogen to the broad-spectrum serum protease plasmin, has been localised to focal contacts, the sites where the integrins are found. It is possible that the integrins can regulate the activity of uPA, and therefore are capable of regulating plasmin production, by bringing into close apposition the components of the

proteolytic pathway and the ECM adhesion pathway. It is a requirement of tumour cell invasion that these cells do not attach too strongly to the underlying ECM and therefore remain static. Instead the infiltrating neoplastic cells must have the machinery to regulate adhesive and de-adhesive events. This regulation, which may occur in different sub-anatomical compartments of the same individual cell, is an important component of any chemotactic or haptotactic migratory response. Altering the composition of the ECM by degrading its constituents may alter the ligands available for particular integrins or alter the strength of the interactions with these adhesive proteins and thus allow movement and migration across and through the tissue stroma.

Immunoglobulin superfamily (IgSF)

The immunoglobulin superfamily of adhesion receptors is composed of numerous molecules of the cell surface which contain the typical immunoglobulin-like domains in the extracellular portion of the molecule⁵³. Their structure is characterized by the repeated immunoglobulin-like domains, which are built from a tightly packed barrel of β strands. A fifty percent of the surface glycoproteins of leukocytes belong to this superfamily. Members of this family are involved in tumour spread and appear to depend upon either loss or gain of functional capacity. For example, in colorectal cancer inactivation of the so-called DCC (deleted in colorectal carcinoma) gene has been reported in roughly 70% of cases, suggesting a fairly major role for the protein encoded by this gene⁵⁴. Other studies in the last years have clearly shown that inactivation of DCC gene may be observed not only in cases of colorectal cancer, but also in breast cancer⁵⁵, prostate cancer, and bladder cancer⁵⁶, endometrial cancer⁵⁷, pancreatic adenocarcinoma⁵⁸, as well as in haematological malignancies⁵⁹. The sequence of the DCC gene predicts for a protein which has strong homology to members of the immunoglobulin superfamily and especially for neural cell adhesion molecules (NCAM). Pierce et al have shown in vitro that DCC functions as NCAM stimulating neurite growth via special intracellular signalling⁶⁰, known that NCAM manifests its action on healthy or regenerated neural cells only without any activity on sick cells⁶¹. While the precise function of this molecule in regulating tumour spread is not known, it is possible that the product of the DCC gene functions in homophilic cell binding. In much the same way as that proposed above for E-cadherin, inactivation or loss of the DCC gene could then result in disruption of cell-cell cohesion between the cells of the primary tumour mass. Cell migration subsequent to this change in cell-cell attachment would then contribute to tumour spread. However, for fully estimating the role of DCC gene, further studies are needed.

The most important members of the immunoglobulin gene superfamily are the ICAM-1 (Intercellular Adhesion Molecule-1)⁶², ICAM-2 (Intercellular Adhesion Molecule-2)^{62,63}, VCAM-1 (Vascular Cell

Adhesion Molecule-1)⁶⁴, PECAM-1 (Platelet Endothelial Cell Adhesion Molecule-1)⁶⁵, MAdCAM-1 (Mucosal Addressing Cell Adhesion Molecule-1)⁶⁶, NCAM (Neural Cell Adhesion Molecule)⁶⁷, and since 1965 the known CEA (CarcinoEmbryonic Antigen)^{68,69}.

ICAM-1 (CD54) is a transmembrane glycoprotein of 90 KDa with fine Ig like extracellular domains⁶². The most important ligands for ICAM-1 are the β_2 integrins LFA 1 (CD11a/CD18) and Mac-1 (CD11b/CD18) which are expressed on leukocytes³⁴. Binding is achieved by 1 and 2 immunoglobulin domains. Thus ICAM-1 mediates the adhesion of leukocytes to ICAM-1 expressing cells. ICAM-1 also binds to fibrinogen, hyaluronase, sialoporphin (CD43), rhinoviruses and plasmodium falciparum-infected erythrocytes. ICAM is expressed on endothelial and epithelial cells, lymphocytes, monocytes, eosinophils, keratinocytes, dendritic cells, haematopoietic stem cells, hepatocytes and fibroblasts. Regulation of ICAM-1 expression is cell specific. ICAM-1 levels are enhanced by inflammatory cytokines and down regulated by anti-inflammatory agents in general⁷³. At inflammatory sites, inflammatory cytokines induce up regulation of ICAM-1 expression on vascular endothelial cells as well as activation of leukocyte integrins LFA 1 and Mac-1. This fact leads to adhesion of leukocytes to the local endothelium which represents an essential step in extravasation of leukocytes to the site of inflammation. Immigration and soluble ICAM-1 has been found in serum, cerebrospinal fluid and bronchoalveolar lavage. Chychezvski et al and Nakae et al in 1995 and 1996, respectively, have shown that soluble ICAM-1 levels are correlated with some endotoxins levels, TNF and cytokines in cases of sepsis^{74,75}. MadCAM-1 is a glycoprotein 58 KDa found on the endothelial cells of some vessels and is implicated in lymphocyte homing to specific tissues such as lymph nodes and mucosa – associated lymphoid tissue^{66,71}. Ligands for MadCAM-1 are integrin $\alpha_4\beta_7$ and L-selectin on leukocyte surface. NCAM (Neural Cell Adhesion Molecule)⁶⁷ is expressed by a variety of cells mainly of neural and mesenchymal origin^{67,76-78}. The observation that NCAM expression is limited in migrating cells, initially gave the impression that this molecule is implicated in malignancy. Indeed Hoki et al showed that loss of NCAM expression in mouse fibroblast cell lines is correlated with expression loss due to contact dependent inhibition of after virus transformation, offering thus a potential mechanism for NCAM involvement in tumorigenesis⁷⁹. However, immunohistochemical studies in a variety of tumours in humans have not until now support this hypothesis. Really, NCAM is present in a numerous of neural, neuroendocrine and mesenchymal tumours in general, in which a reduced/absent expression would be expected. Such tumours are Wilm's tumour, pituitary adenomata and pheochromocytomata as well as small cell lung cancer^{77, 78, 80, 81}. NCAM is a ligand for $\alpha_4\beta_1$ integrin (VLA- 4) found on leukocytes. The interactions, in which members of the IgSF are mediating, are of a homotypic (NCAM molecule is binding to another NCAM molecule) or heterotypic fashion.

One of the major ways that immunoglobulin-like molecules may play a role in modulating tumour spread is by regulating the interaction of circulating tumour cells with host immunocytes. However, it is also possible that members of this family play a major regulatory role in determining the adhesion of circulating cancer cells to capillary endothelium. This process, as well as leukocyte rolling and extravasation are impossible to take place with the mediating of selectins alone; members of the IgSF are always implicated during those processes⁵⁴. An aspect of metastatic dissemination which closely resembles the normal trafficking of immune cells and their extravasation at inflammatory sites in this adhesion of circulating cells to the endothelial cells of arresting vessels. Specific ligands on the endothelial cells capable of interacting with receptors on tumour cells could account, in part, for organ-specific patterns of tumour spread. It is of interest, therefore, that VCAM-1, a member of the immunoglobulin superfamily, which is a cytokine-inducible surface receptor found on the luminal surface of endothelial cells, serves as a ligand for the $\alpha_4\beta_1$ integrin (VLA-4)⁷⁰. This integrin is expressed by several tumour cell types, including rhabdomyosarcoma, neuroblastoma and melanoma. Presumably an interaction between the $\alpha_4\beta_1$ integrin and the VCAM-1 ligand could account for the retention and arrest of cancer cells in distant capillary beds. In malignant melanoma, especially, the results form many experimental studies are in accord with this possibility. Thus correlations have been established between melanoma cell surface levels of $\alpha_4\beta_1$ and the capacity to adhere to activated endothelium. Pre-treatment of mice with cytokines which up-regulate expression of VCAM-1 on endothelial cells has resulted in increased metastatic tumour burden resulting from the intravenous injection of $\alpha_4\beta_1$ -expressing melanoma lines of either murine or human origin. VCAM-1 also binds to leukocyte integrin $\alpha_4\beta_7$ (LPAM-1) on activated peripheral T cells⁷¹. VCAM-1 is a 100-110 kDa transmembrane glycoprotein typically characterized by the presence of seven C₂-type immunoglobulin (Ig) domains⁶⁴. In the extracellular region, there are multiple N-linked glycosylation sites and each C₂ domain is closed by a disulfide bridge. A number of variants of VCAM-1 are known to exist, all of which are likely the result of alternate gene splicing. Cells known to express VCAM-1 include neurons, endothelial cells, smooth muscle cells, fibroblasts and macrophages⁷². VCAM-1 is absent on resting endothelial cells, but can be upregulated by inflammatory mediators such as IL-1 β , IL-4, TNF α and INF- γ ⁷². Soluble VCAM-1 has been identified in culture supernates blood and cerebrospinal fluid. The exact mechanism by which VCAM-1 is generated is unknown. Both, proteolytic cleavage and alternate splicing may occur. However, the exact processes of tumour cell to endothelial cell adhesion may not mimic the normal physiological processes in all aspects. Accordingly, some caution should be exercised with regard to the data derived from experimental analy-

ses and how they may reflect on the clinical situation.

Selectins

The selectins represent a group of adhesion receptors composed of three well-characterised members. These members, termed E-, P- and L-selectin respectively, are expressed on a variety of blood cells or endothelial cells and recognize and bind to carbohydrate moieties present on the interacting cells. E- and P-selectin are expressed by endothelial cells and their cell surface expression can be up-regulated dramatically by exposure to many inflammatory cytokines. L-selectin is expressed by leucocytes. The selectins bear at their N-terminus a calcium-dependent lectin domain which, during the process of leucocyte extravasation, binds to sugar moieties (including members of the 'Lewis' blood group) expressed on the leucocytes. This selectin-sugar interaction serves to draw leucocytes in close to the endothelium, pulling the cells out from normal laminar flow and initiating a process known as 'rolling', whereby the leucocytes roll along the luminal surface of the endothelial cells. This close juxtaposition of the leucocytes, coupled with the slowing down of transit time, allows the stronger integrin-mediated interactions to occur and these stabilise the attachment process. It has been shown that certain epithelial cancers express the sialylated Lewis^x and Lewis^A antigens, which serve as ligands for E-selectin, and that the level of expression found in certain tumour types appears to correlate with the capacity to form distant metastases. Given the apparent similarity between the way in which normal leucocytes extravasate at inflammatory sites and the way in which tumour cells extravasate at distant sites of arrest, it is tempting to speculate that comparable mechanisms are indeed involved in mediating both these processes. Is it the case, therefore, that selectin-sugar interactions pull disseminating tumour cells in to close apposition with the capillary endothelium and allow subsequent integrin-mediated interactions to occur?

To accept this scenario unreservedly is to ignore the fact that the precise resemblance of tumour dissemination to the situation of leucocyte trafficking is doubtful because of the relative dimensions of the circulating cancer cell and the arresting endothelial vessel. Thus, whereas in many instances of inflammatory exudate, the involved blood vessels may be significantly larger than the leucocytes themselves, in many instances of cancer cell arrest the diameter of the arresting vessel actually may be smaller than the malignant cell. There is, then, no requirement for the tumour cell to be pulled out of laminar flow, since it is not actually undergoing laminar flow but rather is forcing its way through a deformable capillary. Such close contact between the two interacting cell types may mean that the integrin-mediated interactions are the most important. For this reason the activation status of the integrins already referred to above, may be the most important determinant in deciding whether or not adhesion occurs.

If tumour cell integrins are constitutively in the active state, then not only may inhibition of apoptosis be affected, but also may be the adhesive behavior of the tumour cells.

CD44

A cell adhesion molecule which does not fall easily into one of the four major groups of receptors and yet which has been closely associated with the ability to metastasize is the hyaluronate-binding protein, CD44. Originally identified as a lymphocyte-homing molecule, CD44 was shown, by some elegant experimental work, to play an important part in the metastatic spread of tumors. Analysis of the surface expression of this molecule on cells from rodent tumors which did or did not metastasize revealed that the expression of different forms of CD44 correlated with the capacity to undergo metastatic spread. Thus, while both metastatic and non-metastatic variants derived from a common parental line could express the so-called standard form of CD44, only the metastatic cells expressed the so-called variant form. This variant form was shown to have an extra inserted region present in the extracellular portion of the molecule. Further studies have not only confirmed that this association between expression of the variant form and metastatic behavior occurs in a range of human tumour types but also have provided an explanation for the origin of this variant form. The structure of CD44 is exceptionally complicated. This complexity is a consequence of splicing from different exons in the gene, resulting in the production of different isoforms. This complexity in the primary protein backbone can then be built upon further by different glycosylation patterns of these isoforms which results in the ability to generate an extensive variety of CD44 molecules.

In its standard form CD44 is an integral cell membrane glycoprotein of 85-95 kDa, arising from post-translational modifications of a 37-kDa core protein, which serves to regulate lymphocyte adhesion to the high endothelial venules during lymphocyte recirculation. The N-terminal region of the molecule has significant homology with the tandem repeat domains of the cartilage link and proteoglycan core proteins, which suggests a role in hyaluronate binding. Indeed, CD44 has been shown to be the major cell surface receptor for hyaluronate in a range of cell types, though this activity can be regulated by differential glycosylation.

The additional inserted region of the variant CD44 is strongly hydrophilic and could affect binding properties of expressing cells, though no clear-cut effect on hyaluronate binding has yet been demonstrated. Expression of the variant forms of CD44 is not restricted solely to tumour cells and, perhaps most interestingly, one of the subsets of cell types in which this form is detected are the activated lymphocytes. There has been considerable controversy over the association between variant exon usage and tumour malignancy but, in certain types, this association does seem real and does pro-

vide prognostic information. Exploration of the generality of the correlation and an elucidation of the underlying mechanistic basis of this association will help explain the precise role of this adhesion receptor in tumour progression. At this time the assumption is that by mimicking the role played in normal lymphocyte migration the CD44 molecule somehow confers metastatic capacity onto normally sedentary cells.

References

- Adams J, Watt F. Regulation of development and differentiation by the extracellular matrix. *Development* 1993; 117: 1183-1198
- Charalabopoulos K., Binolis J., Karkabounas S. Adhesion molecules in carcinogenesis. *Exp Oncol* 2002; 24: 249-257
- Hart IR, Saini A. Biology of tumor metastasis. *Lancet* 1992; B39: 1453-1457
- Bates RC, Lincz LF, Burns GF. Involvement of integrins in cell survival. *Cancer Met Rev* 1995; 14: 191-203
- Frisch SM, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. *Cell Biol* 1994; 124: 619-626
- Hynes RQ. Integrins: a family of cell surface receptors. *Cell* 1992; 48: 549-554
- Hynes RQ. Integrins: versatility, modulation and signaling in cell adhesion. *Cell* 1992; 69: 11-25
- Charalabopoulos K. Integrins as adhesion receptors in neoplasias. *Arch Hell Med* 1998; 15: 166-169
- Juliano RC, Haskill S. Signal transduction from the extracellular matrix. *J Cell Biol* 1993; 120: 577-585
- Charalabopoulos K., Pignatelli M. Adhesion molecules and cancer. *Arch Hell Med* 2001; 18: 16-19
- Zhang Z., Vuori K., Reed JC, Ruoslahti E. The $\alpha_5\beta_1$ integrin supports survival of cells on fibronectin and upregulates Bcl-2 expression. *Proc Natl Acad Sci USA* 1995; 92:6161-6165
- Koch AE, Halloran MM, Haskell CJ, Shah MR, Polverini PJ. Angiogenesis is mediated by soluble forms of E-selectin and vascular cell adhesion molecule1. *Nature* 1995; 376: 517-519
- Brooks PC, Clark RAF, Cheresh DA. Requirement of vascular integrin $\alpha_v\beta_3$ for angiogenesis. *Science* 1994; 264: 569-571
- Brooks PC, Montgomery AMP, Rosenfeld M. Integrin $\alpha_v\beta_3$ antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell* 1994; 79: 1157-1164
- Charalabopoulos K., Papalimneou V, Charalabopoulos A. Adhesion molecules in lung cancer. *Exp Oncol* 2003; 25: 16-21
- Dubaud JL, Dufour S, Hatta K, Takeichi M, Edelman GM, Thiery JP. Adhesion molecules during somatogenesis in the avian embryo. *J Cell Biol* 1987; 104: 1361-1374
- Takeichi M. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* 1991; 251: 1451-1455
- Charalabopoulos K. The structure and function of cadherins; their role in carcinogenesis and other pathologies. *Arch Hell Med* 1999; 16: 127-135
- Ozawa M., Kember R. Molecular organization of the uvomorulin-catenin complex. *J Cell Biol* 1992; 116: 989-996
- Gumbiner BN, Mc Crea PD. Catenins as mediators of the cytoplasmic functions of cadherins. *J Cell Sci* 1993; Suppl H: 155-158
- Sommers CL, Gelmann EP, Kember R, Cavin P, Byers SW. Alterations in beta catenin phosphorylation and plakoglobin expression in human breast cancer cells. *Cancer Res* 1994; 54: 3544-3552
- Behrens J., Frixen V., Schipper J., Weidner M., Birchmeier W. Cell adhesion in invasion and metastasis. *Semin Cell Biol* 1992; 3: 169-178
- Charalabopoulos K, Tsambalas S, Syrigos K, et al. Correlation of E-cadherin expression with clinicopathological data in patients suffering from transitional cell carcinoma of the bladder. *Exp Oncol* 2003; 25: 180-185
- Kljava IJ, Lagenaur C, Bixby JL, Rehr A. Cell adhesion molecules regulating neurite growth from amacrine and rod photoreceptor cells. *J Neurosci* 1994; 14: 5035-5049
- Reiter M. Regulating cell proliferation: as easy as APC. *Science* 1996; 272:974-975
- Kanai V, Ochiai A, Shibata T, et al. C-erb-b-2 gene product directly associates with beta catenin and plakoglobin. *Biochem Res Commun* 1995; 208: 1067-1072
- Su LU, Vogelstein B, Kinzler KW. Association of the APC tumour suppressor protein with catenins. *Science* 1993; 262:1734-1738
- Nigam AK., Savage FJ, Boulos PB, Stamp GWIT, Lin D., Pignatelli M. Loss of cell-cell and cell matrix adhesion molecules in colorectal cancer. *Br J Cancer* 1993; 68: 507-514
- Tsao J, Shibata D. Further evidence that one of the earliest elevations in colorectal carcinogenesis involves APC. *An J Pathol* 1994;45:531-534
- Umbass B, Isaacs WB, Bringuier PP, et al. Decreased E-cadherin expression is associated with poor prognosis in patients with prostate cancer. *Cancer Res* 1994; 54:3929-3933
- Syrigos K, Harrington K, Charalabopoulos K, Karayiannakis A, Wayman J, Pignatelli M. Altered expression of the cadherin/catenin complex in bladder cancer. *Eur J Urol* 2002; 343
- Pignatelli M, Ansari JQ, Guiter P. Loss of membranous E-cadherin expression in pancreatic cancer: correlation with lymph node metastasis, high grade and advanced stage. *J pathol* 1994, 174:243-251
- Jancowski J, Newham PM, Kademir O, Hirano S, Jakeichi M, Pignatelli M. Differential expression of E-cadherin in normal, metastatic and dysplastic esophageal mucosa: a putative biomarker. *Int J Oncol* 1994;4:441-448
- Syrigos K, Karayiannakis A, Charalabopoulos K, Katirtzoglou N, Pignatelli M. Abnormal expression of E-cadherin in dysplastic Barrett's esophagus. *Ann Gastroenterol* 2000; 13(suppl) 168, 92
- Mayer B, Johnson JP, Leite F, et al. E-cadherin expression in primary and metastatic gastric cancer: downregulation correlates with cellular dedifferentiation and granular disintegration. *Cancer Res* 1993; 53:1690-1695
- Charalabopoulos K, Papalimneou V, Evangelou A, et al. Is the E-cadherin downregulation observed in helicobacter pylori infection associated with gastric cancer development? An additional element in the disease jigsaw. *Exp Oncol* 2003, 25:270-273
- Charalabopoulos K, Syrigos K, Papalimneou V, Habib N, Pignatelli M. Role of E-cadherin/catenin complex in primary liver tumours. *Ann Gastroenterol* 2000,13(suppl):177,110
- Matijsen V., Peters HM, Schalkwis KL, Manni JJ, Ruiters DJ. E-cadherin expression in head and neck squamous cell carcinoma is associated with clinical outcome. *Int J Cancer* 1993; 55:580-585
- Vessey CJ, Wilding J, Folarin N, et al. Altered expression and function of E-cadherin in cervical intraepithelial neoplasia and invasive squamous cell carcinoma. *J Pathol* 1995; 176:151-159
- Haas TA, Plow EF. Integrin-ligand interactions: a year in review. *Curr Opin cell Biol* 1994; 6: 656-662
- Albelda SN, Buck CA. Integrins and other cell adhesion molecules. *FASEB J* 1990; 4: 2868-2880
- Elices MJ, Hemler ME. The human integrin VLA-2 is a collagen receptor on some cells and a collagen laminin receptor on others. *Proc Nat Acad Sci USA* 1996; 86: 9906-9910
- Del Buono R, Pignatelli M, Bodmer WF, Wright NA. The

- role of the arginine-glycine- aspartic acid directed cellular binding to type-1 collagen and rat cells in colorectal tumour differentiation. *Differentiation* 1991; 46: 97-103
44. Langnino LR, Gehlsen KR, Wayner E, Carter WG, Engvall E, Ruoslahti E. Endothelial cells use $\alpha_5\beta_1$ integrin as a laminin receptor. *J Cell Biol* 1989; 10q: 2455-2462
 45. Larson RS, Springer TA. Structure and function of leukocyte integrins. *Immunol Rev* 1990; 114: 181-217
 46. Pigott R, Power C. The adhesion molecules facts book. Academic Press, London, UK, 1993; p.p.89-92
 47. Charalabopoulos K. Integrins receptors in neoplasias. *Arch Hell Med* 1998; 15:166-169
 48. Mackay CR, Imhof BA. Cell adhesion in the immune system. *Immunology Today* 1993; 14: 99-102
 49. Poulson R., Pignatelli M., Stetler SW, Liotta LA, Wright PA, Teffers RE. Stromal expression of 72kda type IV collagenase (MMP-2) and TIMP-2 mRNA in colorectal neoplasia. *Am J Pathol* 1992; 141: 389-396
 50. Gogali A, Charalabopoulos K., Constantopoulos S. Integrin receptors in primary lung cancer. *Exp Oncol* 2004; 26:106-110
 51. Albelda SM, Mette SA, Elder DH, et al. Integrin distribution in malignant melanoma: association of the β_3 subunit with tumor progression. *Cancer Res* 1990; 50: 6757-6764
 52. Settor REB, Settor EA, Crehlsen KR, et al. Role of the $\alpha_5\beta_3$ integrin in human melanoma cell invasion. *Proc Natl Acad Sci USA* 1992; 89:1557-1561
 53. Hunkapiller TH, Hood L. Diversity of the immunoglobulin gene superfamily. *Adv Immunol* 1989; 44: 1-63
 54. Vogelstein B., Fearon ER, Hamilton SR, et al. Genetic alterations during colorectal tumor development. *N Engl J Med* 1988; 319: 525-534
 55. Devillee P, Van VM, Knipers DN, Pearson PL, Cornelisse CJ. Somatic genetic changes of chromosome 18 in breast carcinomas: is the DCC gene involved? *Oncogene* 1991; 6:311-315
 56. Wu SQ, Storer BE, Bookland EA, et al. Nonrandom chromosome losses in stepwise neoplastic transformation in vitro of human uroepithelial cells. *Cancer Res.* 1991;51:3323-3326
 57. Imamura T, Arima T, Kato H, Myamoto S, Sasazuki T, Wake N. Chromosomal deletions and K-ras gene mutations in human endometrial carcinomas. *Int J Cancer* 1992;51:47-52.
 58. Hohne MW, Halatsch ME, Kahl GF, Weinel RJ. Frequent loss of expression of the potential tumor suppressor gene DCC in ductal pancreatic adenocarcinoma. *Cancer Res* 1992; 52:2616-2619.
 59. Porfiri E, Secker WI, Hoffbrand AV, Hancoch JF. DCC tumour suppressor gene is inactivated in hematologic malignancies showing monosomy 18. *Blood* 1993; 81:2696-2701
 60. Pierceall WE, Cho KR, Getzenberg RH, et al. NIH3T3 cells expressing the deleted in colorectal cancer tumour suppressor gene product stimulate neurite outgrowth in rat PC12 pheochromocytoma cells. *J Cell Biol* 1994; 124:1017-1027
 61. Figarella-Branger D, Nedelec J, Pellissier JF, Boucraut J, Bianco N, Rougon G. Expression of various isoforms of neural cell adhesive molecules and their highly polysialylated counterparts in diseased human muscles. *J Neurol Sci.* 1990;98:21-36
 62. Cartwright JE, Whitley GS, Johnstone A. The expression and release of adhesion molecules by human endothelial cell lines and their consequent binding of lymphocytes. *Exp Cell Res* 1995; 217:329-335
 63. Cunningham AC, Kirby JA. Regulation and function of adhesion molecules expression by human alveolar epithelial cells. *Immunology* 1995; 86: 279-286
 64. Holness C, Simmons DL. Structural motifs for recognition and adhesion in molecules of the immunoglobulin superfamily. *J Cell Sci* 1994; 107:2065-2070
 65. Muller A. PECAM-1 is required for transendothelial migration of leukocytes. *J Exp Med* 1993; 178:449-460
 66. Buckley CD, Doyannas R, Newport JP, et al. Identification of $\alpha_5\beta_3$ as a heterotypic ligand for CD 31/PECAM-1. *J Cell Sci* 1996;109(pt2):436-445
 67. Thomson JA, Grunnert F, Zimmerman W. Carcinoembryonic antigen gene family; molecular biology and clinical perspectives. *J Clin Lab Anal* 1991; 5:344-348
 68. Levin LV, Griffin TW. Specific adhesion of carcinoembryonic antigen-bearing colorectal cancer cells to immobilized carcinoembryonic antigen. *Cancer Lett* 1991; 60:143-152
 69. Gold P, Freedman SO. Demonstration of tumour specific antigens in human colonic carcinomata by immunological tolerance and absorption techniques. *J Exp Med* 1965; 121:439-443
 70. Zimmerman GA, Prescott SM, McIntyre TM. Endothelial cell interactions with granulocytes: tethering and signaling molecules. *Immunology Today* 1992; 13:93-99
 71. Ruegg C, Postigo AA, Sikorski EE, Butcher EC, Pytela R, Erle DJ. Role of integrin $\alpha_5\beta_1/\alpha_4\beta_1$ in lymphocyte adherence to fibronectin and VCAM-1 and in homotypic cell clustering. *J Cell Biol* 1992; 117:179-189
 72. Shimizu Y, Newman W, Tanaka Y, Shaw S. Lymphocyte interactions with endothelial cells. *Immunology Today* 1992; 13:106-112
 73. Gearing AJH. Soluble forms of vascular adhesion molecules E-selectin, ICAM-1 and VCAM-1: pathological significance. *Ann NY Acad Sci* 1992; 667:324-331
 74. Chyzyewski L, Derek W. Endothelial cell activation in shock. *Rozz Acad Med Bialmyst* 1995; 40:1-12
 75. Nakae H, Endo S, Inada K, Takakawa T, Kasai T. Changes in adhesion molecule levels in sepsis. *Res Commun Mol Pathol Pharmacol* 1996; 91:329-338
 76. Chuong CM, Eidelma GM. Alterations in neural cell adhesion molecules during development of different regions of the nervous system. *J Neurosci* 1984; 4:2354-2368
 77. Hage R, Elbers HR, Brutel dela Riviere A, van den Bosch JM. Neural cell adhesion molecule expression: prognosis in patients with rejected non-small cell lung cancer. *Chest* 1998;114:1316-1320
 78. Molenaar WM, Mantinghe FL. Expression of neural cell adhesion molecules and neurofilament protein isoforms in skeletal muscle tumours. *Hum Pathol* 1998; 29:1290-1293
 79. Aoki J, Umeda M, Takio K, et al. Neural cell adhesion molecule mediated contact dependent inhibition of growth of near diploid mouse fibroblast cell line m55/1m. *J Cell Biol* 1991;115:1751-1761
 80. Carbone DP, Koros AM, Linnoila RI, Jewett P, Gazdar AF. Neural cell adhesion molecule expression and messenger RNA splicing patterns in lung cancer cell lines are correlated with neuroendocrine phenotype and growth morphology. *Cancer Res* 1991; 51:6142-6145
 81. Jin L, Hemperly JJ, Lloyd RV. Expression of neural cell adhesion molecule in normal neoplastic human neuroendocrine tissues. *Ann J Pathol* 1991; 138:961-969