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Tumor and Endothelial Cell Invasion of Basement Membranes

The Matrigel Chemoinvasion Assay As A Tool For Dissecting Molecular Mechanisms

Adriana ALBINI

National Institute for Research on Cancer, Advanced Biotechnology Center, 16132 Genova, Italy

The spread of cancer cells from a primary tumor to distant organs is the major cause of death of cancer patients. Metastatic lesions are often resistent to cancer therapy because of the progressive phenotypic changes that they have undergone. Several genetic and epigenetic factors, both in the cell and in the host, contribute to the development of tumor progression towards metastases. In this review we will analyze the steps involved in tumor metastases, which can be potential targets for anti-metastatic therapy. One of the most critical events in cancer metastasis is the invasion of basement membranes. An assay which we developed over ten years ago, the Matrigel "chemoinvasion" assay, has been a useful tool for studying the mechanisms involved in tumor and endothelial cell invasion of basement membranes and for the screening of anti-invasive agents. Here we will describe the assay and review some of the major results obtained with it. (Pathology Oncology Research Vol 4, No 3, 230–241, 1998)

Keywords: invasion, metastasis, angiogenesis, basement membranes, matrigel, Boyden chamber, chemoinvasion

Introduction

During tumor progression and metastasis, cells undergo a series of modifications which are interrelated and each of which can be rate-limiting (For review see: 1,20,33,140). A recent Nature Commentary[34] suggests that tumor tissue tends towards chaos. Genetic instability favors rapid acquisition of properties conferring tumor cell survival and drug resistance. The metastatic process can be subdivided into discrete steps: the neo-vascularization and increase in size of a primary tumor, the detachment of neoplastic cells and their entry into the circulation via the hematic or lymphatic vessels, their adhesion to endothelium of distant organs, their passage through the capillary basement membrane and proliferation to form a secondary tumor (For review see: 108). Both the host and the tumor cells participate to this process. Several key molecules are involved in this process, some of which are indicated in Table 1.

New, potentially specific and efficient forms of antineoplastic therapy have been developed by taking into account these main events influencing disseminated disease. In order to assess the potential use of an anti-metastatic factor, appropriate models to evaluate malignancy are required.

In vivo the most widely used models are the "experimental" and the "spontaneous" metastasis assays, which assess extravasation and the complete metastatic process, respectively.[48] However it is useful to dissect down the metastatic process into single steps or groups of events. A critical step in metastasis is the invasion of basement membranes.[20,81] Basement membranes are thin continuous sheets of extracellular matrix which represent a barrier to macromolecules and cells. In the past, studies on invasion had been carried out using isolated basement membranes; particularly from the amnion.[58] A breakthrough was the use of a reconstituted membrane, "Matrigel™",[76] which when applied over filters in the Boyden chamber chemotaxis assay, quantitated metastatic cell invasive potential.[6] This in vitro invasion was indeed found to correspond to relative metastatic potential in vivo for a wide variety of cell systems. Here we will refer to this type of assay as "chemoinvasion" assay.[6]

Table 1. Rate limiting steps in tumor progression

Step	Molecules/Mechanism
1. Proliferation	Oncogene activation, suppressor inactivation
2. Loss of cell-cell adhesion	Loss of E-cadherin
3. Matrix degradation	MMP's, uPA/plasmin, cathepsin activity
4. Extravasation and reaching distant sites	Resistance to blood stream and immune control
5. Adhesion to endothelial cells	E-selectin, mucins, V-CAM, I-CAM, β2/β4 integrins
6. Attachment to the extracellular matrix	Integrins (β1, β3, β5), laminin, collagens, fibronectins
7. Basement membrane degradation	gelatinases, uPA/plasmin
8. Migration	Chemotactic growth factors and their receptors, gradients of matrix proteins and their receptors
9. Proliferation at the distant site	Organ specific growth factors
10. Angiogenesis	VEGF, bFGF, HGF, ... stimulation of tyrosine kinase receptors on endothelial cells

The matrigel chemoinvasion assay has been, with its numerous modifications, the most widely accepted system for analysis of tumor cell invasion and its antagonists. Several molecules are altered during the dynamic process of tumor cell invasion of basement membranes. Matrix lytic enzymes are essential to both intravasation and extravasation.[1] The metalloproteinases,[146] serine proteinases[11] and cathepsins[71] have been the most thoroughly investigated in the chemoinvasion assay. Results obtained in the Boyden chamber matrigel assay have been the prelude to successful preclinical studies and clinical trials with protease-, and in particular metalloproteinase-, inhibitors.[24,128] Examination of the effects on invasion by transfected and transduced cells has allowed this to become a rapid test for potential gene therapy agents as well.[38,64,73,138,139]

Endothelial cells, the key cellular element in tumor neovascularization, become able to breach their own basement membrane during angiogenesis, a necessary event to metastasis.[43,44] Similar mechanisms underlie invasion and angiogenesis.[9,40,129] Induction of endothelial cell invasion by tumor cell products and its inhibition has also been studied in the matrigel invasion assay.[134] We will describe the assay from a methodological point of view and review some of the data obtained on inhibitors of invasion with emphasis to the role of lytic enzymes and their inhibitors. Many of these factors have then been applied in clinical therapy.

Transformation and growth

Schematically, the initial growth of a neoplastic cell can be induced by the activation of an oncogene or the inactivation of a tumor suppressor gene, often these two events are interrelated (For review see: 53). Products of oncogenes of several families can contribute to tumor progression. The family of ras genes has been the most studied,[13,94,103] however all oncogenes encoding for proteins involved in the signal transduction cascade can interfere with the invasive mechanisms.[96] The actual critical events tend to differ from tissue type to tissue type.

The mechanisms of action of tumor suppression are varied: chromosome stability, the ability to undergo terminal differentiation and the control of proliferation and survival may be affected. One of the major roles of tumor suppressor genes is in the inhibition of cell proliferation,[23] they can be considered as components of the intracellular signalling pathways which transmit to the replication machinery the growth-inhibitory signals received from its external environment. However, involvement of oncosuppressors in the control of the invasive, malignant phenotype has also been shown.[84,139]

The first oncosuppressor gene identified and cloned, Rb-1, has been demonstrated by our group and by the group of Li, both in 1996,[84,139] to be able to inhibit the ability of tumor cells to invade, in addition to their growth. Melanoma[84,139] and osteosarcoma[84] cells transduced to over express Rb resulted in cells with a strongly reduced invasive potential. The oncosuppressor p16/CDKN2 is also an inhibitor of invasion.[29] This effect does not seem to be simply due to growth reduction. In fact the oncosuppressor p53, although reducing the growth rate of transfected cells, was a weaker inhibitor of invasion.[84] There appears to be a down regulation by specific oncosuppressors of metalloproteinase activity,[29] a phenomenon which will be extensively described later on.

The signaling pathways which mediate the normal functions of various growth factors are altered during tumor progression and neoangiogenesis. Cytokines can induce, besides growth, also migration, invasion and endothelial cell activation.[49,83,116] Inhibition of growth factor interaction with their receptors is a prospective target for anti-metastatic and anti-angiogenic substances, as well as interference with the signaling cascade.[10,135] Several growth factors and/or their receptors, as well as oncogenes involved in the signal transduction cascade, have been causally involved in induction of tumor cell and endothelial cell motility and invasion.[10,78,83] For example, VEGF, a key growth factor, has an important role in metastasis and angiogenesis.[32,69] VEGF has more than one receptor.[133] The higher affinity VEGF receptor is Flt-1, this has been closely linked to endothelial cell differentiation and capillary organization, while a lower affinity receptor, KDR/Flk-1, is responsible

for the induction of the proliferative and invasive phases of angiogenesis. bFGF, with its numerous receptors, TGFs[75,141] HGF with its receptor c-met[15] and many other growth factors are linked to invasion and angiogenesis.[35,148]

Several cytokines act in invasion by inducing increased expression of one or more matrix metalloproteinases,[116] such as IL-1β, bFGF, EGF and PDGF. Cytokines can also reduce matrix metalloproteinase expression, an example of this is TGF-β repression of stromelysin induction, which is mediated at the level of transcription.[91]

Cell-cell adhesion disruption

The suppression of cell-cell adhesion is critical for allowing the escape of the transformed cells during epithelial cell tumor progression to malignancy. An example of this is E-cadherin, a key molecule in maintenance of epithelial cell-cell adhesion.[18] Decreased expression of E-cadherin enhances the invasive and metastatic phenotype of transformed epithelial cells, and this molecule is frequently inactivated in human epithelial cancers.[55,60] Restoration of functional E-cadherin expression has been shown to revert an invasive epithelial tumor cells to a non-invasive phenotype.[18] Cells expressing E-cadherin have an "epithelial" phenotype, those not expressing it have a "mesenchymal phenotype" : E-cadherin appears to mediate the phenomena of epithelial-mesenchymal transition and its alteration is crucial in the transition from adenoma to carcinoma.[105] A well studied example of this is epithelial cell "scattering" and invasion induced by HGF (scatter factor),[89] which can be counteracted by E-cadherin.[19] Both growth suppressive and motility-suppressive functions have been assigned to E-cadherin, mutational analyses suggest that these can be separated.[28] E-cadherin is linked to the cytoskeleton via β-catenin complexes,[90] which is in equilibrium with the product of another gene found altered early in colon carcinogenesis, APC.[62] Disruption of the β-catenin binding domain of E-cadherin has been shown to affect motility.[28] E-cadherin growth suppression appears to be mediated through the p27KIP1 cyclin-dependent kinase inhibitor[126] which in turn affects the phosphorylation state of the Rb gene product.

Extravasation

Interactions between detached tumor cells and the stromal extracellular matrix occur via fibronectin, collagen and other glycoprotein and proteoglycan components.[1,3] The receptors for extracellular matrix proteins belong to the integrin family, a class of cell surface receptors consisting of an α and a β heterodimer.[12] These molecules regulate cell adhesion, migration, growth and survival through complex signaling pathways.[61] The VLA (very late antigen) heterodimers of the β1 subunit are known to

recognize various matrix molecules, often through an RGD (Arg-Gly-Asp) sequence. Tumor cell lines express a wide variety of integrins at their cell surface. RGD containing peptides and anti-integrin antibodies are inhibitors of cell adhesion and migration.[117] Of the numerous integrins known, some have been extensively studied for their role in basement membrane invasion, particularly α3β1, α6β1 and α6β4.

VLA3 (α3β1) is a promiscuous receptor, recognizing fibronectin, collagen type IV and laminin, particularly laminin 5 (epiligrin).[16] α3 expression is enhanced in certain tumors[100] and we have found that α3β1 mediates migration and matrigel invasion of melanoma cells in the Boyden chamber.[97] Interaction of VLA3 with laminin 5 stimulates invasion of malignant glioma cells.[46]. Signaling events induced by anti-α3 integrin antibodies may therefore be involved in invasion of tumor cells of various origin. Stimulation of the α3β1 receptor with antibodies induces MMP-2 activity in rhabdomyosarcoma[79] and glioma[30] cells, indicating that binding of this integrin receptor to its ligand can start the proteolytic cascade necessary for invasion. Current data suggest that inhibition of α3β1 could be of potential use in prevention of tumor cell invasion. α3 and α6β1 integrins both associate with urokinase-type plasminogen activator receptors on cells attached to laminin[144] which could direct proteolysis for tumor cell invasion.

α6β4 is a receptor for the laminin family of matrix proteins which has been correlated with the progression of several tumors, particularly carcinomas.[110] An α6β4 dependent increase in adhesion of colorectal carcinoma cells and invasion of Matrigel has been reported.[27] Likewise, in fibrosarcoma cells, suppression by ribozymes of α6 subunit expression abrogated lung metastasis and matrigel invasion.[145] Up regulation of the α6β4 receptor in breast cancer cells, with TGF-β or hormones, however, resulted in an anti-invasive effect corresponding to differentiation towards a myoepithelial phenotype.[68] The complex role of a6b4 in adhesion, differentiation and migration requires further investigation to determine whether this receptor can be a target of anti-invasive therapy.

Besides integrins, other matrix receptors such as the CD44, the hyaluronan receptor, may have a major role in malignancy. CD44 was originally shown to modulate melanoma cell invasion of collagen gels,[42] and later shown to modulate invasion of matrigel.[77,98] Differing alternate splice variants of CD44 seem to confer invasiveness,[51] however this appears to depend on the cell type. Addition of hyaluronan, the ligand for CD44, to matrigel dose-dependently enhances the invasion of glioma cells[111] although hyaluronan is not generally considered a major component of basement membranes.

The immune system of the host is one of the critical barriers to tumor progression. Very briefly, the immune
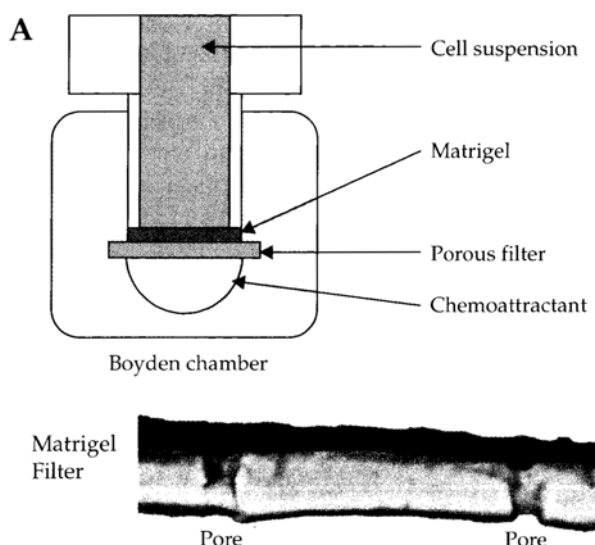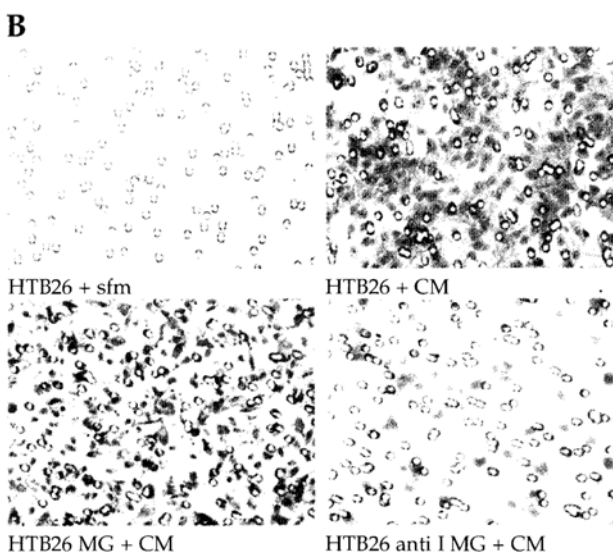
Figure 1a. A schematic diagram of the Boyden chamber chemoinvasion assay. The Boyden Chamber consists of two segments (upper=yellow, lower=light blue) which when fitted together with a filter between them forms the two chambers. In the assay a thin layer of matrigel is coated over the filter which covers the pores as well (right panel).

1b. Light micrographs of HTB26 Breast cancer cells which have crossed through the filter to the lower chamber in the chemotaxis (upper panels) and chemoinvasion (lower panels) assays. + sfm = serum-free medium: no chemoattractant, few cells cross the filter; + CM = with fibroblast conditioned medium, cells readily migrate to the filter underside. MG + CM = chemoinvasion in the presence of matrigel and conditioned medium, the invasive tumor cells cross to the lower chamber, though fewer than with chemotaxis alone. anti I MG + CM = chemoinvasion in the presence of matrigel and conditioned medium; the cells have been treated with anti integrinantil antibodies, invasion is substantially reduced.

system may exert both non-specific and specific anti-tumor effects. Among the non-specific effector cells, monocytes/macrophages, and different types of lymphoid cells (NK, CD8+ T-lymphocytes) have been shown to play a role in tumor cell destruction (reviewed in 85,137). Tumor specific cytotoxic T lymphocytes (CTLs) recognize "tumor specific antigens". CTL's may preferentially accumulate at the tumor site as tumor infiltrating lymphocytes. These CD8+ T-lymphocytes and natural killer (NK) cells can be differentiated to lymphokine-activated killer (LAK) cells by short term culture in IL-2 retaining the capability of specifically lysing tumor cells.

The immune cells infiltrating the tumor mass may also have an opposite role as macrophages are able to produce a wide variety of cytokines and are thought to sustain tumor angiogenesis.[56,82,107,112,125,143] At the same time the release of inflammatory cytokines and oxygen radicals can participate in tissue damage and matrix degradation causing the loss of integrity of basement membranes. Therefore the immune system plays complex and contradictory roles in tumor progression.

Interactions with the endothelium of the target organ

After a transformed cell has detached and migrated, extravasation from the circulation occurs. Following growth, escape from the primary tumor and host immune surveillance, survival of mechanical stress in the circulation, a malignant cell which has reached the target organ encounters the vascular endothelium of the organ. Cell adhesion molecules (CAMs) expressed on the endothelial cell surface mediate attachment of malignant cells, some of these may be site-specific.[49,147] It is generally believed that the initial interaction occurs during tumor cell rolling on top of endothelium through low affinity interactions, once the cell is retarded in its motion, higher affinity interactions (for instance through VCAM-integrin $\alpha4\beta1$ interactions) take place, arresting the tumor cell on the endothelial cell surface.[49] These events mirror those of normal leukocytes during their trafficking out of the blood stream.[147]

Metastatic cells tightly adhering to the endothelium induce endothelial cell retraction, cell surface uPA and plasmin[36] and/or soluble factors[80] may be involved. Certain cytokines, such as IL-1, may enhance these interactions and therefore favor metastasis.[50] Numerous endothelial cell-adhesion molecules, such as VCAM-1, ICAM-1 and E-selectin, are up-regulated on activated endothelial cells. Endothelial cell activation, for example exposure to IL-1α, has been shown to increase expression of these cell-adhesion molecules,[31] and IL-1α expressing tumor cells show increased metastatic potential. In parallel, ligands for cell binding molecules on endothelial cell surface are increased on neoplastic cells. An example is E-selectin, which binds sialyl-Lewis(a) and sialyl-Lewis(x) carbohydrates of

A

Plate cells on Matrigel

Medium

Cells

Matrigel

48 hours

Tumor cells grow
formimg branching
colonies invading
matrigel

Endothelial cells form a
capillary-like network on
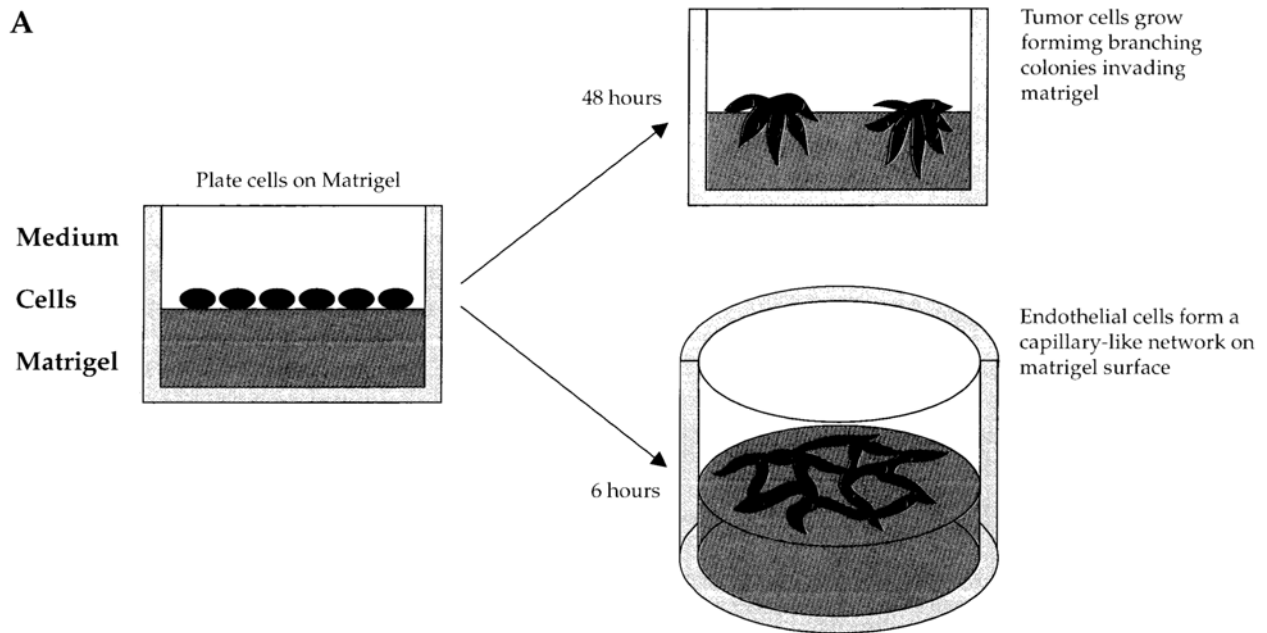matrigel surface

6 hours

Figure 2a. A schematic diagram of the 3-dimensional matrigel invasion assay. A thick layer of matrigel is placed in the culture well and allowed to polymerize. This is then covered by a cell suspension either in media or in more matrigel.

mucins.[70] Over-expression of mucins on tumor cells has been shown to increase adhesion to endothelial cells and increase invasion in the chemoinvasion assay.[142] This corresponded to an increased metastatic potential, even though adhesion to laminin was reduced. Over-expression of ICAM-1, VCAM-1 and ELAM-1 might influence tumor progression in colorectal cancer.[92] High expression of the VLA4 integrin, ligand for VCAM-1, in melanoma cells has

B

Low metastatic OS cells

High metastatic OS cells

HIV-Tat-treated HUVEC

HIV-tat+anti-Tat-treated HUVEC

been associated with increased metastatic potential[57] although experimental over-expression appears to give the opposite results.[109]

Basement membrane invasion and metastasis

Endothelial basement membranes are barriers normally impermeable to migrating cells, however metastatic cells can breach them to enter the parenchyma of the target organ and proliferate, forming a secondary tumor or metastasis.[87] Metastatic cells have been found to express significant amounts of matrix degrading enzymes, particularly metalloproteinases,[113] serine proteases,[11] cathepsins and others. Although several enzymes are involved in degradation of basement membrane, it appears that collagenase IV/gelatinase enzymes as well as serine proteases are the key enzymes for basement membrane invasion and they are produced in significantly greater quantity during metastasis.

Alterations in the balance between proteolytic enzymes and their inhibitors (see below) appear to be a key feature of invasion. Although tumor cells themselves can show
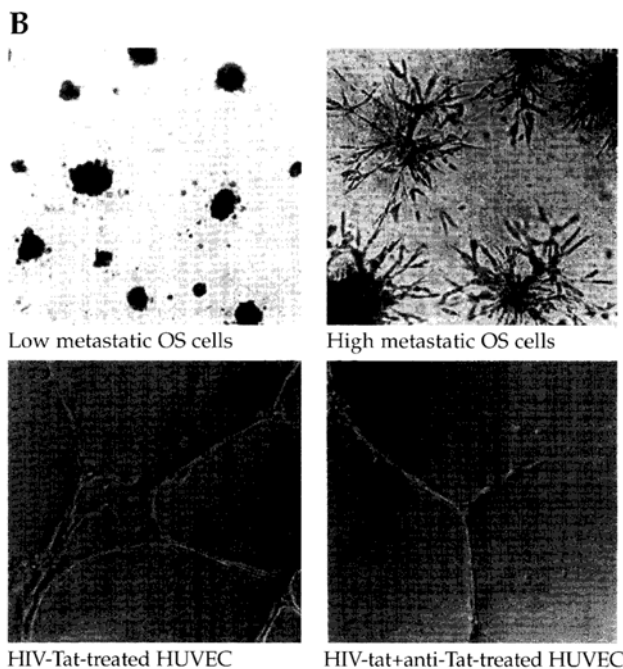
2b. Light micrographs of the organization of cells in the 3-dimensional matrigel invasion assay. Low metastatic osteosarcoma cells (Os) form large round colonies, while high metastatic cells form the characteristic branching, invasive colonies. Endothelial cells (HUVE) form interconnected networks in the matrigel, this is enhanced in the presence of factors which stimulate angiogenesis (in this case HIV-Tat). Blocking of the action of the factor with specific antibodies results in reversal of the

increased protease production, often these are derived from the host tissue in response to tumor cell stimulation.[104] For the matrix metalloproteinases, sarcomas generally appear to show higher intrinsic metalloproteinase production, whereas for carcinomas, metalloproteinase production appears to be mostly derived from host stromal fibroblasts.[136]

Key enzymes for the process of invasion are the type IV collagenases: MMP-2 (gelatinase A)[37] and MMP-9 (gelatinase B),[74] while others may be important in activation of these or other proteases, such as MT-MMPs (transmembrane cell surface MMPs)[120] and stromelysins.[26] Most matrix metalloproteinases are synthesized as latent proenzymes. Cleavage of an N-terminal segment results in activation of the enzyme. This N-terminal peptide contains a free cysteine residue which appears to chelate the metal ion in the active site, rendering the enzyme inactive.[128,129] In fact, addition of exogenous synthetic N-terminal peptide has been shown to inhibit MMP activity and invasion in vitro.[95] Even an excess of cysteine alone has been shown to block MMP activity and reduce tumor metastasis in vivo.[5] The C-terminal end has an important function in TIMP binding.[45] Factors which increase MMP-2 expression also increase invasion and metastasis, for example IL-8.[88] Although over expression of MMP-2 is most often observed in many tumors, MMP-9 also plays a critical role in tumor cell invasion.[74] MT-MMP has been reported to be a specific activator of MMP-2,[121] such that carcinoma cells expressing MT-MMP on their surfaces can bind and activate MMP-2 derived from the host stroma. At least 4 different MT-MMPs have been identified so far.[120]

The metalloproteinases interact with another major proteinase system regulating invasion: the plasminogen activator system.[11,93,99] This consists of plasminogen activator (uPA, also produced as a latent proenzyme), its receptor (uPAR) and its inhibitor (PAI's). uPA cleaves plasminogen to plasmin, which has a broad substrate specificity and degrades many components of the extracellular matrix. uPA can also directly activate metalloproteinases.[72] Inhibitors of plasmin have been found to inhibit invasion into matrigel, while inhibitors of uPA delay invasion and stimulators of uPA production enhanced invasion (reviewed in 11). uPA must be bound to cell surface uPAR to affect invasion; interruption of this interaction leads to decreased invasion. A peptide from uPA, ATF has been reported to have chemotactic and chemoinvasive capability.[130] A complex relationship exits between uPA, uPAR and PAI-1. For example, uPAR binds vitronectin, this binding is enhanced by uPA and uPA-PAI-1 complexes, but is inhibited by PAI-1 alone (see 11). Such complex interactions may explain why conflicting data have been obtained with PAI regarding its role in invasion.

Inhibitors of metalloproteinases have been proven to be particularly effective in regulating invasion and metastasis. An imbalance between metalloproteinases and

their natural tissue inhibitors (TIMP's) is a major factor inducing tumor cell invasion.[52,146] There are so far four natural inhibitors of the MMP's: TIMP-1, TIMP-2 and TIMP-3 and TIMP-4. TIMPs 1, 2 have been shown to inhibit tumor cell invasion in vitro and to reduce metastases in vivo.[7,38,39,64,73,138] However, these molecules also display other activities as well, TIMP-2 has been reported to affect adhesion[114] and apoptosis[138] of melanoma cells. TIMP-1 effects both cell growth and survival.[127] A striking example of non MMP related activities is the potent anti-tumor activity induced by transfection of TIMP-3, apparently due to an enhancement of apoptosis.[2,14,17] Therefore MMPs and TIMPs are no longer considered to be involved only in matrix degradation and its regulation.[26]

In addition to the natural TIMP inhibitors of MMP's, a series of synthetic pharmacological inhibitors of MMPs have been developed.[24,131] Of these, the prototype is Marimastat, whose predecessor was batimastat (BB94). These compounds have been shown to inhibit invasion in vitro and metastases in vivo.[24] Marimastat has shown suppression of tumor invasion metastasis and angiogenesis in phase I and II clinical trials, and is entering phase III. Toxicity has been limited to skeletal muscle and is reversible.[24] Other compounds are under investigation.[106] Application as therapy of anti-proteolytic factors seems to be very promising.

Angiogenesis in tumor progression and anti-angiogenic approaches

Neo-vascularization is rare under normal physiological conditions in adults, and when it occurs, in wound healing or in the uterus during the oestrous cycle, it is strictly regulated. Naturally occurring inhibitors exert a rapid controlling influence over such neo-angiogenesis. Vascularization of tumors is necessary to their invasive growth and metastatic diffusion.[44] The early phase of angiogenesis, the degradation of basement membrane, which allows migration of endothelial cells towards the tumor, has mechanisms similar to those mentioned for metastatic cells.[86,129] The identification of soluble factors stimulating the angiogenic cascade,[25] as well as inhibiting it, have been made in vitro using the chemoinvasion assay. Inhibition of integrin mediated cell adhesion and migration,[21] as well as the use of metalloproteinase inhibitors,[132] targets both metastatic and endothelial cells. One of the major integrins involved in endothelial cell adhesion and migration is $\alpha_v\beta_3$.[21] Antibodies to this integrin are promising antiangiogenic agents[22] and are in clinical trials.[106] Protease inhibitors, developed as 'bullets' directed to the tumor cells, proved to be very effective inhibitors of tumor associated neo-vascularization.[131]

Certain molecules seem to be specifically aimed towards the activated endothelium. One of the best known and studied molecules came from the observation of in

vitro endothelial cell destruction by a fungal contamination. The pioneer studies of Ingber yielded a novel class of potentially useful anti-angiogenic factors,[65] fumagillin and its derivatives (AGM-1470, chloroacetylcarbamoyl) which inhibit endothelial cell proliferation even in the presence of saturating levels of basic fibroblast growth factor (bFGF).[65] Angiostatin and endostatin, the most recent members of the anti-angiogenic drugs,[101,102] are both derived from the urine or serum of mice affected with tumors and confirm the hypothesis that tumor cells produce factors to retard the growth of their own metastasis.

Inhibitors of angiogenic growth factors, growth factor receptors and their interaction, such as antibodies, small peptidomimetics and sulphonate compounds have shown beneficial effects.[106] Most of these compounds have proven to inhibit endothelial cell invasion of basement membranes.

Furthermore, molecules with a long story of biomedical applications have had a 'revival' as antiangiogenic compounds. Class I interferons have anti-angiogenic effects by controlling either bFGF dependent endothelial cell stimulation or metalloprotease production,[43] and they are good inhibitors of endothelial cell invasion in vitro.[66] Differentiative agents, such as retinoids, are also capable of preventing tumor progression, modulating changes in cell morphology, adhesion, growth and invasion not only of tumor[59] but also that of endothelial cells.[66]

The method

Matrigel preparation and composition

Matrigel is prepared from the murine EHS tumor grown in vivo as previously reported.[76] Tumors are removed from the mice, snap-frozen in liquid nitrogen and stored at –80°C until a sufficient quantity is obtained. The frozen material is then homogenized in 3.4 M NaCl with 50 mM Tris, 4 mM EDTA and 2 mM NEM and the insoluble material pelleted and saved. This is repeated 3 times, followed by extraction twice with 2M urea with 50 mM Tris and 150 mM NaCl. The soluble material from these extracts is saved and dialyzed against 50 mM Tris, 150 mM NaCl and eventually DMEM at 4°C. The preparation is sterilized by addition of a few drops of chloroform in the final dialyses. The final extract is stored frozen at concentrations ranging from 12–18 mg/ml. Commercially prepared matrigel is generally supplied in aliquots at 10–12 mg/ml (Becton Dickinson, Bedford, MA).

The chemoinvasion assay

For the chemoinvasion assay originally described we use Boyden chambers.[6] Polycarbonate filters (8 or 12 µm pore, depending on the cell size PVP-free from Nuclepore,

Concorezzo, Milan, Italy) are coated with Matrigel diluted with water (10–100 µg/filter). Since matrigel tends to polymerize at temperatures above 4°C, the dilutions in water are carried out on ice. We first perform a 1/2 dilution, to obtain a homogenous stock, and then the final dilution. Filters are placed on sterile tissue culture plates under a laminar flow hood, and liquid matrigel (50 µl for 13 mm diameter filters) of diluted matrigel are pipetted onto the filter. During this step care must be taken to insure that the matrigel solution is applied even over most of the surface of the filter, leaving a small border of uncoated filter to prevent leakage off the filter. The matrigel coated filters are then dried under laminar flow. The filters are re-hydrated just before performing the assay using cold serum free D'MEM. This procedure leads to an even reconstitution of the matrigel coating on the filter surface.[6] The amount of matrigel to be applied must be determined empirically for each experimental system. Generally, the density of the coating influences migration time, and more aggressive metastatic cells are able to cross higher amounts of matrigel. A suitable filter pore size (5, 8, 10, 12 µM) for the cell type must also be used, this is generally what gives good selectivity in chemotaxis assays. Numerous substances can be used as chemoattractants in the chemoinvasion assay. Good standard references are fibroblast conditioned medium for tumor cells, reproducing an attraction induced by stromal products,[6] or Kaposi's Sarcoma cell conditioned medium for endothelial cells, mimicking a highly angiogenic environment.[4] Purified single growth factors are also frequently used, such as EGF for epithelial cells or vascular endothelial growth factor (VEGF) for endothelial cells. Serum-free medium containing 0.1% bovine serum albumin (BSA) is used as negative control. Often chemotaxis assays are done in parallel to determine if treatments affect chemotaxis itself or if they are specific for invasion. An invasion index (invasion/chemotaxis) can be calculated and indicate the specific contribution of matrix degradation using the following formula:

$$\text{Invasion index} = \frac{\text{Invaded Cells}}{\text{Migrated Cells}} \times 100$$

Under control conditions, without stimulation from a 'chemoinvasive' agent in the lower compartment, cells do not invade. Cells are harvested with trypsin (or just EDTA), washed with serum-free DMEM with 0.1% BSA and placed in the upper compartment of the Boyden chamber (usually about 1.3×10^5 cells in 400–800 µl). Chambers are then incubated at 37°C in 5% CO_2 for 6 h. For "checkerboard" analysis of invasion increasing concentrations of the same chemoattractant are also added to the cells. Invasion inhibitors acting on the chemoattractant are added to the lower compartment, while inhibitors acting on the cells or cell products, for instance blocking

protease secretion or activation, are generally added along with the cells. Biological response modifiers, such as interferon, often require a pretreatment of the cells (24 hrs or longer) before chemoinvasion is performed. At the end of the incubation time, cells remaining on the upper surface of the filter are mechanically removed by wiping them with a cotton swab or stripping on a glass slide. The cells migrated to the under surface are quantitated after staining (with toluidine blue, hematoxylin/eosin or others). In the original assay quantitation was performed by microscope counting five to ten random fields for each filter. Assays are generally performed in triplicate and repeated two or three times.

Various alternatives have been proposed such as colorimetric detection of the staining, image analysis, and metabolic labeling with MTT or similar compounds.[47,63,118,122] Alternatives to matrigel have also been applied,[124] as have alternatives to Boyden chambers.[41,58,115] If the assay is performed under sterile conditions, the invaded cells can be recovered and expanded, generally resulting in a more invasive population as compared to the initial population.[8,67,123]

An alternative to Boyden chambers and single filters are either the multiwell chambers or the transwell™ chambers, coated with matrigel.[115] Transwell™ chambers have the advantage of sterility and "industrial" standardization, however the efficiency of invasion is lower (thicker coating requires longer assay times) and is more costly. Boyden chambers are recyclable and if enough are available it is easy to process about 50 samples per day.

Metastatic cells migrate and invade through the matrigel, while benign counterparts are less or not invasive. Highly migratory but not invasive cells, such as fibrobasts, are able to migrate in the absence of matrigel but do not cross the coated basement membrane barrier, a phenomenon which requires active proteolysis.

Endothelial cells can be assessed using the same protocol. Generally the coating of matrigel required to block invasion in the absence of a stimulus is lower, since the endothelial cell "aggressiveness" is far below that of highly metastatic cells.

Growth on Matrigel

Matrigel can also be used for morphological studies on tumor and vascular cell invasion.[6,54] Matrigel is thawed at 0°C in an ice-water bath, and 0.5 ml of a concentrated solution (10 mg/ml) are pipetted into 13 mm/diameter tissue culture wells, avoiding even small bubbles. The matrigel is then polymerized for 1 hr at 37°C. Once polymerization had occurred, 5×10^4 cells in 1 ml of final volume are carefully pipetted on top of the gel. These suspensions are mixed several times to ensure separation into single cells before plating. The plates are then incubated at 37°C

in a 5% CO_2, humidified atmosphere. The assays are photographed and monitored with an inverted microscope, a modification using MTT as a colorant has been recently described.[119]

Non tumorigenic cells generally do not grow. Low metastatic tumor cells form large round colonies, while high metastatic cells form branching, invasive colonies. Endothelial cells form interconnected networks in the matrigel, enhanced by factors which stimulate angiogenesis. Blocking of the action of the factor with anti-angiogenic drugs inhibits the capillary-like morphology.

References

1. *Ahmad A, Hart IR:* Mechanisms of metastasis. Crit Rev Oncol Hematol 26:163-173, 1997.
2. *Ahonen M, Baker AH, Kahari VM:* Adenovirus-mediated gene delivery of tissue inhibitor of metalloproteinases-3 inhibits invasion and induces apoptosis in melanoma cells. Cancer Res. 58:2310-2315, 1998.
3. *Akiyama SK, Olden K, Yamada KM:* Fibronectin and integrins in invasion and metastasis. Cancer Metastasis Rev 14:173-189, 1995.
4. *Albini A, Barillari G, Benelli R, et al:* Angiogenic properties of human immunodeficiency virus type 1 Tat protein. Proc Natl Acad Sci USA 92:4838-4842, 1995.
5. *Albini A, D'Agostini F, Giunciuglio D, et al:* Inhibition of invasion, gelatinase activity, tumor take and metastasis of malignant cells by N-acetylcysteine. Int J Cancer 61:121-129, 1995.
6. *Albini A, Iwamoto Y, Kleinman HK, et al:* A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. Cancer Res 47:3239-3245, 1987.
7. *Albini A, Melchiori A, Santi L, et al:* Tumor cell invasion inhibited by TIMP-2. J Natl Cancer Inst 83:775-779, 1991.
8. *Albini A, Muller PK, Parodi S:* A method to select cell populations with enhanced chemotactic activity. Bioscience Rep. 4:311-318, 1984.
9. *Alessandro R, Kohn EC:* Molecular genetics of cancer. Tumor invasion and angiogenesis. Cancer 76:1874-1877, 1995.
10. *Alessandro R, Spoonster J, Wersto RP, et al:* Signal transduction as a therapeutic target. Curr Top Microbiol Immunol 213:167-188, 1996.
11. *Andreasen PA, Kjoller L, Christensen L, et al:* The urokinase-type plasminogen activator system in cancer metastasis: a review. Int J Cancer 72:1-22, 1997.
12. *Aota S, Yamada KM:* Integrin functions and signal transduction. Adv Exp Med Biol 669-682, 1997.

13. *Arbiser JL, Moses MA, Fernandez CA, et al:* Oncogenic H-ras stimulates tumor angiogenesis by two distinct pathways. Proc Natl Acad Sci USA 94:861-866, 1997.

14. *Baker AH, Zaltsman AB, George SJ, et al:* Divergent effects of tissue inhibitor of metalloproteinase-1,-2, or -3 overexpression on rat vascular smooth muscle cell invasion, proliferation, and death in vitro. J Clin Invest 101:1478-1487, 1998.

15. *Bardelli A, Comoglio PM:* Scatter factor receptors are key players in a unique multistep program leading to invasive growth. Ciba Found Symp 212:133-144, 1997.

16. *Berditchevski F, Tolias KF, Wong K, et al:* A novel link between integrins, transmembrane-4 superfamily proteins (CD63 and CD81), and phosphatidylinositol 4-kinase. J Biol Chem 272:2595-2598, 1997.

17. *Bian J, Wang Y, Smith RM, et al:* Suppression of in vivo tumor growth and induction of suspension cell death by tissue inhibitor of metalloproteinases (TIMP)-3. Carcinogenesis 17:1805-1811, 1996.

18. *Birchmeier W:* E-cadherin as a tumor (invasion) suppressor gene. Bioessays 17:97-99, 1995.

19. *Birchmeier W, Brinkmann V, Niemann C, et al:* Role of HGF/SF and c-Met in morphogenesis and metastasis of epithelial cells. Ciba Found Symp 212:230-240; discussion 240-246, 1997.

20. *Boyd D:* Invasion and metastasis. Cancer Metast Rev 15: 77-89, 1996.

21. *Brooks P, Clark A. Cheresh D:* Requirement of vascular integrin $\alpha_v\beta_3$ for angiogenesis. Science 264:569-571, 1994.

22. *Brooks P, Montgomery A, Rosenfeld M, et al:* D. Integrin $\alpha_v\beta_3$ antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. Cell 79:1157-1164, 1994.

23. *Brown MA.* Tumor suppressor genes and human cancer. Adv Genet 36:45-135, 1997.

24. *Brown PD.* Matrix metalloproteinase inhibitors in the treatment of cancer. Med Oncol 14:1-10, 1997.

25. *Bussolino F, Albini A, Camussi G, et al:* Role of soluble mediators in angiogenesis. Eur J Cancer 2401-2412, 1996.

26. *Chambers AF, Matrisian LM:* Changing views in the role of matrix metalloproteinases in metastasis. J Natl Cancer Inst 89:1260-1270, 1997.

27. *Chao C, Lotz MM, Clarke AC, et al:* A function for the integrin alpha6beta4 in the invasive properties of colorectal carcinoma cells. Cancer Res 56:4811-4819, 1996.

28. *Chen H, Paradies NE, Fedor-Chaiken M, et al:* E-cadherin mediates adhesion and suppresses cell motility via distinct mechanisms. J Cell Sci 110:345-356, 1997.

29. *Chintala SK, Fueyo J, Gomez-Manzano C, et al:* Adenovirus-mediated p16/CDKN2 gene transfer suppresses glioma invasion in vitro. Oncogene 15:2049-2057, 1997.

30. *Chintala SK, Sawaya R, Rao, JS.* Modulation of matrix metalloprotease-2 and invasion in human glioma cells by alpha3beta1 integrin. Cancer Lett 103:201-208, 1996.

31. *Chirivi RG, Chiodoni C, Musiani P, et al:* IL-1alpha gene-transfected human melanoma cells increase tumor-cell adhesion to endothelial cells and their retention in the lung of nude mice. Int J Cancer 67:856-863, 1996.

32. *Claffey KP, Robinson GS:* Regulation of VEGF/VPF expression in tumor cells: consequences for tumor growth and metastasis. Cancer Metastasis Rev 15:165-176, 1996.

33. *Cockett MI, Murphy G, Birch ML, et al:* Matrix metalloproteinases and metastatic cancer. Biochem Soc Symp 63:295-313, 1998.

34. *Coffey DS:* Self-organization, complexity and chaos: the new biology for medicine. Nat Med 4:882-885, 1998.

35. *Colville NP, Willoughby DA:* Growth factors in angiogenesis: current interest and therapeutic potential. Mol Med Today 3:14-23, 1997.

36. *Conforti G, Dominguez JC, Ronne E, et al:* Cell-surface plasminogen activation causes a retraction of in vitro cultured human umbilical vein endothelial cell monolayer. Blood 83:994-1005, 1994.

37. *Corcoran ML, Hewitt RE, Kleiner DJ and WG, S-S.* MMP-2: expression, activation and inhibition. Enzyme Prot 49:7-19, 1996.

38. *DeClerck YA, Perez N, Shimada H, et al:* Inhibition of invasion and metastasis in cells transfected with an inhibitor of metalloproteinases. Cancer Res 52:701-708, 1992.

40. *Ellis, LM and Fidler, IJ:* Angiogenesis and metastasis. Eur J Cancer 32A:2451-2460, 1996.

41. *Erkell LJ, Schirrmacher, V:* Quantitative in vitro assay for tumor cell invasion through extracellular matrix or into protein gels. Cancer Res 48;6933-6937, 1988.

42. *Faassen AE, Schrager JA, Klein DJ, et al:* A cell surface chondroitin sulfate proteoglycan, immunologically related to CD44, is involved in type 1 collagen-mediated melanoma cell motility and invasion. J Cell Biol 116:521-531, 1992.

43. *Fidler IJ, Kumar R, Bielenberg DR, et al* Molecular determinants of angiogenesis in cancer metastasis. Cancer J Sci Am S58-66, 1998.

44. *Folkman, J:* Angiogenesis and angiogenesis inhibition: an overview. Exs 79:1-8, 1997.

45. *Fridman R, Fuerst TR, Bird RE, et al:* Domain structure of human 72-kDa gelatinase/type IV collagenase. Characterization of proteolytic activity and identification of the tissue inhibitor of metalloproteinase-2 (TIMP-2) binding regions. J Biol Chem 267:15398-15405, 1992.

46. *Fukushima Y, Ohnishi T, Arita N, et al:* Integrin alpha3beta1-mediated interaction with laminin-5 stimulates adhesion, migration and invasion of malignant glioma cells. Int J Cancer 76:63-72, 1998.

47. *Ghola A, Eckert K, Maurer, HR:* A rapid and sensitive fluorometric screening assay using YO-PRO-1 to quantify tumor cell invasion through matrigel. Clin Exp Metastasis 14:451-458, 1996.

48. *Giavazzi R:* The nude mouse in oncology research. In: Metastatic Models (Eds: Boven, E and Winograd, B), CRC Press, Inc, London, 1991, pp. 117-132.

49. *Giavazzi R:* Cytokine-mediated tumor-endothelial cell interaction in metastasis. Curr Top Microbiol Immunol 213:13-30,1996.

50. *Giavazzi R, Garofalo A, Bani MR, et al:* Interleukin1-induced augumentation of experimental metastases from a human melanoma in nude mice. Cancer Res 50:4771-4775, 1990.

51. *Giunciuglio D, Culty M, Fassina G, et al:* Invasive phenotype of MCF-10A cells overexpressing c-Ha-ras and c-erb-B2 oncogenes. Int J Cancer 63:815-822, 1995.

52. *Gomez DE, Alonso DF, Yoshiji H, et al:* Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. Eur J Cell Biol 74:111-122, 1997.

53. *Grander, D:* How do mutated oncogenes and tumor suppressor genes cause cancer? Med Oncol 15:20-26, 1998.

54. *Grant DS, Tashiro K, Segui-Real B, et al:* Two different laminin domains mediate the differentiation of human endothelial cells into capillary-like structures in vitro. Cell 58:933-943, 1989.

55. *Guilford P, Hopkins J, Harraway J, et al:* E-cadherin germline mutations in familial gastric cancer. Nature 392:402-405, 1998.

56. *Harmey JH, Dimitriadis E, Kay E, et al:* Regulation of macrophage production of vascular endothelial growth factor (VEGF) by hypoxia and transforming growth factor beta-1. Ann Surg Oncol 5:271-278, 1998.

57. *Hart IR, Birch M, Marshall, JF:* Cell adhesion receptor expression during melanoma progression and metastasis. Cancer Metastasis Rev 10:115-128, 1991.

58. *Hendrix MJC, Seftor EA, Seftor REB, et al:* Comparison of tumor cell invasion assay: human amnion versus reconstituted basement membrane barriers. Invas Metast 9:278-297, 1989.

59. *Hendrix MJC, Wood WR, Seftor EA, et al:* Retinoic acid inhibition of human melanoma cell invasion through a reconstituted basement membrane and its relation to decreases in the expression of proteolytic enzymes and motility factor receptor. Cancer Res 50:4121-4130, 1990.

60. *Hiraguri S, Godfrey T, Nakamura H, et al:* Mechanisms of inactivation of E-cadherin in breast cancer cell lines. Cancer Res 58:1972-1977, 1998.

61. *Howe A, Aplin AE, Alahari SK, et al:* Integrin signaling and cell growth control. Curr Opin Cell Biol 10:220-31, 1998.

62. *Ilyas M, Tomlinson IP:* The interactions of APC, E-cadherin and beta-catenin in tumour development and progression. J Pathol 182:128-137, 1997.

63. *Imamura H, Takao S, Aikou, T:* A modified invasion-3(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assay for quantitating tumor cell invasion. Cancer Res 54:3620-3624, 1994.

64. *Imren S, Kohn D, Shimada H, et al:* Overexpression of tissue inhibitor of metalloproteinases-2 by retroviral-mediated gene transfer in vivo inhibits tumor growth and invasion. Cancer Res 56:2891-2895, 1996.

65. *Ingber D, Fujita T, Kishimoto S, et al:* Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth. Nature 348:555-557, 1990.

66. *Iurlaro M, Benelli R, Masiello L, et al:* Beta Interferon inhibits HIV-1 Tat induced angiogenesis: synergism with 13-cis Retinoic Acid. Eur J Cancer 34:570-576, 1998.

67. *Jacob K, Wach F, Knuchel R, et al:* Characterization of selected strongly and weakly invasive sublines of a primary human melanoma cell line and isolation of subtractive cDNA clones. Int J Cancer 60:668-675, 1995.

68. *Jones JL, Royall JE, Critchley DR, et al:* Modulation of myoepithelial-associated alpha6beta4 integrin in a breast cancer cell line alters invasive potential. Exp. Cell Res. 235:325-333, 1997.

69. *Joukov V, Kaipainen A, Jeltsch M, et al:* Vascular endothelial growth factors VEGF-B and VEGF-C. J Cell Physiol 173:211-215, 1997.

70. *Kannagi R:* Carbohydrate-mediated cell adhesion involved in hematogenous metastasis of cancer. Glycoconj J 14:577-584, 1997.

71. *Kawada A, Hara K, Kominami E, et al:* Expression of cathepsin D and B in invasion and metastasis of squamous cell carcinoma. Br J Dermatol 137:361-366, 1997.

72. *Keski OJ, Lohi J, Tuuttila A, et al:* Proteolytic processing of the 72,000-Da type IV collagenase by urokinase plasminogen activator. Exp Cell Res 202:471-476, 1992.

73. *Khokha R, Zimmer MJ, Graham CH, et al:* Suppression of invasion by inducible expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) in B16-F10 melanoma cells. J. Natl Cancer Inst 84:1017-1022, 1992.

74. *Kim J, Yu W, Kovalski K, et al:* Requirement for specific proteases in cancer cell intravasation as revealed by a novel semiquantitative PCR-based assay. Cell 94:353-362, 1998.

75. *Klein S, Roghani M, Rifkin, DB:* Fibroblast growth factors as angiogenesis factors: new insights into their mechanism of action. Exs 79:159-192, 1997.

76. *Kleinman HK, McGarvey ML, Hassell JR, et al:* Basement membranes complexes with biological activity. Biochemistry 25:312-318, 1986.

77. *Knutson JR, J. Lidia, Fields, GB, McCarthy, JB:* CD44/ chondroitin sulfate proteoglycan and alpha2 beta1 integrin mediate human melanoma cell migration on type IV collagen and invasion of basement membranes. Mol Biol Cell 7:383-396, 1996.

78. *Kohn EC, Liotta, LA:* Molecular insights into cancer invasion: strategies for prevention and intervention. Cancer Res 55:1856-1862, 1995.

79. *Kubota S, Ito H, Ishibashi Y, et al:* Anti-alpha3 integrin antibody induces the activated form of matrix metalloprotease-2 (MMP-2)with concomitant simulation of invasion through matrigel by human rhabdomyosarcoma cells. Int J Cancer 70:106-111, 1997.

80. *Kusama T, Nakamori S, Ohigashi H, et al:* Enhancement of in vitro tumor-cell transcellular migration by tumor- cell-secreted endothelial-cell-retraction factor. Int J Cancer 63:112-118, 1995.

81. *Lawrence JA, Steeg PS:* Mechanisms of tumor invasion and metastasis. World J Urol 14:124-130, 1996.

82. *Leek RD, Lewis CE, Whitehouse R, et al:* Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. Cancer Res 56:4625-4629, 1996.

83. *Levine MD, Liotta LA, Stracke, ML:* Stimulation and regulation of tumor cell motility in invasion and metastasis. Exs 74:157-179, 1995.

84. *Li J, Hu SX, Perng GS, et al:* Expression of the retinoblastoma (RB) tumor suppressor gene inhibits tumor cell invasion in vitro. Oncogene 13:2379-2386, 1996.

85. *Lindauer M, Stanislawski T, Haussler A, et al:* The molecular basis of cancer immunotherapy by cytotoxic T lymphocytes. J Mol Med 76:32-47, 1998.

86. *Liotta L, Steeg P, Stetler-Stevenson, W:* Cancer metastasis and angiogenesis: An imbalance of positive and negative regulation. Cell 64:327-336, 1991.

87. *Liotta LA:* Tumor invasion and metastases - Role of the extracellular matrix: Rhoads memorial award lecture. Cancer Res. 46:1-7, 1986.

88. *Luca M, Huang S, Gershenwald JE, et al:* Expression of Interleukin-8 by human melanoma cells up-regulates MMP-2 activity and increases tumor growth and metastasis. Am J Pathol 151:1105-1113, 1997.

89. *Maggiora P, Gambarotta G, Olivero M, et al:* Control of invasive growth by the HGF receptor family. J Cell Physiol 173:183-186, 1997.

90. *Mareel M, Boterberg T, Noe V, et al:* E-Cadherin/Catenin/Cytoskeleton complex: a regulator of cancer invasion. J Cell Physiol 173:271-274, 1997.

91. *Matrisian LM, Ganser GL, Kerr LD, et al:* Negative regulation of gene expression by TGF-beta. Mol Reprod Dev 32:111-120, 1992.

92. *Maurer CA, Friess H, Kretschmann B, et al:* Over-expression of ICAM-1, VCAM-1 and ELAM-1 might influence tumor progression in colorectal cancer. Int J Cancer 79:76-81, 1998.

93. *Mazzieri R, Masiero L, Zanetta L, et al:* Control of type IV collagenase activity by components of the urokinase- plasmin system: a regulatory mechanism with cell-bound reactants. Embo J 16:2319-232, 1997.

94. *Meade TL, Boukamp P, Fusenig NE, et al:* Differential expression of matrix metalloproteinases in activated c-ras- Ha-trans-

fected immortalized human keratinocytes. Br J Cancer 77:724-730, 1998.

95. *Melchiori A, Albini A, Ray JM, et al:* Inhibition of tumor cell invasion by a highly conserved peptide sequence from the matrix metalloproteinase enzyme prosegment. Cancer Res 52:2353-2356, 1992.

96. *Melchiori A, Carlone S, Allavena G, et al:* Invasiveness and chemotactic activity of oncogene transformed NIH/3T3 cells. Anticancer Res 10:37-44, 1990.

97. *Melchiori A, Mortarini R, Carlone S, et al:* The $\alpha_3\beta_1$ integrin is involved in melanoma cell migration and invasion. Exp Cell Res 218:233-2421995.

98. *Merzak A, Koocheckpour S, Pilkington, GJ:* CD44 mediates human glioma cell adhesion and invasion in vitro. Cancer Res 54:3988-3992, 1994.

99. *Mignatti P, Rifkin, DB:* Plasminogen activators and matrix metalloproteinases in angiogenesis. Enzyme Protein 49:117-137, 1996.

100. *Natali PG, Nicotra MR, Bartolazzi A, et al:* Integrin expression in cutaneous malignant melanoma: association of the alpha 3/beta 1 heterodimer with tumor progression. Int J Cancer 54:68-72, 1993.

101. *O'Reilly MS, Boehm T, Shing Y, et al:* Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell 88:277-285, 1997.

102. *O'Reilly MS, Holmgren L, Chen C, et al:* Angiostatin induces and sustains dormancy of human primary tumors in mice. Nature Med 2:689-692, 1996.

103. *Oft M, Peli J, Rudaz C, et al:* TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. Genes Dev 10:2462-2477, 1996.

104. *Ohtani H:* Stromal reaction in cancer tissue: pathophysiologic significance of the expression of matrix-degrading enzymes in relation to matrix turnover and immune/inflammatory reactions. Pathol Int 48:1-9, 1998.

105. *Perl AK, Wilgenbus P, Dahl U, et al:* A casual role for E-cadherin in the transition from adenoma to carcinoma. Nature 392:190-193, 1998.

106. *Pluda JM:* Tumor-associated angiogenesis: mechanisms, clinical implications, and therapeutic strategies. Semin Oncol 24:203-218, 1997.

107. *Polverini PJ:* Role of the macrophage in angiogenesis-dependent diseases. In: Regulation of Angiogenesis (Eds: Goldberg, IP and Rosen, ER), Birkhäuser Verlag, Basel, 1997, pp.11-28.

108. *Price JT, Bonovich MT, Kohn EC:* The biochemistry of cancer dissemination. Crit Rev Biochem Mol Biol 32:175-253, 1997.

109. *Qian F, Vaux DL, Weissman IL:* Expression of the integrin alpha 4 beta 1 on melanoma cells can inhibit the invasive stage of metastasis formation. Cell 77:335-347, 1994.

110. *Rabinovitz I, Mercurio AM:* The integrin alpha 6 beta 4 and the biology of carcinoma. Biochem Cell Biol 74:811-21, 1996.

111. *Radotra B, McCormick D:* Glioma invasion in vitro is mediated by CD44-hyaluronan interactions. J Pathol 181:434-438, 1997.

112. *Ramos MA, Kuzuya M, Esaki T, et al:* Induction of macrophage VEGF in response to oxidized LDL and VEGF accumulation in human atherosclerotic lesions. Arterioscler Thromb Vasc Biol 18:1188-1196, 1998.

113. *Ray JM, Stetler SW:* The role of matrix metalloproteases and their inhibitors in tumour invasion, metastasis and angiogenesis. Eur Respir J 7:2062-2072, 1994.

114. *Ray JM, Stetler-Stevenson WG:* Gelatinase A activity directly modulates melanoma cell adhesion and spreading. EMBO J 14:908-917, 1995.

115. *Repesh LA:* A new in vitro assay for quantitating tumor cell invasion. Invas Metast 9:192-208, 1989.

116. *Ries C, Petrides PE:* Cytokine regulation of matrix metalloproteinase activity and its regulatory dysfunction in disease. Biol Chem Hoppe Seyler 376:345-355, 1995.

117. *Ruoslahti E:* Integrins as signaling molecules and targets for tumor therapy. Kidney Int 51:1413-1417, 1997.

118. *Saito K, Oku T, Ata N, et al:* A modified and convenient method for assessing tumor cell invasion and migration and its application to screening for inhibitors. Biol Pharm Bull 20:345-348, 1997.

119. *Sasaki CY, Passaniti, A:* Identification of anti-invasive but noncytotoxic chemotherapeutic agents using the tetrazolium dye MTT to quantitate viable cells in matrigel. Biotechniques 24:1038-1043, 1998.

120. *Sato H, Okada Y, Seiki M:* Membrane-type matrix metalloproteinases (MT-MMPs) in cell invasion. Thromb Haemost 78:497-500, 1997.

121. *Sato H, Takino T, Okada Y, et al:* A matrix metalloproteinase expressed on the surface of invasive tumor cells. Nature 370:61-65, 1994.

122. *Schonermark MP, Bock O, Buchner A, et al:* Quantification of tumor cell invasion using confocal laser scan microscopy. Nat Med 3:1167-1171, 1997.

123. *Seftor EA, Seftor RE, Hendrix MJ:* Selection of invasive and metastatic subpopulations from a heterogeneous human melanoma cell line. Biotechniques 9:324-331, 1990.

124. *Siegal GP, Wang MH, Rinehart CA, et al:* Development of a novel human extracellular matrix for quantitation of invasiveness of human cells. Cancer Lett. 69:123-132, 1993.

125. *Smith SK:* Angiogenesis. Semin Reprod Endocrinol 15:221-227, 1997.

126. *St Croix B, Sheehan C, Rak JW, et al:* E-Cadherin-dependent growth suppression is mediated by the cyclin- dependent kinase inhibitor p27(KIP1). J Cell Biol 142:557-571, 1998.

127. *Stetler-Stevenson M, Mansoor A, Lim M, et al:* Expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in reactive and neoplastic lymphoid cells. Blood 89:1708-1715, 1997.

128. *Stetler-Stevenson W, Hewitt R, Corcoran M.* Matrix metalloproteinases and tumor invasion: from correlation and causality to the clinic. Semin Cancer Biol 7:147-154, 1996.

129. *Stetler-Stevenson WG, Corcoran ML:* Tumor angiogenesis: functional similarities with tumor invasion. EXS 79:413-418, 1997.

130. *Stoppelli MP, Corti A, Soffientini A, et al:* Differentiation-enhanced binding of the amino-terminal fragment of human urokinase plasminogen activator to a specific receptor on U937 monocytes. Proc Natl Acad Sci U S A 82:4939-4943, 1985.

131. *Talbot DC, Brown PD:* Experimental and clinical studies on the use of matrix metalloproteinase inhibitors for the treatment of cancer. Eur J Cancer 32:2528-2533, 1996.

132. *Taraboletti G, Garofalo A, Belotti D, et al:* Inhibition of angiogenesis and murine hemangioma growth by batimastat, a synthetic inhibitor of matrix metalloproteinases. J Natl Cancer Inst 87:293-298, 1995.

133. *Terman BI, Dougher VM:* Biological properties of VEGF/VPF receptors. Cancer Metastasis Rev 15:159-163, 1996.

134. *Thompson E, Nakamura S, Shima T, et al:* Supernatants of acquired immunodeficiency syndrome-related Kaposi's sarcoma cells induce endothelial cell chemotaxis and invasiveness. Cancer Res. 51:2670-2671, 1991.

135. *Timar J, Raso E, Fazakas Zs, et al:* Multiple use of a signal transduction pathway in tumor cell invasion. Anticancer Res 16:3299-3306, 1996.

136. *Tryggvason K, Hoyhtya M, Pyke, C:* Type IV collagenases in invasive tumors. Breast Cancer Res Treat 24:209-218, 1993.

137. *Tsai V, Kawashima I, Keogh E, et al:* In vitro immunization and expansion of antigen-specific cytotoxic T lymphocytes for adoptive immunotherapy using peptide-pulsed dendritic cells. Crit Rev Immunol 18:65-75, 1998.

138. *Valente P, Fassina GF, Melchori A, et al:* TIMP-2 over-expression reduces invasion and angiogenesis and protects B16F10 melanoma cells from apoptosis. Int J Cancer 75:246-253, 1998.

139. *Valente P, Melchiori A, Paggi MG, et al:* Rb-1 oncosuppressor gene over-expression inhibits tumour progression and induces melanogenesis in metastatic melanoma cells. Oncogene 13:1169-1178, 1996.

140. *Woodhouse EC, Chuaqui RF, Liotta LA:* General mechanisms of metastasis. Cancer 80:1529-1537, 1997.

141. *Wright JA, Huang A:* Growth factors in mechanisms of malignancy: roles for TGF-beta and FGF. Histol Histopathol 11:521-536, 1996.

142. *Xie S, Luca M, Huang S, et al:* Expression of MCAM/MUC18 by human melanoma cells leads to increased tumor growth and metastasis. Cancer Res. 57:2295-2303, 1997.

143. *Xiong M, Elson G, Legarda D, et al:* Production of vascular endothelial growth factor by murine macrophages: regulation by hypoxia, lactate, and the inducible nitric oxide synthase pathway. Am J Pathol 153:587-598, 1998.

144. *Xue W, Mizukami I, Todd RF, et al:* Urokinase-type plasminogen activator receptors associate with beta1 and beta3 integrins of fibrosarcoma cells: dependence on extracellular matrix components. Cancer Res 57:1682-1689, 1997.

145. *Yamamoto H, Irie V, Fukushima Y, et al:* Abrogation of lung metastasis of human fibrosarcoma cells by ribozyme-mediated suppression of integrin alpha6 subunit expression. Int J Cancer 65:519-524, 1996.

146. *Yu AE, Hewitt RE, Kleiner DE, et al:* Molecular regulation of cellular invasion—role of gelatinase A and TIMP-2. Biochem Cell Biol 74:823-31, 1996.

147. *Zetter BR:* Adhesion molecules in tumor metastasis. Semin Cancer Biol 4:219-229, 1993.

148. *Zetter BR:* Angiogenesis and tumor metastasis. Annu Rev Med 49:407-424, 1998.