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 resistant 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\(^1\) 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 anti-neoplastic 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.\(^2\,\) 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.\(^3\) 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.\(^4\) A breakthrough was the use of a reconstituted membrane, “Matrigel\(^5\)^8\), which when applied over filters in the Boyden chamber chemotaxis assay, quantitated metastatic cell invasive potential.\(^5\) 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

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

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, 26,137 to be able to inhibit the ability of tumor cells to invade, in addition to their growth. Melanoma 20,136 and osteosarcoma 24 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 oncogenesis of metalloproteinase activity, 76 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 neangiogenesis. Cytokines can induce, besides growth, also migration, invasion and endothelial cell activation. 29,33,116 Inhibition of growth factor interaction with their receptors is a prospective target for anti-metastatic and antiangiogenic 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. 10,78,85 For example, VEGF, a key growth factor, has an important role in metastasis and angiogenesis. 52,69 VEGF has more than one receptor. 13 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/Flik-1, is responsible
for the induction of the proliferative and invasive phases of angiogenesis. bFGF, with its numerous receptors, TGFs, HGF with its receptor c-met and many other growth factors are linked to invasion and angiogenesis.

Several cytokines act in invasion by inducing increased expression of one or more matrix metalloproteinases, 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.

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. 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. Restoration of functional E-cadherin expression has been shown to revert an invasive epithelial tumor cells to a non-invasive phenotype. 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. A well studied example of this is epithelial cell "scattering" and invasion induced by HGF (scatter factor), which can be counteracted by E-cadherin. Both growth suppressive and motility-suppressive functions have been assigned to E-cadherin, mutational analyses suggest that these can be separated. E-cadherin is linked to the cytoskeleton via β-catenin complexes, which is in equilibrium with the product of another gene found altered early in colon carcinogenesis, APC. Disruption of the β-catenin binding domain of E-cadherin has been shown to affect motility. E-cadherin growth suppression appears to be mediated through the p27KIP1 cyclin-dependent kinase inhibitor, 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. 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. These molecules regulate cell adhesion, migration, growth and survival through complex signaling pathways. 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. 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). α3 expression is enhanced in certain tumors and we have found that α3β1 mediates migration and matrigel invasion of melanoma cells in the Boyden chamber. Interaction of VLA3 with laminin 5 stimulates invasion of malignant glioma cells. 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 and glioma 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 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. An α6β4 dependent increase in adhesion of colorectal carcinoma cells and invasion of Matrigel has been reported. Likewise, in fibrosarcoma cells, suppression by ribozymes of α6 subunit expression abrogated lung metastasis and matrigel invasion. 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. The complex role of α6β4 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 and later shown to modulate invasion of matrigel. Differing alternate splice variants of CD44 seem to confer invasiveness, 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 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
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”, CTLs 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.56,62,107,112,125,143 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 α4β1 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 plasminogen and/or soluble factors 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
mucins. Over-expression of mucins on tumor cells has been shown to increase adhesion to endothelial cells and increase invasion in the chemoinvasion assay. 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. High expression of the VLA4 integrin, ligand for VCAM-1, in melanoma cells has been associated with increased metastatic potential, although experimental over-expression appears to give the opposite results.

**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. Metastatic cells have been found to express significant amounts of matrix degrading enzymes, particularly metalloproteinases, serine proteases, 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 (HUVECs) 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. 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.

Key enzymes for the process of invasion are the type IV collagenases: MMP-2 (gelatinase A) and MMP-9 (gelatinase B), while others may be important in activation of these or other proteases, such as MT-MMPs (transmembrane cell surface MMPs) and stromelysins. 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. In fact, addition of exogenous synthetic N-terminal peptide has been shown to inhibit MMP activity and invasion in vitro. Even an excess of cysteine alone has been shown to block MMP activity and reduce tumor metastasis in vivo. The C-terminal end has an important function in TIMP binding. Factors which increase MMP-2 expression also increase invasion and metastasis, for example IL-8. Although over expression of MMP-2 is most often observed in many tumors, MMP-9 also plays a critical role in tumor cell invasion.

MT-MMP has been reported to be a specific activator of MMP-2, 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.

The metalloproteinases interact with another major proteinase system regulating invasion: the plasminogen activator system. This consists of plasminogen activator (uPA, also produced as a latent proenzyme), its receptor (uPAR) and its inhibitor (PAI), uPAR cleaves plasminogen to plasmin, which has a broad substrate specificity and degrades many components of the extracellular matrix. uPA can also directly activate metalloproteinases. 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. A complex relationship exists 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. 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. However, these molecules also display other activities as well. TIMP-2 has been reported to affect adhesion and apoptosis of melanoma cells. TIMP-1 effects both cell growth and survival.

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. Therefore MMPs and TIMPs are no longer considered to be involved only in matrix degradation and its regulation.

In addition to the natural TIMP inhibitors of MMP’s, a series of synthetic pharmacological inhibitors of MMPs have been developed. 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. 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. Other compounds are under investigation. Application as therapy of anti-proteolytic factors seems to be very promising.

**Angiogenesis in tumor progression and anti-angiogenic approaches**

Neovascularization 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. 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. The identification of soluble factors stimulating the angiogenic cascade, as well as inhibiting it, have been made in vitro using the chemo-invasion assay. Inhibition of integrin mediated cell adhesion and migration, as well as the use of metalloproteinase inhibitors, targets both metastatic and endothelial cells. One of the major integrins involved in endothelial cell adhesion and migration is αβ. Antibodies to this integrin are promising antiangiogenic agents and are in clinical trials. Protease inhibitors, developed as 'bullets' directed to the tumor cells, proved to be very effective inhibitors of tumor associated neo-vascularization.

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 Ingram yielded a novel class of potentially useful anti-angiogenic factors,\(^6\) tumaglin and its derivatives (AGM-1470, chloroacetylecarbamoyl) which inhibit endothelial cell proliferation even in the presence of saturating levels of basic fibroblast growth factor (bFGF).\(^6\)

Angiostatin and endostatin, the most recent members of the anti-angiogenic drugs,\(^10,10^\) 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 sulphamate compounds have shown beneficial effects.\(^10^6\) 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,\(^13\) and they are good inhibitors of endothelial cell invasion in vitro.\(^26\) Differentiative agents, such as retinoic acids, are also capable of preventing tumor progression, modulating changes in cell morphology, adhesion, growth and invasion not only of tumor\(^29\) 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.\(^66\) Tumors are removed from the mice, snap-frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) 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 D’MEM at \(4^\circ\text{C}\). 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.\(^8\) 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 \(4^\circ\text{C}\), 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 ensure that the matrigel solution is applied evenly 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; 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 x 10^6 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.\(^\text{47,48,116,122}\) Alternatives to matrigel have also been applied,\(^\text{123}\) as have alternatives to Boyden chambers.\(^\text{41,59,115}\) 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.\(^\text{8,65,123}\)

An alternative to Boyden chambers and single filters are either the multiwell chambers or the transwell™ chambers, coated with matrigel.\(^\text{115}\) 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 fibroblasts, 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.\(^\text{5,54}\) 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, 5x10⁴ 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% CO₂, humidified atmosphere. The assays are photographed and monitored with an inverted microscope, a modification using MTT as a colorant has been recently described.\(^\text{119}\)

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.

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