The plasmin cascade and matrix metalloproteinases in non-small cell lung cancer

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There are several steps involved in the metastatic spread of cancer from the primary tumour to a secondary remote site. Firstly, the cancer cells have to escape from the primary site and then intravasate into the blood or lymphatic circulation. The cells must survive transportation in the circulation before extravasating at a distant site. These cells then need to establish themselves at the new site before growth and replication can occur to form a metastatic colony. During this time the malignant cell has to avoid destruction by the host immune system. Vital to the growth of both the primary and metastatic disease is the capacity of the tumour to induce breakdown of the extracellular matrix (ECM) and the formation of a blood supply through new blood vessel formation, a process known as angiogenesis.

The ECM is a framework of proteins and proteoglycans secreted by and surrounding stromal fibroblasts. It gives structural support to cells and plays a central role in cell adhesion, differentiation, proliferation, and migration. The ECM is separated from epithelial cells by a basement membrane which is made up of type IV collagen and creates a scaffold upon which heparan sulphate, laminin, and other components are arranged. The turnover of the ECM usually occurs slowly in mature tissues but is accelerated in wound healing, arthritic joint destruction, uterine involution, and malignancy. All benign disorders demonstrate a continuous basement membrane whereas a consistent finding in invasive tumours is penetration of the basement membrane. For penetration of the basement membrane and intravasation to occur there must be alteration of the cell-cell and cell-matrix attachment followed by a proteolytic alteration of the ECM and migration of the tumour cell through the modified matrix. Malignant cells demonstrate repeated attachment and release from the ECM as well as enhanced proteolysis and migration through matrix barriers. There are several families of enzymes capable of degrading the ECM including the serine proteases, cathepsins, and the matrix metalloproteinases. uPA consists of two polypeptide chains linked by a disulphide bridge. The C-terminal sequence contains the serine protease domain whilst the N-terminal sequence consists of a growth factor domain and a kringle. uPA expression is regulated by hormones, growth factors and cytokines. The conversion of single chain pro-uPA to active two chain uPA is catalysed by plasmin, coagulation factor XIIa, cathepsins B and L, and prostatic specific antigen. The growth factor domain of uPA binds to a urokinase receptor (uPAR), a protein attached to the cell membrane by a glycosyl phosphatidylinositol anchor. uPAR is capable of binding both pro-uPA and uPA. uPAR binding uPA increases plasmin activity at the cell surface. Plasmin bound to the cell surface is protected from inhibition by α2-antiplasmin and this helps to restrict proteolysis to this site.

The family of serine protease inhibitors (serpins) includes α2-antiplasmin and the plasminogen activator inhibitors PAI-1 and PAI-2. These inhibitors contain a reactive centre peptide loop that acts as a pseudo-substrate for proteases. The loop forms a 1:1 stable stoichiometric complex with the protease and inactivates it. After binding, PAI-2 is cleaved and inactivated. PAI-1 and PAI-2 can combine with the uPA/uPAR complex although at a slower rate than with uPA alone. This new complex inhibits ECM degradation. The uPA/uPAR/PAI complex is endocytosed and degraded when associated with a member of the low density lipoprotein receptor family. Matrix metalloproteinases and their inhibitors

The matrix metalloproteinases (MMPs) are a family of zinc atom dependent endopeptidases that are capable of digesting ECM and basement membrane components under physiological conditions. They all contain a catalytic domain that can degrade at least one component of the ECM. They also have a distinctive PRCGVPD sequence in the pro-enzyme domain that, in the latent state, has an unpaired cysteine residue that can occupy a coordination position on the zinc atom. The disruption of this sulphur-zinc “bond” activates the enzyme. Tissue inhibitors of metalloproteinases (TIMPs) are specific endogenous inhibitors of MMPs. MMP family members differ structurally from each other by the presence or absence of other domains that affect substrate specificity, inhibitor binding, and ECM binding. Most MMPs are secreted as latent pro-enzymes that require activation by the cleavage of an N-terminal sequence and are classically grouped by their substrate specificity.
Table 1 Matrix metalloproteinase (MMP) family

<table>
<thead>
<tr>
<th>Major subsets</th>
<th>Name</th>
<th>MMP no.</th>
<th>Major substrates</th>
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<tr>
<td>Classic matrix metalloproteinase (MMP) members: Collagenases</td>
<td>Interstitial collagenase (fibroblast type)</td>
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<tr>
<td>Stromelysins</td>
<td>Stromelysin-1</td>
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<td>Laminin, fibronectin, proteoglycans, type IV collagens</td>
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<tr>
<td></td>
<td>Stromelysin-2</td>
<td>MMP-10</td>
<td>Types I and IV collagens, gelatin</td>
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<td></td>
<td>Matrilysin</td>
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<td>Gelatinases</td>
<td>Gelatinase A</td>
<td>MMP-2</td>
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<td>Gelatinase B</td>
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<td>Elastases</td>
<td>Metalloelastase</td>
<td>MMP-12</td>
<td>Elastin</td>
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<tr>
<td></td>
<td>?</td>
<td>MMP-13</td>
<td>Elastin</td>
</tr>
<tr>
<td>Novel matrix metalloproteinase family members: RXKR secreted type</td>
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<td>MMP-11</td>
<td>Laminin, fibronectin, proteoglycans, alpha 1-antitrypsin</td>
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<td>RXKR membrane type</td>
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<td>MMP-14</td>
<td>Gelatinase A, fibroblast collagens, ECM glycoproteins, proteoglycans</td>
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<tr>
<td></td>
<td>MT2-MMP</td>
<td>MMP-15</td>
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<td>MT3-MMP</td>
<td>MMP-16</td>
<td>Gelatinase A</td>
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<td>MT4-MMP</td>
<td>MMP-17</td>
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Adapted from Stetler-Stevenson et al. and Chambers et al.

There is also a subgroup of MMPs that are cell membrane bound and a subgroup that are secreted in an activated form. These latter two subgroups contain a 10 amino acid furin-type protease recognition sequence RXKR. Membrane type 1-MMP (MT1-MMP) has a trans-membrane domain and is capable of activating progelatinase A in a manner similar to the uPA/uPAR system. Between them the MMPs are capable of degrading all the constituents of the ECM (table 1).

The transcription of most MMPs is regulated by a number of agents including growth factors, hormones, oncogene products, and tumour promoters. Induction is mediated by pro-angiogenic factors such as interleukin 1β (IL-1β), platelet-derived endothelial cell growth factor (PD-ECGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and tumour necrosis factor-alpha (TNFα) and is suppressed by transforming growth factor-beta (TGF-β). Regulation of MMP activity is also dependent on the activation of their secreted latent pro-enzymes. Two pathways for the in vivo activation of MMPs have been postulated. The plasmin cascade may activate gelatinase B and stromelysin-1 either via the uPAR at the cell surface or at a site distant to the place of secretion. Plasmin cleaves 84 N-terminal amino acids from latent stromelysin-1 to activate it and also 81 N-terminal amino acids from latent interstitial collagenase to form partially active collagenase which is made fully active when stromelysin-1 cleaves a further 15 amino acids from its C-terminal (fig 1). On the other hand, gelatinase A appears to be activated by a cell mediated pattern. The complex nature of the ECM means that remodelling will require the combined activity of several enzymes and the co-expression of MMPs may enhance ECM degradation and therefore increase the metastatic potential.

All members of the MMP family can be regulated by their endogenous inhibitors, the TIMPs. These inhibitors are secreted by many cells in culture including fibroblasts, endothelial cells, chondrocytes, and vascular smooth muscle. The rate of ECM turnover is regulated by the balance between activated MMP levels and free TIMP levels. This balance is important in both physiological and pathological states. Four TIMPs have been characterised, the core size proteins of which are all approximately 21 kDa and contain 12 cysteine residues. The N-terminal is inhibitory whilst the C-terminal has the binding specificity. TIMP-1 and TIMP-2 are secreted from cells and remain soluble whereas TIMP-3 binds to the ECM with high affinity.

TIMP-1 is a 28 kDa protein that forms a high affinity non-covalent 1:1 complex with all active MMPs but preferentially with activated interstitial collagenase, stromelysin-1 and gelatinase A. TIMP-1 also forms a complex with progelatinase A that blocks its activation by stromelysin. TIMP-2 preferentially forms a 1:1 complex with gelatinase A and its C-terminal will strongly bind progelatinase A. TIMP-3 is a 24 kDa protein with inhibitory activities against gelatinases A and B, collagenase, and stromelysin-1, similar to the action of TIMP-1. TIMP-4 is a 23 kDa protein that can bind the C-terminal of gelatinase A and also progelatinase A. The transcription of TIMPs is regulated by hormones, cytokines, anti-inflammatory agents, and tumour promoters.
Invasion, migration and metastases
Successful tumour invasion necessitates three
important steps. (1) The attachment of a cell to
the ECM must be weakened whilst a proteolytic
defect is formed in the ECM. (2) The
detached cell has to migrate through this
defect. (3) Malignant cells must either have
sufficient proteinase activity to break through
collagenous protein barriers or acquire proteinase
activity from host stromal cells. They may also
benefit from proteinases derived from immune
cells that infiltrate tumour tissue.
Serine proteinases are capable of activating
MMPs, allowing them to degrade the ECM.
They also directly degrade components of the
ECM. MMPs are the only known family of
enzymes that can denature and digest fibrillar
collagens. As the metastatic cascade requires
modification of the matrix it is proposed that
serine proteinases and MMPs play a significant
role.
Serine proteinases and MMPs are frequently
produced by stromal cells, possibly as a host
response to the tumour. In situ hybridisation
studies show higher levels of stromal MMPs
close to tumour cells with lower levels further
away.49 This suggests the presence of tumour
cell-derived factors that are diffusible. Stromal
expression of MMPs occurs particularly
around tumour groups at the advancing edge of
the tumour.46 As well as expression of MMPs in
fibroblasts, they are also expressed in the
endothelial cells of blood vessels adjacent to
tumour cells.40 41 The expression pattern of ser-
ine proteinases and MMPs varies between
tumours of similar and dissimilar tissue types.
When found at the surface of cancer cells they
may cause migration and invasion, in the vicin-
ity of endothelial cells they may be associated
with angiogenesis, and in the stroma and at the
surface of fibroblasts they can lead to stromal
remodelling.
The uPA system is capable of stimulating cell
migration and invasion.42 43 uPA, plasminogen,
uPAR, and PAI-1 are all upregulated during
normal cell migration with uPA and uPAR,
being concentrated at the leading edge.44 The
coopexpression of uPA, uPAR, and PAI-1 are
necessary for optimal invasion in cultured lung
cancer cells45 whilst PAI-1 has been shown to
reduce tumour invasion.45 The migration of
cells is decreased by PAI-1 independent of its
ability to inhibit plasminogen activation.46 uPAR
expression correlates with invasive activity47
whilst t-2-antiplasmin and PAI-2 inhibited invasion.49 50

The uPA/uPAR complex is localised at
adhesion sites51 where there are higher concen-
trations of integrins. This leads to uPAR/vitronectin
and uPAR/integrin interactions
concentrated at cell:cell and cell:stroma
contact sites. Proteolysis at these sites can lead to
the release of cells and allow invasion. PAI-1
can inhibit migration by inhibiting proteolysis
or inhibiting uPAR/vitronectin binding. PAI-1 may
also be pro-migratory if it protects
vitronectin from uPA destruction. PAI-1 may
therefore act for or against migration and cell
invasion dependent on its local expression and
concentration and those of other members of
the plasmin cascade.52 Additionally, uPAR is
involved in chemotaxis53 and can mediate a
mechanical force across the cell membrane to
the cytoskeleton.54 As well as its protease activ-
ity, the uPA/uPAR complex transduces signals
via intracellular tyrosine phosphorylation55 and
by induction of c-fos gene expression.56 The
binding of ligands to integrins leads to signal
transduction57 and, as uPAR is capable of bind-
ing vitronectin and various integrins, it may
initiate cell signalling and migration. The plas-
minogen system therefore has a major role in
invasion and migration.
uPA is usually absent from resting epithe-
lium but is upregulated by angiogenic factors
such as VEGF.58 Overexpression of uPA occurs
in non-small cell lung cancer59 60 as well as
other solid tumours.61 62 uPA transcripts are
present in a high percentage of lung cancers,
principally within the stromal cells (fibroblasts,
perivascular cells and inflammatory cells), but
also within a smaller percentage of the cancer
cells.57 uPA protein is detected mainly in the
stromal cells of lung cancers. PAI-1 is found in
endothelial cells in colon carcinoma64 and the
endothelial and cancer cells in breast
tumours.55
There is considerable evidence to suggest that
MMPs are actively involved in the
metastatic process. MMP concentrations relate
to metastatic potential in vitro65 whilst syn-
thetic MMP inhibitors like batimastat decrease
the spread of cancer in vivo.66 Transfection with
cDNA for gelatinase A, gelatinase B, and
MT1-MMP also increases metastasis in vari-
ous cancer cell lines.66 67 Overexpression of
TIMP-1 and TIMP-2 leads to decreased ang-
giogenesis, endothelial cell migration and
invasion68 71 and antisense downregulation
of TIMP-1 mRNA increases invasion.72
High levels of MMP expression are found in
non-small cell carcinoma73–78 and in many
malignant tissue types including breast,79
ovarian,80 prostatic,81 gastric,82 colonic,83
thyroid,84 and squamous cell carcinomas of the
head and neck.75 No single MMP is overex-
pressed in all tumour cells nor consistently
overexpressed in every tumour of a single
histological classification. The number of
MMP family members detected tends to
increase with increased tumour stage and the
levels of any individual MMP tends to be
higher the more advanced the tumour stage.
Stromal expression of MMPs and serum
proteinases predominates over the cancer cell
cancer cell expression in a pattern seen with many types of
cancer.79 82 84 “This suggests a supportive or
pro-active role for the stroma in cancer, probably as a host response to the tumour.
The ratio of active gelatinase A to latent pro-
gelatinase A increases as the tumour grade
increases in breast cancer.85 The activation rate
of progelatinase A is significantly higher in
advanced breast cancer with lymph node
spread.86 The presence of progelatinase A may
mark the malignant potential of a cell. Experi-
ments in human lung carcinoma cell lines show
an infiltrating capacity that correlates with a
high expression of gelatinase A mRNA.87
Increasing levels of gelatinase A are found as
lesions progress from dysplasia through carcinoma in situ to frankly invasive carcinoma of breast and prostate. Although gelatinase A is the most commonly expressed MMP in non-small cell lung cancer, further studies are required to see if levels of gelatinase A expression increase as dysplasia changes to carcinoma. Gelatinase A is mainly expressed in stromal cells closest to cancer cells but also some tumour cells in the peripheral regions of cancer colonies. No difference has been found in the levels of gelatinase A and the histological subtype, but the level of gelatinase A is associated with the invasive behaviour and metastatic potential of most histological types of lung neoplasm.

The expression of gelatinase A can frequently be superimposed on to that of MT1-MMP. This suggests that the transcription of these two genes may be coordinated and that MT1-MMP then activates progelatinase A allowing ECM degradation. No association between gelatinase A and MT1-MMP in normal lung parenchyma has been found. The correlation of MT1-MMP with gelatinase A activation in pulmonary carcinomas again suggests that MT1-MMP is a tumour specific activator of progelatinase A. MT1-MMP mRNA and its protein are present in the stroma of adenocarcinoma and both the stroma and tumour cells of squamous cell cancer. Activation of gelatinase A involves binding of a progelatinase A/TIMP-2 complex to a cell surface receptor followed by cleavage by MT1-MMP. This restricts gelatinase A activity to the pericellular region of stromal matrix degrading enzymes, limiting gelatinase A activity to be directed towards the invasive edge of breast and colon cancers.

Collagenase-1 and stromelysin-1 and stromelysin-3 have been identified in non-small cell lung cancer but not in non-neoplastic lung. Stromelysin-1 transcripts are detected especially in squamous cell lung carcinoma, again more commonly in fibroblasts. Stromelysin-3 has been described in bronchial epithelial cells in vitro and ex vivo wound repair models. It is expressed in bronchial dysplasia and in situ carcinomas which have an increased likelihood of developing invasive squamous cell carcinoma. This suggests that induction of stromelysin-3 in fibroblasts may be an early event in malignant transformation. Stromelysin-3 and uPA are frequently co-expressed, suggesting cooperation in ECM breakdown with stromelysin-3 degrading α1-antitrypsin allowing uPA and the other serum proteases to degrade the matrix.

Matrilysins differ from the other MMPs because its expression is in epithelial rather than mesenchymal-derived cells. Matrilysins have been found in both benign and malignant stages of many adenocarcinomas with increased levels in the malignant tissue. Enhancement of matrilysin expression in tumour cell lines increases invasive potential and tumour formation when inoculated into immunosuppressed host animals, and there is a significant reduction in the number and size of intestinal adenomas in matrilysin-null mice compared with controls. This may suggest that matrilysin is responsible for the aggressive behaviour of tumours, especially in the early stage. Matrilysins are expressed frequently in non-small cell lung cancer, mainly in the cytoplasm of cancer cells but also in fibroblasts. High levels of matrilysin mRNA are found in squamous and adenocarcinoma whilst low levels are found in non-neoplastic lung tissue. One report favours expression in adenocarcinoma over squamous cells. Matrilysins positive tumour cells are more common in the peripheral areas of the tumour than in the centre, suggesting a possible role in tumour cell invasion of the ECM and angiogenesis. Not surprisingly, low TIMP expression correlates with increased invasiveness in various murine and human cell lines but not all malignancies have raised MMP activity with decreased TIMP activity. In several reports raised levels of TIMPs have been found in malignant tissue. TIMP-1 expression is associated with the progression of colorectal tumours from adenoma to invasive adenocarcinoma. Rat embryo fibroblasts
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transfected to overexpress TIMP-2 have a reduced in vivo growth rate and decreased local invasion after subcutaneous injection into nude mice. They also show decreased lung colonisation when injected intravenously.171 Spontaneous loss of the metastatic phenotype in a human epidermoid carcinoma line is accompanied by an increase in TIMP-2 expression.175 TIMP-1, TIMP-2 and TIMP-3 are present in both tumour and non-neoplastic lung. The frequency of TIMP-1 and TIMP-2 expression is significantly higher in non-neoplastic lung whereas TIMP-3 expression tends to increase with malignancy.99 TIMP-1 and TIMP-2 mRNA is found mainly in the stroma surrounding tumour cells, especially in well differentiated tumours. TIMP-3 occurs primarily in stromal cells and occasionally in adenocarcinoma cells. The maximal expression of TIMP-3 in colorectal adenocarcinomas occurs in the stroma associated with the invasive edge of moderately and poorly differentiated tumours.170 This pattern of expression is interesting as TIMP-3 has been shown to be anti-angiogenic.110

Growth

Plasmin and uPA are involved in the activation of specific growth factors including hFGF,111 hepatocyte growth factor,112 and TGF-β.113 TGF-β activation increases expression of PAIs115 resulting in a negative feedback loop. The classical view of MMPs and their involvement in malignancy is their role in intravasation and extravasation via their proteolytic effect on physical barriers. Mounting evidence shows that MMPs can induce growth and tumorigenicity in various cell types. Transfection of cDNA for matrilysin into colonic carcinoma cells decreases fibroblast collagenase production.124 The classical view of MMPs and their involvement in malignancy is their role in intravasation and extravasation via their proteolytic effect on physical barriers. Mounting evidence shows that MMPs can induce growth and tumorigenicity in various cell types. Transfection of cDNA for matrilysin into colonic carcinoma cells decreases fibroblast collagenase production.124

overexpression of TIMP-1, TIMP-2, and TIMP-3 genes leads to overproduction of their corresponding proteins and to inhibition of melanoma cell invasion.177 TIMP-1 and TIMP-2 act solely by MMP inhibition, whilst the effect of TIMP-3 is more potent. At 24 hours raised TIMP-3 expression leads to a marked decrease in invasion and cell adhesion. At 72 hours there is an increase in apoptosis and a decrease in viable cells.127 An increase in programmed cell death by TIMP-3 also occurs in vascular smooth muscle128 and in colon carcinoma cells.129 130 This may be due in part to stabilisation of TNF-α receptors on the cell surface. Apoptosis was neither caused by TIMP-1 or TIMP-2 nor by synthetic MMP inhibition, which indicates that MMP inhibition alone does not lead to apoptosis.125 It appears that loss of cell adhesion to the ECM may be the important factor. Recent evidence shows that overexpression of TIMP-2 leads to decreased apoptosis but increased necrosis, which suggests that it too plays a part in tumour cell survival.69

PAI-2 is found in both cytosolic and secreted forms. The cytosolic form of PAI-2 is probably involved in apoptosis independent of its ability to inhibit uPA. PAI-2 is similar in form to CrmA (a viral serpin)131 and CrmA can inhibit interleukin-1β converting enzyme (ICE), a potent inducer of apoptosis.132 This finding suggests that PAI-2 may inhibit apoptosis. The balance between proliferation and apoptosis defines whether a tumour grows or remains dormant. If the major role of PAI-2 is to inhibit apoptosis, its expression in a tumour would tip the balance in favour of proliferation and tumour growth. The addition of recombinant PAI-2 leads to a decrease in growth of uPA producing tumours in SCID mice.142 Melanoma cell lines overexpressing PAI-2 injected into SCID mice develop fewer metastases than PAI-2 negative controls.143 In these studies inhibition of the plasmin cascade and invasion appears more important than the ability to inhibit apoptosis.

Apoptosis

Apoptosis (programmed cell death) is suppressed in the presence of an intact ECM basement membrane.125 Increased stromelysin-1 expression leads to apoptosis and this can be blocked by MMP inhibition.119 MMPs may therefore be involved in apoptosis by their ability to degrade the ECM. p53 is a promoter of apoptosis, a regulator of the cell cycle, and a tumour suppressor gene. The promoter region for the gene encoding gelatinase A contains a p53 binding site and is subject to regulation by wild-type but not mutant p53 in vivo and in vitro.120 In the absence of the p53 binding site, p53 leads to repression of the gelatinase A promoter. This links p53, MMPs, and apoptosis. Perhaps in carcinogenesis mutant p53 has no regulatory control over gelatinase A.

Adenovirus mediated transfer of TIMP-1, TIMP-2, and TIMP-3 act solely by MMP inhibition, whilst the effect of TIMP-3 is more potent. At 24 hours raised TIMP-3 expression leads to a marked decrease in invasion and cell adhesion. At 72 hours there is an increase in apoptosis and a decrease in viable cells.127 An increase in programmed cell death by TIMP-3 also occurs in vascular smooth muscle128 and in colon carcinoma cells.129 130 This may be due in part to stabilisation of TNF-α receptors on the cell surface. Apoptosis was neither caused by TIMP-1 or TIMP-2 nor by synthetic MMP inhibition, which indicates that MMP inhibition alone does not lead to apoptosis.125 It appears that loss of cell adhesion to the ECM may be the important factor. Recent evidence shows that overexpression of TIMP-2 leads to decreased apoptosis but increased necrosis, which suggests that it too plays a part in tumour cell survival.69

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Angiogenesis
The process of angiogenesis has three steps that are similar to those involved in invasion: proliferation of endothelial cells, the breakdown of the ECM, and endothelial cell migration. The level of angiogenesis is a well recognised prognostic indicator in many tumour types, including non-small cell lung carcinoma. The quantity of vessels increases as bronchial epithelium progresses from normal tissue through to carcinoma in situ. The association between angiogenesis and growth factors in non-small cell lung cancer has been studied demonstrating relationships with VEGF, bFGF, and PD-ECGF. This correlates with the pro-angiogenic properties of these growth factors. A strong inverse relationship between bcl-2 and angiogenesis has also been found. As normal lung epithelium expresses bcl-2, this suggests that loss of bcl-2 activity may allow angiogenesis to occur. It is logical that proteases play an important role in angiogenesis. The levels of uPA in breast cancer correlate with vascular grade and this will probably be the case in non-small cell lung cancer. The expression of gelatinase A and B in endothelial cells is also important in angiogenesis. Gene targeting has led to the development of gelatinase A-deficient mice that develop normally. Gelatinase A-deficient mice show decreased angiogenesis compared with normal mice when injected with B16-BL6 melanoma cells and a slower growth and decreased colonies in those injected with Lewis lung carcinoma cells. B16-BL6 melanoma cells do not express gelatinase A in vitro, so the presence of gelatinase A activity in these tumours in the normal mice but not in the gelatinase A-deficient mice suggests that this has derived from the host tissue. Lewis lung carcinoma cells can produce gelatinase A yet they still demonstrate poorer growth in the gelatinase A-deficient mice. Host produced gelatinase A must therefore play a critical role in tumour invasion and angiogenesis. As there is only partial suppression of angiogenesis in gelatinase A-deficient mice, presumably the other proteases partly compensate for the lack of this MMP. Gelatinase A leads to endothelial cell tube formation on matrigel and this is blocked by TIMP-1 and TIMP-2, yet TIMP-1 is capable of promoting endothelial cell proliferation. Angiogenesis is decreased by the TIMP-2 inhibition of MMPs and uPAR antagonists are also capable of decreasing angiogenesis.

A major event in angiogenesis is stimulation of ECM modelling. bFGF released into the ECM is often bound to the heparan sulphate proteoglycans on laminin, fibronectin, and collagen. ECM damage or proteolysis will therefore release bFGF which is proangiogenic and upregulates uPA, PAI-1, and gelatinase expression and represses TIMP-2 expression. This may be important in physiological wound healing and in tumours with raised MMP levels. MMPs also process precursors into bioactive forms. Gelatinase A (but not gelatinase B) is capable of cleaving the ectodomain of fibroblast growth factor receptor 1 (FGFR-1) and therefore of modulating the angiogenic activity of bFGF. MMPs also act on TNF-α and its receptor. VEGF induces the expression of interstitial collagenase at the exclusion of other MMPs and interstitial collagenase is an absolute requirement for angiogenesis. The addition of VEGF to dermal microvascular endothelial cells increases gelatinase A levels and decreases gelatinase A inhibition by decreasing TIMP-2 levels. Although interstitial collagenase levels remained unchanged, there was decreased inhibition as TIMP-1 levels were reduced. These changes in respective levels of MMPs and their inhibitors could contribute to the role played by VEGF in angiogenesis.

MMPs are traditionally thought to be pro-angiogenic whilst TIMPs are antagonistic. There is evidence that the plasmin cascade and MMP activity may also have an anti-angiogenic component. Gelatinase B, matrilysin, and a macrophage metalloelastase hydrolyse plasminogen to angiostatin, a potent inhibitor of angiogenesis. Endostatin is a similar anti-angiogenic substance formed by collagen type XVIII proteolysis. Kringle 5 of human plasminogen also selectively inhibits endothelial migration. These anti-angiogenic breakdown products may serve as a negative feedback loop. Angiostatin can be produced by the primary tumour and must pass through the circulation as it can be isolated from urine. Excision of the primary tumour can remove this inhibitory effect and lead to rapid growth of previously dormant metastases which is a well-recognised observation.

The fumagillin analogue TNP-470 inhibits the proliferation of growth factor stimulated endothelial cells and inhibits bFGF induced tubule formation. Lung metastases in mice remain dormant under suppression of angiogenesis by TNP-470. Removal of suppression leads to rapid growth of the micrometastases. Interestingly, whilst the rate of proliferation of cells is equal in dormant and growing metastases, the rate of apoptosis in dormant metastases is three times that of growing metastases. This suggests that inhibitors of angiogenesis may control the growth of metastases by indirectly increasing apoptosis, presumably by starving the tumour of nutrients and oxygen.

Prognosis
The best predictor for outcome in non-small cell lung cancer is the International Staging System. Subjects with the same stage of disease can have markedly different rates of disease progression. This finding suggests that these tumours, despite having the same histological subtype, are biologically different. Immunohistochemical investigation may reveal other prognostic markers and may suggest subgroups that could benefit from adjuvant therapy after surgical resection. Raised levels of uPA have been found in many malignant tissue types and this confers a poor prognostic significance in breast, bladder, gastric, colonic, and cervical cancer.
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Associated with a poor prognosis but ELISA shown that uPA in non-small cell lung cancer is present in lung cancer with lower levels being present in node negative breast cancer. Immunochemical studies have shown that uPA in non-small cell lung cancer is associated with a poor prognosis but ELISA studies have not shown a prognostic role for uPA.

The presence of uPAR correlates with decreased survival in squamous cell carcinoma. uPAR is also associated with a poor prognosis in colon and breast cancer.

Raised levels of PAI-1 expression is associated with a poor outcome in lung adenocarcinoma. Raised levels of PAI-1 suggest a more aggressive phenotype and give a poor prognosis.

Significantly higher mRNA levels of both gelatinase A and gelatinase B are found in stage III non-small cell lung cancer than in stages I and II, with no difference in histological subtype. This association with tumour grade also occurs in breast and colorectal tumours. MT1-MMP is expressed significantly more frequently in stage III non-small cell lung cancer than in stages I and II and increased MT1-MMP mRNA correlates with the presence of lymph node secondaries in lung tumours. Higher numbers of stromelysin-3 positive fibroblasts and the co-expression of stromelysin-1, stromelysin-3, and uPA correlate with increased tumour size and with lymph node spread.

TIMP-3 is more frequently expressed in stage III non-small cell lung cancer, along with many MMPs, whereas TIMP-1 and TIMP-2 show an early substantial (although non-significant) decline as the tumour stage increases. The decrease in TIMP-1 and TIMP-2 as the stage progresses gives support to the theory that the metastatic potential is dependent on the balance between MMP and TIMP activity. This is at odds with other studies showing that a high TIMP-1 expression is associated with a poor prognosis in non-small cell lung cancer, and that increased stromelysin-3 and TIMP-2 expression occurs in poorly differentiated squamous cell carcinoma and in those with nodal metastases. In both breast and bladder cancer raised TIMP-1 mRNA and TIMP-2 protein are associated with a poor prognosis.

There is raised TIMP-1 expression in colorectal cancers with secondary spread and TIMP-1 expression correlates with the more aggressive phenotype in bladder cancer. Raised levels of TIMPs in malignancy may occur as a response to raised levels of MMPs. The ability of TIMPs to stimulate cell proliferation may account for raised levels being present in advanced disease.

**Therapy**

Synthetic inhibitors of metalloproteinases have been developed and are undergoing clinical trials. Batimastat (BB94) is a hydroxamine analogue that gives widespread inhibition of MMPs. Batimastat decreases in vitro ECM degradation and invasion of endothelial cells through an artificial membrane. It decreases the metastatic spread of murine melanoma and human mammary carcinoma cells in mice. The effects of MMP inhibitors on growth in vivo may be related to their effects on tumour angiogenesis. MMP inhibitors block angiogenesis assayed in chick and rat models of neovascularisation. Batimastat has low oral bioavailability but has been used in clinical trials to treat malignant ascites and pleural effusions. Malignant pleural effusions contain high concentrations of gelatinases A and B so metalloproteinase inhibitors appear to offer a new way of approaching this common problem.

Marimastat (BB-2516) is an orally active synthetic MMP inhibitor that decreases lung and mammary cancer growth in animal models. It has been used in phase 1, 2 and 3 studies leading to a fall in the rate of rise of tumour markers in a dose dependent manner.

CT-1746 is a gelatinase selective MMP inhibitor that reduces tumour spread and metastasis, converting aggressive colonic cancer into a more indolent disease in a nude mice model. It has been used in conjunction with cyclophosphamide to inhibit the growth and metastatic spread of Lewis lung carcinoma in mice. Together they have a greater effect than when given separately. D-penicillamine can inhibit the activation of prometalloproteinases and offers another way of decreasing MMP activity.

Angiostatin and endostatin are capable of decreasing the growth of primary tumours and metastases in vivo. After treatment is stopped the tumours grow again but repeated therapy can lead to dormancy without the development of drug resistance.

Tumour cells and endothelial cells can stimulate the growth of each other. Combination treatment regimes with traditional cytotoxic chemotherapy and anti-angiogenic drugs could affect both of these distinct cell populations. Long term treatment with MMP inhibitors may be used in patients with low or non-existent metastatic disease. Inhibition of MMP expression by antisense techniques may also have a clinical role. The pharmaceutical use of TIMPs may not be as straightforward as they lack bioavailability and may stimulate cell growth. Trials of these agents will evaluate whether our increased knowledge of the molecular biology of malignancy can be put into clinical practice.
Conclusions

The plasmin cascade, MMPs, and TIMPs are involved in invasion, migration, growth and angiogenesis and have a putative role in apoptosis. The prognostic significance of proteinase expression and association with angiogenesis (a recognised prognostic marker in its own rights) needs to be evaluated further. Immunostaining of tissue from surgical resections of non-small cell lung cancer and assessing prognostic markers may highlight a subgroup of patients who could benefit from adjuvant chemotherapy or radiotherapy. The use of synthetic proteinase inhibitors may lead to a new approach in the management of non-small cell lung cancer. Further study in conjunction with established cytotoxic agents. Further research into the molecular biology of lung cancer may highlight other pathways that can be manipulated for clinical gain.

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