Enzymatic function of multiple origins regulates the progression of colorectal cancer and the development of metastases

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Abstract

Enzymes play a crucial role in the progression of colorectal cancer and the development of metastases. They facilitate malignant cell invasion through the degradation of the extracellular matrix, the rupture of the basement membrane and the derangement of cell-cell adhesion. Furthermore, they promote tumour cell migration and support the evolution of metastatic lesions in the liver and other organs, through multiple molecular mechanisms, including growth factor release and angiogenesis. Urokinase plasminogen activator system, matrix metalloproteinases, heparanase and autocrine motility factor constitute important enzymatic complexes which assist colorectal cancer growth, with potential clinical applications in the diagnosis and treatment of the disease. Hippokratia 2009; 13 (1): 23-31

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Colorectal cancer is one of the commonest malignant neoplasms worldwide, with over 1 million new cases and around half a million cancer-related deaths reported in 2000. It is considered the second most lethal cancer type in the developed world and the main cause of death is the development of liver metastases. Most patients who suffer from the disease, succumb to the effects of distant metastatic lesions rather than the primary carcinoma itself.

The invasion of foreign tissue by malignant cells involves their interaction with the extracellular matrix (ECM) and the basement membranes. The latter in particular, create connective tissue barriers at various steps of the metastatic process: escape from the primary tumour site, intravasation, extravasation, muscular and perineural infiltration. Basement membranes consist of collagen-type IV meshwork, laminin and heparan sulphate proteoglycans (HSPGs) and do not present pores, which would facilitate cell migration. Thus, tumour cells need to degrade these component constituents, in order to promote metastasis; then they are able to reach local vessels, intravasate and create new colonies in distant tissues.

The proteolytic activity of malignant cells involves four classes of proteases: serine-, thiol-, aspartylproteases and metalloproteinases. In addition to enhanced protease activity, high heparanase levels also correlate with tumour aggressiveness.

High motility of malignant cells is another necessary feature in the tissue invasion and it appears to be promoted by a variety of compounds such as host and tumour secreted factors, ECM components, growth factors and hyaluronan. Motility stimulated by all these components may be defined as chemokinesis (random cell movements), chemotaxis (directed cell movements in response to concentration gradients of soluble compounds) and aptotaxis (directed cell migration towards insoluble ECM proteins).

Urokinase plasminogen activator (uPA) system

The uPA system consists of the urokinase-type plasminogen activator (uPA), its receptor (uPAR), the tissue-type plasminogen activator (tPA), the plasminogen (Plg) and plasminogen activator inhibitors 1 and 2 (PAI-1 and PAI-2). This biological system is implicated in multiple cell activities, including cytokine release, protease expression, adhesion, chemotaxis, proliferation and neutrophil activation for oxidant production. Consequently, it modulates growth, invasion, inflammation, angiogenesis and metastasis of multiple tumour types.

uPA is a serine protease, which is secreted as an inactive single-chain zymogen named pro-uPA. When bound to uPAR, pro-uPA is cleaved by plasmin into active double-chain uPA that converts plasminogen into plasmin. The latter degrades the extracellular matrix and the basement membrane, acting either directly or through the activation of other proteases, such as pro-metalloproteinases and pro-collagenases. This sequence of actions eventually results in cancer invasion and progression. After hepatic resection, due to colorectal cancer metastases, uPA activity was found to be increased in the remnant parenchyma, leading to liver regeneration.
through ECM production and hepatocyte proliferation. A 50% resection was observed to be the threshold for intense uPA activity. uPAR is a cysteine rich 50-60kDa extracellular glycoprotein without any transmembrane domain. Normal tissues present little or no uPAR, although exceptions occur: The receptor is expressed in haematopoietic cells, including monocytes, neutrophils, eosinophils, T-lymphocytes, natural killer cells, dendritic cells; also in smooth muscle cells, hepatocytes, fibroblasts and placental cells. It is also over-expressed in various tumour cells, including colon cancer, liver, breast, lung, stomach, ovary, prostate and bone; additionally in several tumour assisting cells, such as endothelial cells, macrophages and fibroblasts.

A cohort study including 354 Swedish and 255 Danish patients with colorectal cancer, evaluated preoperative plasma levels of soluble uPAR. It was discovered by ELISA that the receptor’s concentration was significantly higher in Dukes’ D patients. The study concluded that uPAR preoperative measurement could be a prognostic parameter, aiding clinical decisions on effective treatment. Another study on 100 colorectal carcinoma patients, reported that high antigen levels of uPAR in tumour tissue correlated with liver metastases and involvement of lymph nodes. Similarly, a Danish study, including 591 patients with colorectal cancer, evaluated preoperatively soluble uPAR and indicated a substantial increase in patients with high mortality. It was concluded that uPAR blood test independently predicted survival. Nowadays gene therapy is practiced towards down regulation of uPAR expression in various types of tumours.

PAIs are anti-proteases that inhibit uPA and tPA. Both PAI-1 and PAI-2 belong to the serine protease inhibitor superfamily and are involved in cancer growth and metastasis. PAI-1 is the major inhibitor of the uPA system, is synthesised primarily in endothelial cells, modulates the fibrinolytic activity in the vasculature and is widely expressed throughout tumours. PAI-2 is presented in the epidermis, monocytes and macrophages, as well as in the plasma and the placenta of pregnant women. Apart from anti-protease activity, PAI-2 presents anti-viral activity.

Initially, it was expected that PAI-1 would prevent tumour growth through the inhibition of uPA. However, multiple research studies associated this molecule with poor prognosis of multiple types of cancer, increased cancer invasion and neoangiogenesis. Also, experiments where this inhibitor was absent or blocked showed that tumour growth and invasion was reduced. Bajou et al demonstrated in vivo and in vitro that PAI-1 played a key role in tumour progression, controlled proteolysis and regulated cell migration during angiogenesis. When host cells expressed the inhibitor in normal concentration, angiogenesis was promoted. On the contrary, in the case of host absence of PAI-1, the production by tumour cells could not induce angiogenesis. Similarly, in vitro studies with smooth muscle cells concluded that PAI-1 inhibited cell attachment. Upon administrating a PAI inhibitor, smooth muscle cell adhesion was restored and angiogenesis was reduced in vivo. A clinical study on 206 patients with colorectal cancer, evaluated the association of PAI-1 genotypes with the disease prognosis. It was concluded that there was an increase of 4G/4G genotype in Dukes’ C and D cancer, although not statistically significant.

The ability of PAI-1 to modify cell adhesion, regulating attachment and detachment, has been suggested in order to explain its tumour promoting role. This unexpected action appears a significant observation, which is not related to its role as a protease inhibitor. Specifically, PAI-1 can detach cells from fibronectin and type I collagen. This detachment presupposes the formation of uPA and uPAR complexes on the cell surface and their binding to matrix-engaged integrins. The subsequent binding of PAI-1 to uPA and uPAR promotes the disengagement of integrins from the ECM, thus causing cell detachment. The de-adhesive properties of this inhibitor may be regulated by cytokines, growth factors and hormones, which remain to be precisely investigated.

Figure 1: The role of urokinase-type plasminogen activator (uPA), uPA receptor (uPAR) and plasmin in the metastatic process. Regulation of extracellular matrix (ECM) degradation, neoangiogenesis, invasion and metastasis by uPA, uPAR and plasmin.

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Matrix metalloproteinases (MMPs)

The collagenous structure of the extracellular matrix and basement membrane, as well as the dynamic balance between fibrogenesis and fibrolysis, are very important for cancer progression. MMPs are molecules which control these procedures and are implicated in cancer cell migration. They compose a family of zinc endopeptidases, which degrade extracellular matrix, but also non-matrix proteins. MMPs include more than 24 enzymes, with epilysin (MMP-28) being the newest member of the family (Figure 2).

Their structure, activity and substrate preferences vary considerably and are classified into numerous separate subgroups (Table 1). Structurally, MMPs present three common domains: a pre domain (N-terminal signal sequence), a catalytic domain (N-terminal propeptide) and a hemopexin-like domain (C-terminal Hpx). The catalytic domain contains cysteine with the Zn ion and keeps pro-MMP inactive. Metalloproteinases get activated, when the propeptide is removed by another MMP or other protease.

Their actions are numerous. They contribute to the wound healing process, morphogenesis and tissue remodelling after injury, like acute myocardial infarction. Additionally, they play a key role in rheumatoid arthritis, neural diseases and cancer growth. Their ability to degrade the ECM and the basement membrane is frequently utilized by tumour cells to invade the tissues.

The degradative ability of MMPs also facilitates cancer evolution via growth factor release and/or angiogenesis. As they produce ECM-fragments, growth factors such as tumour necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β) and insulin like growth factor (IGF) may be activated leading to tumour progression; concurrently, these factors or the ECM degradation itself, may directly or indirectly promote angiogenesis. Although, multiple ECM fragments like tumstatin, MMP fragments such as the hemepxin like domain named PEX and plasminogen fragments like angiostatin may inhibit tumour angiogenesis through various mechanisms, including restriction of endothelial cell proliferation or migration.

Cell adhesion plays a crucial role in malignant progression. Specifically, cadherins and catenins promote cell anchorage and any modification in their status may facilitate cell migration. Moreover, several studies have presented a functional link between E-cadherin and MMPs, where E-cadherin suppresses the activity of these proteinases in vivo and in vitro conditions. Miyaki et al using murine experimental models reported that COKFu colon carcinoma cell lines without the expression of E-cadherin showed very low or no activity of a 62kDa gelatinase and subsequent decreased invasion, in comparison with highly E-cadherin-expressing cells. They concluded that E-cadherin not only is involved in cell-cell adhesion, but also regulates proteinase secretion, suppressing tumour invasion and growth.

ECM includes various types of collagen, but the most common is collagen type IV. Therefore, MMP-2, -9 and -11, which regulate this type of collagen, have been studied for clinical purposes. However, the results were not clear; high levels were recorded in patients with various adenocarcinomas, with or without liver metastases, as well as in patients with liver metastases and resected primary tumour. In this last group, increased expression of MMP-2 was attributed to previous stimulation of stromal...
Metalloproteinase functions are regulated by endogenous inhibitors such as α2-macroglobulin and specific inhibitors, named tissue inhibitors of metalloproteinases (TIMPs). Pathological conditions occur, when MMPs and TIMPs act independently. Synthesis of MMP inhibitors is an ambitious target for cancer treatment, though with dismal outcomes. Eighty two percent of numerous synthesized matrix metalloproteinase inhibitors (MMPIs) had no clinically significant effect, seven remain in clinical trials and only one (Periostat) has been approved for clinical use by the FDA.

Asano et al investigated the expression of multiple MMPs and their inhibitors in 112 colorectal cancer tissue samples and 11 metastatic liver lesions. They indicated a significant higher expression of MMP-1, -3, -7, -9, -10 and -11 in the tumours in comparison with normal tissue. In the metastatic foci they demonstrated lower levels of MMP-1 and -11 and higher of TIMP-1 comparing with primary cancer. They also announced that the lower expression of MMP-15 was an important risk factor for early recurrence of the disease.

Heparanase

Heparan sulphate proteoglycans (HSPGs) are macromolecules, which consist of a protein core linked with...
heparin sulphate chains. They can be traced at the cytoplasm, the cell surface or may be secreted into the ECM\textsuperscript{36}. Heparan sulphate (HS) chains present an extensive structural heterogeneity and they are considered the most “information dense” biopolymer in nature\textsuperscript{37}. They may interact with a surprising multiplicity of molecules (>100), such as ECM proteins, growth factors, chemokines, cytokines, morphogens, coagulation factors, enzymes and their inhibitors (Table 2)\textsuperscript{38,39}. The biodiversity of HS and the variety of core proteins are responsible for the multiple biological functions of HSPGs. These macromolecules regulate the stability and insolubility of ECM and therefore contribute to cell adhesion and locomotion.

Furthermore, they are present in blood vessels and capillaries interfering in angiogenesis. In general, they control intracellular and extracellular responses and stand at the intersection of numerous signalling pathways\textsuperscript{38,40}.

Various enzymes may transform the HSPG structure and thus regulate their function: sheddases cleave the proteoglycans from the cell surface, heparanase degrades HS into biologically active fragments and endosulfatases specifically remove 6-O sulphates from HS chains\textsuperscript{36}. The most studied and interesting of these enzymes in cancer research remains heparanase.

Heparanase is an endo-β-D-glucuronidase, rare in normal tissue, but frequently expressed in highly metastatic tumours. This enzyme cleaves HS at specific sites, creating fragments of considerable size (5 to 10 kD, 10 to 20 sugar units long), which may be more biologically active than their ancestor heparin sulphate chains\textsuperscript{36,39}.

By cleaving the HS chains, heparan degrades HSPGs and subsequently ECM, facilitating tumour cell locomotion, invasion and metastasis. Additionally, the enzyme releases factors, which are bound with HS chains, mediating cell behaviour. Angiogenic growth factors such as fibroblast growth factor 2 (FGF-2) and vascular endothelial growth factor (VEGF) constitute apparent examples of trapped molecules in the ECM, which are released and activated through this enzymatic action\textsuperscript{41}.

Heparan’s increased expression is associated with a high metastatic capacity of various tumour cell lines and tissues including brain, oral cavity, nasopharynx, thyroid, oesophagus, stomach, gall bladder, pancreas, ovary, endometrium, bladder, prostate, multiple myeloma and acute myeloid leukaemia, liver and colon\textsuperscript{39}. Various proposed mechanisms attempt to explain how heparan assists the metastatic process. Degradation of HS chains destroys natural biological barriers and facilitates tumour migration and invasion. Also, the release of HS-bound growth factors, involved in angiogenesis, such as basic fibroblast growth factor (bFGF) and VEGF may assist the creation of neovessels, which supply growing tumours or metastatic foci\textsuperscript{39,42}. The upregulation of cyclooxygenase-2 through heparanase action promotes neangiogenesis in oesophageal cancer\textsuperscript{43}, invasive breast cancer\textsuperscript{44} and sigmoid-ring cell stomach cancer\textsuperscript{45}. Furthermore, the enhancement of syndecan-1 synthesis and shedding from the cell surface, may constitute another mechanism which induces metastasis, as this proteoglycan promotes growth and angiogenesis of tumours\textsuperscript{36,46}.

Heparanase is related to colon cancer progression and metastasis. Friedmann et al studied the expression of heparanase in 16 patients with colon adenocarcinoma and concluded that the most poorly differentiated carcinomas showed the highest expression of the enzyme; high expression was also noted in lung, lymph node, liver metastases, as well as in the accompanying stromal fibroblasts. In contrast, heparanase expression was low in normal tissue\textsuperscript{46}. Immunohistochemical studies by Elkin et al on human colon adenocarcinoma tissue revealed that

| Table 2: Heparan sulphate binding interactions\textsuperscript{38,40}. |
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| **Group** | **Molecules** |
| Matrix components | • Fibronectin<br>• Thrombospondin<br>• Laminin<br>• Vitronectin<br>• Fibrillar collagens |
| Enzymes | • SOD<br>• LPL<br>• Proteinases |
| Protease inhibitors | • PN-1<br>• HCII (Serpin peptidase inhibitor D1)<br>• Antithrombin III<br>• Protein C Inhibitor<br>• MPI |
| Growth factors | • FGF<br>• HGF<br>• VEGF<br>• TGF-β<br>• Amphiregulin<br>• HB-EGF |
| Miscellaneous | • Apolipoproteins<br>• NCAM<br>• Viral proteins<br>• HBP-Tc |

heparanase was specifically expressed in newly formed capillaries and small blood vessels, while no traces of the enzyme were detected in mature vessels\(^{42}\).

Heparanase inhibitors were used in several studies to inhibit the enzyme’s activity. Heparin and heparin species containing at least 16 sugar units both at the N and O positions showed specific heparanase inhibition. Also, other sulphated polysaccharides, such as dextran sulphate, carrageenan lambda and laminarin sulphate exercised similar inhibitory action\(^{46,49}\). By preventing heparanase functions, these compounds reduced the incidence of experimental metastases, neoangiogenesis and tumour cell colonisation of normal organs, like liver and lungs\(^{59,60}\).

Today, many new laboratory techniques are developed in order to better observe and understand heparanase biological activities. New ELISA methods and mass spectroscopy are currently used for quantitative and qualitative analysis of the enzyme\(^{45,51}\). Several research groups are testing heparanase inhibitors with the aim of creating an effective antitumour drug. Phosphomannopentaose sulphate (PI-88) is already being evaluated in a phase II clinical trial, in patients with advanced malignancies\(^3\). However, due to its multiple natural functions the inhibition of heparanase, apart from tumour growth, affects embryonic development, immune surveillance, anticoagulant activities, inflammation and healing process. This becomes evident by the therapeutic amplitude of PI-88, which includes antiangiogenic, antiviral, antimalarial, antiproliferative and anticoagulant activities. Multiple ongoing studies attempt to answer the existing queries and accurately control the heparanase biological functions\(^{52,50,52}\).

**Autocrine motility factor (AMF)**

This 55kDa protein was initially isolated from A2058 melanoma cells, as a tumour secreted cytokine that stimulates direct and random migration\(^{51}\). Experiments using molecular cloning and sequencing suggested that AMF is a multifunctional member of the ectoenzyme/exoenzyme family\(^{54}\). This cytokine was identified as the glycolytic enzyme phosphoglucone isomerase (PGI)\(^{53}\), as neuroleukin (NLK), inducing growth of embryonic spinal and sensory neurons\(^{55,56}\) and as maturation factor, mediating human myeloid leukaemia cell differentiation\(^{57}\). Additionally, AMF exhibits the function of a sperm antigen-36 and a myofibril-bound serine proteinase inhibitor\(^{58}\).

AMF promotes cell motility via binding to a cell surface receptor named AMFR/gp78. This is a seven-transmembrane glycoprotein of 78 kDa\(^{59,60}\). When AMF reacts with its receptor, the latter is internalised, stimulates a pertussis toxin-sensitive G protein, activates protein kinase C (PKC) and inositol phosphate is produced; the receptor also undergoes phosphorylation. These molecular alterations are implicated in normal and tumour cell locomotion\(^{54,58}\). Multiple studies have revealed that the expression of AMF and its receptor is associated with increased tumour penetrating ability to normal tissues\(^{61,63}\). From the clinical aspect, the presence of this motility factor in urine and serum marks a worse prognosis, indicating progression in gastrointestinal, kidney and breast cancer\(^{63}\), as well as in colorectal cancer\(^{46,62}\). Nakamori et al studied the expression of AMFR in 118 patients who underwent surgical resection of colorectal cancer. They reported that patients with AMFR-positive tumours had significantly poorer survival rate. Among 101 patients who had curative resection, a significant difference in disease free survival was demonstrated among positive and negative tumours, as well\(^{64}\).

Carcinoma-derived AMF promoted angiogenesis, influencing endothelial cells. In vitro experiments with human umbilical vein endothelial cells (HUVECs) demonstrated that AMF induced an extensive expression of AMFR, which was hardly detected in untreated cells. This expression was associated with endothelial cell motility\(^{34}\). Moreover, HUVECs underwent morphogenesis and formed capillary-like tubes, by anastomosing one another. In vivo experiments in mice showed that AMF over-expressing tumour cell were able to provoke the development of new capillary blood vessels, which in turn could be prevented by specific AMF inhibitors\(^{58,65,66}\).

Vascular endothelial growth factor (VEGF) is one of the most important angiogenic factors and AMF increases its activity by promoting the expression of Flt1, a transmembrane VEGF receptor on endothelial cells. Flt-1 expression is achieved through the activation of phosphatidylinositol 3’-kinase (PI3K) in endothelial cells through a paracrine way. This angiogenic effect, reported in various in vitro and in vivo experiments with tumour cells, assisted the metastatic process\(^8,67,68\).

An anti-apoptotic action has also been attributed to AMF. Transfected mouse fibroblasts with the AMF gene demonstrated an aggressive ability for invasion and apoptotic resistance via Akt/protein kinase B (Akt/ PKB)\(^34\). Akt/PKB inactivated caspase-9 and BAD, which belong to Bcl-2 family and therefore apoptosis was suppressed\(^{44,69}\). Haga et al also concluded that AMF-transfected cells were apoptosis resistant. They used mitomycin C to induce apoptosis, a chemotherapeutic drug with anticancer action, and showed that AMF over-expressing cells had increased survival to apoptotic signals\(^{34}\).

In general, AMF is a multifactorial protein, which interferes with tumour growth and metastasis, through multiple mechanisms (Figure 3). Numerous experiments, in vitro and in vivo, with multiple cancer types, including colorectal cancer, revealed some of its tumour regulatory actions. However, many aspects need to be elucidated and current studies are focused on the evaluation of AMF’s application in cancer diagnosis and treatment\(^{48}\).

**Conclusions**

Tumours, including colorectal cancer, progress using a variety of enzymatic systems. The functions of these enzymes are multipotent and involve interactions with the extracellular matrix and the basement membrane,
tissue remodelling, alterations in the cell adhesion and migration, vessel formation and the regulation of apoptosis. Accumulating clinical and experimental data show that enzymes significantly affect not only the invasion of colorectal cancer cells in the primary site, but also the development of distant metastatic lesions, predominantly in the liver.

Urokinase plasminogen activator, matrix metalloproteinases, heparanase and autocrine motility factor, attract great research interest, due to their enzymatic activity in various stages of colorectal cancer. Clinical studies attempt to exploit current knowledge toward the discovery of effective pharmaceutical factors, which could modulate the activity of these enzymes in favour of patients. However, as they intervene in numerous pathological and physiological functions within the human body, therapeutic applications are slowly developed, because they require time-consuming and cautiously designed clinical trials.

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