Summary. The involvement of matrix metalloproteinase (MMPs)-2 and -9, also known as gelatinases, in cancer cell migration and invasion has been well documented, although it is not yet clear how they facilitate metastasis formation in the course of malignancies. The idea that gelatinases are responsible for degradation of extracellular matrix (ECM) components and breakdown of basement membrane (BM) tissue boundaries has turned out not to be entirely correct. An action by remodelling the ECM components of the BM exposing new cryptic sites, or releasing growth factors, cytokines, or active ECM proteolysed fragments seems to be nearer to the truth. On the other hand, tissue inhibitors of gelatinase activity (TIMP-2), are involved both in the MMP-2 activation process, in concert with membrane type 1-MMP (MT1-MMP), and in the inhibition of gelatinolytic activity. Therefore proteolysis, the central step for cancer metastasis, should occur as a result of an imbalance between MMP-2 and TIMP-2. Many studies have reported the importance of this balance in patients with different malignancies, and considerable effort is currently being spent on the study of molecules that can shift the balance in favour of inhibition of MMP proteolytic activity. In this review we focus on the role of gelatinase activity in cancer invasion, addressing the following issues: how and where proteolysis occurs in cancer tissues, how it can be regulated, what the clinical implications are of the studies reported in literature so far, and finally what the future developments in this field that could have an impact on the management of patients affected by malignancies may be.

Key words: Gelatinases, Matrix metalloproteinases, Cancer, Metastasis, Inhibitors

Introduction

In cancer, the occurrence of metastasis severely affects prognosis and survival, and is often the underlying cause of death. Unfortunately, no therapies are so far available to prevent or block the dissemination of cancer cells either through the blood circulation or in the surrounding tissues. For a number of researchers, the investigation of the mechanisms by which cancer cells cross the basement membrane (BM) tissue boundaries and penetrate the surrounding tissues to spread out, or diffuse to other sites through the blood circulation, has been one of the hot issues in the last decade.

Matrix metalloproteinases (MMPs) have been largely implicated in a number of physiological and pathological conditions where remodelling of extracellular matrix (ECM) components occurs (Fig. 1). Most of the studies of gelatinase activity have been focused on their ability to degrade the BM and the surrounding tissues, allowing cancer cell motility and metastasis formation.

While the mechanisms that modulate cancer cell motility are not yet entirely clear, MMPs are known to be able to facilitate tumor invasion and/or formation either by proteolysis of BM ECM components or by mediating the epithelial mesenchymal transaction (EMT). Along these lines, based on a number of studies mostly in vitro, new therapeutic approaches are currently being explored. In this review we briefly outline the important biological findings that are laying the strategic bases for future applications in several fields of clinical practice.

Gelatinase activation, and inhibition

MMPs are a family of Zinc (Zn) endopeptidases secreted as pro-enzymes, with proteolytic activity toward ECM components. At least 20 components of this family have so far been characterized, and grouped in six subsets based on substrate specificity and/or structure homology: interstitial collagenases, gelatinases, membrane type MMPs (MT-MMPs), stromelysin,
stromelysin-3, and metalloelastase (Nagase and Woessner Jr., 1999). Gelatinases, previously named Collagenases IV and now known as MMP-2 and MMP-9 of 72 and 92 Kd, respectively, will be discussed here in more detail. The activation of these enzymes requires the presence of both tissue type-1 of MMP (MT1-MMP) and tissue inhibitor-2 of MMP (TIMP-2), although their mechanisms still need to be fully elucidated. MT1-MMP is a peculiar type of MMP, since it is a transmembrane receptor that binds MMPs in the extracellular region while the intracellular tail is in contact with cellular structures. Why TIMP-2 is essential to allow MMP-2 activation is not yet clear, but it is known that the C-terminus of MMP-2 forms a non-covalent complex with TIMP-2, while the free part of MT1-MMP can remove the pro-domain portion of the molecule (Itoh et al., 1995; Kinoshita et al., 1998). A possible explanation is that MT1-MMP is the receptor for TIMP-2, and this complex would therefore be a receptor for pro-MMP-2 (Strongin et al., 1995). In addition, other proteases, such as thrombin, or plasminogen, have been reported to be involved in the activation of MMP-2 (Mazzieri et al., 1997; Davis et al., 2001; Lafleur et al., 2001). It is known that active MMP-2 is coupled at the invadopodia of the cells, where it is concentrated in a strategic position for optimal focusing of its activity (Sato et al., 1994; Mueller et al., 1999; Liotta and Kohn, 2001). However, the MMP-2 receptor is still unknown, although it has been reported that integrin αvβ3 might act to link MMP-2 (Brooks et al., 1996).

The activity of MMP-2 is inhibited by TIMP-2, so that too extensive and uncontrolled proteolytic action is avoided. On this basis, TIMP-2 plays a central role because of its dual effect: activation and inhibition of MMP-2. This dual function is a controversial and intriguing issue, and it has been suggested that at low concentration TIMP-2 works as an activator of MMP-2, whereas at higher concentration it exerts an inhibitory effect. The ideal molar ratio between MT1-MMP, TIMP-2, and MMP-2 for enzyme activation is 1:1:1 (Nagase, 1998).

### Proteolysis as a result of the MMP/TIMP balance

As stated above, proteolysis is the result of a balance between MMP-2 and TIMP-2 activity, and occurs in a very precise and focused manner so that it facilitates cancer cell motility, but still provides efficient adhesion. Increased production of MMP-2 and/or MMP-9, and decreased production of TIMP-2 both result in the enhancement of cell motility and invasion. Overexpression of MMP-2, and/or MMP-9 has been largely reported in several types of cancer such as melanoma, lung, gastric, breast and liver tissues (Ray and Stetler-Stevenson, 1995; Tokuraku et al., 1995; Mori et al., 1997; Kupferman et al., 2000; Giannelli et al., 2001a,b). On the other hand, the delivery of TIMP-2 by adenovirus to the liver of nude mice with colorectal liver metastasis produced a significant reduction of the metastasis compared to controls (Brand et al., 2000).

The concept of proteolysis is undergoing profound changes in these years. In the past it was considered to be closely related to tumor invasion because it was thought that degradation of ECM components could cause the formation of "holes" or complete dissolution of the BM structure so that cancer cells could fall through and then metastasize. Lately instead, gelatinases have been hypothesized to act not only by breaking down tissue boundaries, but also by revealing new cryptic sites as a consequence of proteolysis (Breuss et al., 1995; Giannelli et al., 1997, 1999); this issue has recently been reviewed (Liotta and Kohn, 2001). This is the case of Laminin-5 (Ln-5), a Ln isoform that promotes static adhesion and hemidesmosome assembling when it is expressed in intact form, and strong migration and invasion after being processed by either MMP-2 and/or MT1-MMP (Giannelli et al., 1997, 2001a,b; Koshikawa et al., 2000). Furthermore, we also have to consider that ECM components are assembled in a network, and proteolysis of one component induces rearrangements of the structural conformation of the molecule, if not the entire network, with a consequent new interaction with cells. In the case of Ln-5, proteolysis of the γ2 chain causes a rearrangement of the molecule, so that an additional functional site becomes available on the a chain, at a quite distant site from the cleavage site of the γ chain (Giannelli et al., 1997). Therefore, we cannot rule out that the rearrangement of the molecule induces a twist in Ln-5, altering interaction with the cells to a three-dimensional structure. Alternatively, the cleaved form of Ln-5 might couple other ECM components differently, not excluding other Ln-5 molecules. This is in agreement with other studies showing that another ECM component, Fibronectin, might display different biological functions when it is stretched (Zhong et al., 1998). The E8 domain on the long arm of Ln-1 promotes migration of olfactory neuronal cells via α6β1 integrin, whereas treatment of Ln-1 with antibodies against the

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**Fig. 1.** Published studies on MMPs. The percentage of the MMP studies published in the literature is higher in cancer than in development and remodelling of tissues, as calculated from 1989 up to 2001.
Localization of MMPs in tumor tissue

The study of MMP-2 and MMP-9 tissue localization supports the idea that proteolysis occurs in a focused manner. Several studies have reported that MMP-2 and/or MMP-9 are expressed in tumor tissue, but also in the surrounding stroma (Fig. 2). In both cases, gelatinases are concentrated along the BM, where proteolysis of ECM components is required to allow cancer cell metastasis. Consistently, gelatinases have been found to be up-regulated at the advancing edge of invasive malignancies, where they are also co-expressed with MT1-MMP, TIMP-2, and with some ECM proteins, suggesting their interaction in cancer cell invasion and metastasis (Chen, 1992; Birkedal-Hansen, 1995; Kohn and Liotta, 1995). In particular, MMP-2 has been found to be expressed by stromal cells (fibroblasts, macrophages, mesenchymal cells) of the surrounding peritumoral tissues (Stetler-Stevenson et al., 1993; Coussens and Werb, 1996). Also in breast tissue remodelling, MMP-2 is produced mainly by myoepithelial and fibroblast cells although it is focused at the BM levels where mammary epithelial cells of the terminal buds penetrate the fat pad during branching morphogenesis (Giannelli et al., 1999). Lately, the role of the stroma has been revised, as in the case of the mammary gland where the fat pad is not considered just as a container for gland development but participates actively in tissue remodelling and homeostasis, probably being a target for hormones, growth factors and MMPs that can modulate adipocyte differentiation (Neville et al., 1998; Giannelli et al., 1999; Alexander et al., 2001).

Therefore, stromal tissue actively participates in proteolysis and tissue remodelling during malignacies, also because after crossing the BM, cancer cells use MMP proteolytic activity to penetrate the surrounding tissues. There is no doubt that the surrounding microenvironment may influence cancer growth and development, although this idea derives from indirect evidence and/or experience, rather than from demonstration in experimental models. For instance, if we agree with the idea that the ECM soup modulates several cellular functions such as growth, differentiation, survival, apoptosis, etc., we can hypothesize that in cancer an altered cross-talk occurs between cells and ECM components. This is an important issue that needs to be elucidated in the near future, and there are at least two independent techniques that can support such studies: in situ zymography and laser capturing microdissection (LCM) followed by microarray assays.

In situ zymography enables the localization of MMPs by immuno-staining, and also reveals their gelatinolytic activity. Several studies have so far reported gelatinase immuno-staining in different tissues, but one of the common problems raised is regarding the state of activity of such enzymes. In situ zymography allows investigators to solve this problem, as shown for instance in glioblastoma and thyroid tumors (Nakada et al., 1999; Nakamura et al., 1999).

LCM followed by microarrays is surely one of the most fascinating and powerful techniques for the future. Briefly, the laser cells are dissected by surrounding tissues, and collected in appropriate tubes. Then, on these cells a number of genes or proteins can be investigated at the same time using micro-chip probes that have lately become commercially available. The advantage of this technique is that it elevates morphological studies to biomolecular levels, making it possible to distinguish which cells are to be investigated within tissues (Bichsel et al., 2001; Gillespie et al., 2001). Particular interest is given to the cancer cells located at the advancing edge, where the tumor is engaged with the surrounding tissues that it needs to penetrate in order to metastasize (Fig. 3). Such studies represent the main current hope of fighting cancer metastasis and targeting new therapies. In hepatocellular carcinoma tissues, the total MMP-2 levels are similar between metastatic and non metastatic patients, but in metastatic patients MMP-2 is mainly localized at the advancing front of the malignancy, suggesting its involvement in tumor progression. This observation is even more remarkable since TIMP-2 is up-regulated in patients without metastasis and down-regulated in patients with metastasis (Giannelli et al., 2001a,b). In short, a bulk of data indicates the advancing edge of cancer as the most important target in a new era of scientific technological research.

TIMP-2 and other synthetic inhibitors of gelatinases as therapeutic agents

The idea of using gelatinase inhibitors in cancer is emerging in parallel with the growing literature on enzyme activity in malignancies. Some of the more commonly used gelatinase inhibitors are Batimastat (BB-94), and more recently Marimastat, that mimic the cleavage site of Collagen competing with other ECM and working as suicide inhibitors. These inhibitors have
been shown to inhibit migration and invasion in experimental models in vitro, and also metastasis formation in nude mice (Giannelli et al., 1997; Bu et al., 1998; Brand et al., 2000). However, after the initial enthusiasm the clinical use of gelatinase inhibitors in human subjects has been limited because of its side-effects. Nevertheless, by phage display libraries new inhibitors that are specific for gelatinases but not for other MMPs have been individuated; these show activity in blocking cancer cell migration and invasion in both in vitro and animal models, while no data are yet available in human subjects (Koivunen et al., 1999). Furthermore, in nude mice matrilysin antisense oligonucleotide has been shown to significantly reduce colorectal liver metastasis formation in a dose-dependent manner in the liver (Hasegawa et al., 1998). A more sophisticated approach is the use of gene therapy that might be useful to deliver TIMP-2 directly inside the cancer cells. In nude mice it has been shown to dramatically reduce the occurrence of colorectal liver metastasis, underlining the critical role of gelatinases in cancer spread (Brand et al., 2000). However, all these new inhibitors have to be tested in vivo to verify the presence of possible side-effects, while it has recently been proposed that epigallocatechin-3-gallate, a flavonol present in green tea, is a potent inhibitor of gelatinases, thus explaining the beneficial effects of green tea in countering cancer development (Yang and Wang, 1993; Garbisa et al., 2001). This compound is of particular interest as it is easy and practical to administer: two cups of green tea provide therapeutic serum concentrations of epigallocatechin-3-gallate without side-effects (Garbisa et al., 2001).

It is clear from the above that the main problems with inhibitors are their side effects and/or the difficulty in selectively targeting cancer tissues.

**Clinical applications**

The clinical implications of the huge number of studies, and the related question of how patients may benefit, are of extreme importance to all clinicians as well as investigators. First of all, there is large consensus in the literature on the increased levels of MMP-2 and/or
MMP-9 in patients with cancer, this confirming the findings obtained in experimental models in vitro. The first but most important step consists of the ongoing studies aiming to gain insight into the pathogenesis of cancer metastasis, and according to these findings, new therapeutic approaches can then be identified and targeted. Many efforts have been made to relate gelatinase levels with histopathological findings, or with tumor grading, but no correlations have been reported.

In melanoma tumors, MMP-2 staining has been found to be related with differentiation of the cancer cells, while higher levels correlate with worse prognosis, so the authors suggest the use of MMP-2 as a prognostic factor (Vaisanen et al., 1998). However, in another study MMP-2 was not found to be a prognostic factor, and was of limited clinical value in patients with melanoma (Vuuristo et al., 2000). However, in these kinds of studies, sampling and reagents are very crucial and might account for such different results. In non-small cell lung carcinoma, increased levels of MMP-2 were detected. No correlation was found with other clinicopathological parameters, but this could be used in patients without lymph-node involvement as an independent prognostic factor (Passlick et al., 2000). Others have also reported a significant increase of MMP-9 in lung carcinoma although serum levels did not correlate with the tissue levels of the enzyme (Iizasa et al., 1999), but this finding might be explained by the participation of stromal tissue in cancer metastasis, as considered above. We found a significant correlation between serum and tissue levels of MMP-2 in patients with metastatic and non-metastatic hepatocellular carcinoma (HCC) (Gianelli et al., 2001a,b). In prostate cancer, low levels of gelatinases have been reported in fresh prostate cancer tissue, but also in organ-cultured specimens (Varani et al., 2001). In this study, the authors attribute their results to the low invasive phenotype of prostate cancer, while other investigators found a significant increase of MMP-2 levels compared with patients with benign prostatic hyperplasia, the prostate volume being measured by ultrasonography (Gohji et al., 1998). Recently, MMP-2 and MMP-9 activity was investigated in the urine of patients with bladder cancer and was found to correlate with tumor progression (Gerhards et al., 2001).

Conclusions

A bulk of data in the literature show that gelatinases are clearly implicated in cancer metastasis. Therefore, new therapies to inhibit gelatinase activity at the advancing edge of malignancies in cancer tissues in order to prevent or block cancer cell invasion and metastasis are being addressed. However, the presence of side effects and/or low specificity of these drugs severely affects their clinical use; more studies are needed to better focus mechanisms which regulate gelatinase production and/or activation. Useful approach in the therapy of cancer metastasis is based on the possibility of individuating drugs better targeting the right sites and featuring fewer side effects. In this regard, gene therapy seems to be a powerful but not so immediate approach, because of difficulties related to the virus carrier. Inhibitors without side effects may be a more feasible approach in the near future, including vegetable inhibitors of gelatinases such as epigallocatechin-3-gallate in green tea. Finally, an alternative approach might be represented by the use of molecules which modulate gelatinases or TIMP-2 production, in order to reach a correct balance between enzymes and inhibitors.

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