Tissue inhibitor of metalloproteinases-1 in breast cancer

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Abstract

Whether patients diagnosed with primary breast cancer are offered adjuvant systemic therapy following surgical removal of the tumor is based on prognosis. Prognosis is estimated in every patient using established prognostic variables. Unfortunately, when using the currently available prognostic parameters a significant proportion of patients are over-treated. Thus, in order to improve stratification of breast cancer patients, additional prognostic factors need to be identified. Tissue inhibitor of metalloproteinases-1 (TIMP-1) is one of the promising candidates for new prognostic markers in breast cancer, as a number of studies have demonstrated an association between high tumor-tissue levels of TIMP-1 mRNA as well as TIMP-1 protein and a poor prognosis of breast cancer patients. TIMP-1 is a member of the TIMP family, currently comprising four members (TIMP-1–4), and its main function is inhibition of the activity of various matrix metalloproteinases (MMPs). The association between high levels of protease inhibitor and poor prognosis may be somewhat surprising, as proteolytic activity plays a pivotal role in cancer cell invasion and metastasis. However, the recent discovery of other biological functions of TIMP-1 such as growth-stimulating functions, as well as anti-apoptotic and pro-angiogenetic effects, may in part explain this paradox. The purpose of this review is to give an update on the current status of TIMP-1 in breast cancer, emphasizing the prognostic utility of the inhibitor. In addition, the suggested tumor-stimulatory roles of TIMP-1 will be outlined.

Introduction

Breast cancer is the leading type of cancer among women today and the incidence of this disease is increasing in all industrialized countries. In spite of the increasing incidence, mortality has been rather stable for several years. As a result of this tendency still more women are living with breast cancer and accordingly substantial efforts are allocated to the development of effective management of breast cancer patients.

Whether patients diagnosed with primary breast cancer are offered adjuvant systemic treatment in addition to loco-regional therapy of the tumor is determined by a number of prognostic variables such as nodal status, tumor size, grade of malignancy, age and hormone receptor status, with nodal status being the most important (Danish Breast Cancer Cooperative Group Guidelines 2004, www.dbcg.dk; Goldhirsch et al. 2003). Using these prognostic variables, adjuvant systemic therapy is offered to the majority of node-negative patients (~60%) and to all node-positive patients, i.e. to a prognostically heterogenous group with risks ranking from 10 to 80%. With the adjuvant systemic therapy approximately 30–40% of the expected deaths can be avoided (Early Breast Cancer Trialists’ Collaborative Group 1998a, 1998b); however, in absolute terms the mortality reduction ranks from only a few percent in the lower-risk group and up to 25% in the higher-risk group. Thus, although adjuvant systemic therapy has led to a significant improvement in the survival in the breast cancer population a large proportion of the patients are over-treated. Since the
treatment causes several adverse side effects and inflicts considerable costs on the society, additional prognostic factors need to be identified in order to improve risk classification and thereby a more rational management of breast cancer patients.

Several promising prognostic markers are emerging and currently under investigation. Besides a brief description of some of the most important of these, this review will focus on uPAR of metalloproteinase-1 (TIMP-1), which has recently been discovered to carry valuable prognostic information in primary breast cancer.

Emerging candidates for new prognostic factors in breast cancer

Several promising prognostic markers, which may be useful in breast cancer, are currently under investigation (reviewed in Thomssen et al. 2003, Coradini & Daidone 2004, Mirza et al. 2002, Hayes et al. 2001). Common to all these emerging new prognosticators are their implication in the main phenotypic alterations characterizing breast cancer cells such as unrestricted proliferation (thymidine-labelling index, mitotic index, Ki-67, cyclins, erbB2 and c-myc), limitless replicative potential (telomerase), evasion of apoptosis (Bel-2, Bax, p53), sustained angiogenesis (vascular endothelial growth factor (VEGF) and neovascularization) and invasion and metastasis (cathepsin D, E-cadherins, urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor type 1 (PAI-1; Hanahan & Weinberg 2000). In addition, the recent development of gene expression microarrays (genomic profiling) capable of evaluating the expression of thousands of genes simultaneously makes it possible to identify distinct patterns of gene expression, which correlate with prognosis (Perou et al. 1999, 2000, Sørlie et al. 2001, West et al. 2001, Zajchowski et al. 2001, Van’t Veer et al. 2002, Cleator & Ashworth 2004). Genomic profiling thus makes a more detailed prognostic profiling of the disease possible compared with the standard methodologies, which rely on a few prognostic markers to estimate prognosis. Despite the identification of these putative markers few are currently used in clinical practice, the main reason being that only few studies have demonstrated conclusive evidence of their clinical utility. Further studies are still needed to elucidate their prognostic value. Among the factors investigated the most promising have been demonstrated to be the factors involved in the processes of invasion and metastasis.

Tumor invasion and metastasis are the primary determinant of patient outcome and concordantly molecules involved in these processes are obvious candidates for new prognostic markers in breast cancer. Proteolytic enzyme systems are responsible for the turnover of the extracellular matrix (ECM) in normal physiological conditions such as embryo development, morphogenesis and tissue remodelling (i.e. wound healing). In addition, it is well established that deregulation of these enzyme systems is associated with the malignant progression of cancer cells. It is believed that overexpression of proteolytic enzymes in the tumors render the cells capable of breaking down the ECM and basement membrane (BM), which under normal conditions maintain the integrity of the tissues, thereby allowing cells to invade the surroundings. In this regard, the uPA system, which is known to play a crucial role in cancer metastasis, has gained much attention (Andreasen et al. 1997). Numerous groups have independently shown that high levels of uPA and, paradoxically, high concentrations of its inhibitor PAI-1 correlate with poor prognosis in breast cancer (reviewed in Schmitt et al. 1997). The surprising finding concerning PAI-1 has recently been partly explained by the demonstration of an anti-apoptotic (Kwaan et al. 2000) as well as a pro-angiogenic (Bajou et al. 1998) effect of this protein. In fact, uPA and PAI-1 are among the first prognostic indicators to have their clinical value confirmed in evidence-level 1 studies (Hayes et al. 1996), and they are now considered for the routine assessment of prognosis in patients with primary breast cancer (Jänicke et al. 2001, Duffy 2002, Look et al. 2002). By using uPA and PAI-1 in stratification of node-negative breast cancer patients approximately 50% of these patients can be considered at low risk of experiencing recurrence of disease and accordingly may be spared the toxicity of adjuvant systemic therapy (Jänicke et al. 2001). Thus, using uPA and PAI-1 in prognostic stratification an improvement is achieved although complete separation of high- and low-risk node-negative women is still not possible. To this end additional prognostic factors are needed.

Other proteolytic enzymes known to play a pivotal role in cancer dissemination are the matrix metalloproteinases (MMPs). MMPs are a family of zinc-dependent endopeptidases, which together are able to cleave virtually every component of the ECM including BM (reviewed in Stamenkovic 2000, Egeblad & Werb 2002). The expression and activation of MMPs are increased in almost all human cancers compared with normal tissues and this increase has been associated with a poor patient prognosis (Chenard et al. 1998, Nakopoulou et al. 2002, Ranuncolo et al. 2003, Talvensaari-Mattila et al. 2003, Leppä et al. 2004). In addition to the MMPs, one of the four natural
inhibitors of the MMPs, tissue inhibitor of metalloproteinases type-1 (TIMP-1), has also been shown to be upregulated in numerous cancers including breast cancer and surprisingly, as was the case for PAI-1, this upregulation has in several cases been shown to be associated with a poor patient prognosis (Ree et al. 1997, McCarthy et al. 1999, Nakopoulou et al. 2002a, Schrohl et al. 2003, 2004). The upregulation of TIMP-1 in cancer together with the association to a poor prognosis could reflect the fact that the balance between expression of MMPs and TIMP-1, although still favouring MMPs, is at a higher overall level than in non-cancerous conditions. High TIMP-1 levels would therefore be associated with a worse prognosis but would not be the cause of it (Egeblad & Werb 2002).

However, several lines of evidence have recently implied that TIMP-1 is a multifunctional protein that in addition to its MMP-inhibitory effect also possesses distinct tumor-stimulatory functions. Indication of a prognostic significance of TIMP-1 has resulted in extensive investigation of this new putative prognostic marker in breast cancer. In the following sections the proposed cancer-related functions of TIMP-1 will be outlined.

**TIMP-1**

TIMP-1 is a 28.5 kDa glycoprotein that forms non-covalent 1:1 stoichiometric complexes with MMPs, thereby inhibiting the proteolytic activity of these enzymes. It is a member of the TIMP family, currently comprising four members (TIMP-1, -2, 3- and -4), which exhibit approximately 50% sequence similarity, is expressed by a wide range of cells and is present in most tissues and body fluids. Common to all members of the TIMP family is the presence of 12 cysteine residues in conserved regions forming six disulfide bonds that fold the protein into a two-domain structure. The presence of a large number of disulfide bonds renders the TIMP molecules resistant to extremes of temperature and pH, and denaturing conditions (reviewed in Lambert et al. 2004, Brew et al. 2000).

High levels of TIMP-1 mRNA as well as TIMP-1 protein have been demonstrated in several types of cancer, including breast cancer, and this has been associated with a poor prognosis of the patients (Ree et al. 1997, McCarthy et al. 1999, Nakopoulou et al. 2002a, Schrohl et al. 2003, 2004). As for PAI-1, this paradox points to TIMP-1 as a multifunctional protein, which in addition to the MMP-inhibitory effect has distinct tumor-promoting functions. Proposed functions include growth promotion and anti-apoptotic effects as well as both anti- and pro-angiogenic functions, as will be described in the following sections.

**TIMP-1 functions**

**TIMP-1 and MMP inhibition**

Endogenous inhibitors such as TIMP-1 tightly control MMP activity. TIMP-1 binds non-covalently in a 1:1 stoichiometric fashion to the active form of MMPs thereby inhibiting the proteolytic activity of these enzymes. In addition, TIMP-1 can form complexes with pro-MMP-9 (Duffy et al. 2000). In spite of the fact that the interaction between TIMP-1 and MMPs is non-covalent the complex has proved to be rather stable (Weligus et al. 1985, Murphy et al. 1991). Mutational studies have shown that the N-terminal domain of TIMP-1 is critical for the inhibitory action. In a study conducted by Murphy and co-workers (1991) it was demonstrated that a truncated version of TIMP-1 containing only the N-terminal part of the protein retained the ability to inhibit MMPs as effectively as wild-type protein. The importance of the N-terminal domain of TIMP-1 in MMP inhibition is supported by the fact that point mutations introduced in this region of the protein abolish the MMP-inhibitory capacity (Chesler et al. 1995). Multiple sites in the N-terminal domain are probably involved in the interaction with MMPs. For instance, residues around the disulfide bond Cys-1–Cys-70 have been demonstrated to be crucial for the inhibitory activity of TIMP-1 (Caterina et al. 1997).

The mechanisms of MMP inhibition by TIMP-1 have been the subject of excessive investigations. In 1997, the first structure of complete TIMP-1 in complex with the catalytic domain of human MMP-3 was published (Gomis-Rüth et al. 1997). Subsequently the X-ray structure of TIMP-2 in complex with membrane type 1 MMP has been described (Fernandez-Catalan et al. 1998). These publications have revealed several features concerning the interaction between TIMPs and the MMPs. TIMP-1 has the shape of an elongated wedge and MMPs contain a small active-site cleft, which harbours the catalytic zinc atom (Maskos & Bode 2003). In complex with MMPs, the edge of the TIMP-1 molecule binds and occupies the entire length of the active-site cleft of MMPs (Gomis-Rüth et al. 1997, Fernandez-Catalan et al. 1998). In addition, the structural studies have supported the previous observation that TIMP-1 interacts with MMPs predominantly through its N-terminal moiety. Furthermore, these studies have identified segments of the TIMP-1 protein participating in the interaction with MMPs as...
TIMP-1 and stimulation of proliferation

TIMP-1 was originally purified as a protein possessing erythroid-potentiating activity, thus stimulating the proliferation of early erythroid precursor cells and the growth of the K-562 human erythroleukemia cell line (Westbrook et al. 1984, Avalos et al. 1988). The identity of this protein as TIMP-1 was later established (Docherty et al. 1985). Subsequently the cell-growth-promoting effects of TIMP-1 has been extended to a wide range of normal cell types as well as neoplastic cell types (Bertaux et al. 1991, Hayakawa et al. 1992).

The molecular basis of growth control by TIMP-1 remains to be fully elucidated. However, several models have been proposed concerning the mechanisms by which TIMP-1 mediates growth promotion. These will be discussed below.

Several lines of evidence indicate that TIMP-1-mediated stimulation of cell proliferation is independent of its MMP-inhibitory effect. In support of this view are findings demonstrating that the two major activities of TIMP-1, i.e. inhibition of MMPs and stimulation of growth, are functionally distinct as well as possibly structurally separate. Chesler et al. (1995) showed that a mutated form of TIMP-1 lacking regions necessary for MMP inhibition was capable of stimulating the growth of erythroid precursors as effectively as non-mutated TIMP-1. Furthermore, it has been demonstrated that reduction/alkylation of TIMP-1, which eliminates the proteinase-inhibitory activity (Hayakawa et al. 1994), does not affect the growth promoting effect of TIMP-1 (O’Shea et al. 1992). These results indicate that the influence of TIMP-1 on the growth of these cells is independent of its ability to inhibit MMPs and that stimulation of growth and MMP inhibition are structurally distinct activities of the protein. In addition to the findings mentioned above, binding sites specific for TIMP-1 have been identified on the surface of several human cell types responding to this protein including keratinocytes, erythroid precursors, breast carcinoma cells and K-562 cells (Avalos et al. 1988, Bertaux et al. 1991, Luparello et al. 1999, Ritter et al. 1999).

These observations have lead to the currently prevailing view that TIMP-1 stimulates cell proliferation through a direct interaction with an as-yet unidentified cell-surface receptor involving no mediation by the MMP system. Binding of TIMP-1 to this receptor would then in turn initiate an intracellular signalling cascade resulting in a cellular response of proliferation. In support of such a mechanism, Luparello and co-workers (1999) investigated the proliferative effect of TIMP-1 on two different breast carcinoma cell lines, BC-3A and BC-61, endowed with different malignant potential. Data from this study indicate that only the more tumorigenic cell line (BC-61) was responsive to TIMP-1 treatment by increasing its proliferative rate in a dose-dependent manner (Luparello et al. 1999). The same authors explained this phenomenon by the identification of TIMP-1-binding proteins uniquely present on BC-61 cells. These TIMP-1-binding proteins were shown not to possess any gelatinolytic activity and therefore they did not represent membrane-associated MMPs. These findings support the hypothesis of an actual receptor-mediated action of TIMP-1. Moreover, the study implies that heterogeneity of the response to TIMP-1 could be expected for cells with differential expression of this receptor, i.e. TIMP-1 may preferentially bind to neoplastic cells selectively expressing TIMP-1 receptors. Thus, the appearance or upregulation of a TIMP-1 receptor could be associated with the degree of malignancy.

The identification of the intracellular signalling pathway initiated by TIMP-1 upon binding to the putative membrane receptor has been the emphasis of numerous studies. However, controversy still exists as to how TIMP-1 transduces its growth signal into the cell. For example, binding of a fusion protein of human TIMP-1 and enhanced green fluorescent protein to the surface of MCF-7 breast carcinoma cells has been demonstrated to result in enhanced proliferation of these cells (Ritter et al. 1999). Interestingly, this binding was followed by translocation of TIMP-1 to the nucleus. These results raise the possibility that following binding to the cell surface TIMP-1 translocates to the nucleus and in turn directly affects replication and/or transcription leading to cell proliferation. Likewise, accumulation of TIMP-1 has also been shown in the nuclei of human gingival fibroblasts, reaching a maximum in the S-phase of the cell cycle (Zhao et al. 1998).

An alternative mechanism of action is that TIMP-1 binds to the cell surface and initiates a signal transduction cascade mediating the growth signal into the cell. Thus, it has been found that following binding of TIMP-1 to the cell surface an increase in tyrosine-specific phosphorylation of cellular proteins, which is a widely recognized intracellular signal used by several
growth factors, was induced (Luparello et al. 1999). Likewise, a TIMP-1-mediated increase in tyrosine-targeted phosphorylation resulting in growth promotion of osteosarcoma cells have been demonstrated (Yamashita et al. 1996). These studies suggest a crucial role for tyrosine kinases in the signal transduction of TIMP-1. Furthermore, demonstration of the activation of one of the known mitogen-activated protein (MAP) kinases, extracellular-signal-regulated kinase 2 (ERK2), following treatment with TIMP-1 suggests that a MAP kinase signalling pathway could be implicated in TIMP-1-dependent growth signalling (Yamashita et al. 1996). The recent finding that TIMP-1 is able to activate Ras (stimulate the complex formation between Ras and GTP) as well as to enhance the binding between Ras-GTP and Raf-1 has extended the model (Wang et al. 2002). The fact that Ras is a well-established activator of the MAP kinase cascade supports the hypothesis that the proliferative effect of TIMP-1, as for several other known growth factors, is mediated by tyrosine-specific phosphorylation through the MAP kinase pathway.

Whether TIMP-1 enters the cell and directly influences the nucleus or whether TIMP-1 merely stays outside the cell and initiates a signalling cascade thereby indirectly transmitting the growth signal to the nucleus remains to be elucidated.

An alternative model suggests that the apparent proliferative action of TIMP-1 is dependent on its ability to inhibit MMPs, rather than to stimulate a cell-surface receptor by a process independent of its MMP-inhibitory effect; that is, an indirect action. In this regard, it has been demonstrated that TIMP-1 and the synthetic inhibitor of MMPs, GM6001, stimulated proliferation of the cancer cell line MDA-MB-435 with similar kinetics, indicating that the two inhibitors stimulate proliferation using the same mechanisms (Porter et al. 2004). Furthermore, a GM6001 derivative incapable of inhibiting MMPs had no effect on proliferation, confirming that the growth-stimulatory function of GM6001 was dependent on its ability to inhibit MMPs. By employing several specific signal transduction inhibitors the researchers showed that stimulation of growth by TIMP-1 occurred through the MAP kinase/ERK kinase (MEK)/ERK and p38 kinase pathways. The authors proposed mechanisms by which TIMP-1 mediated MMP inhibition could stimulate growth. One of these putative mechanisms involved the possibility that TIMP-1 prevents the degradation of a newly synthesized growth factor by constitutively active MMPs thereby allowing the growth factor to stimulate the cells. Although indicative, results from this study do not provide conclusive evidence that TIMP-1 stimulates cell growth through the inhibition of MMPs. It cannot be excluded that the synthetic MMP inhibitor tested and TIMP-1, although seemingly similar, work through different mechanisms. The idea of the existence of a TIMP-1 receptor can therefore not be abandoned based on this study alone and finally, it should be emphasized that the two overall models (direct or indirect mechanisms of TIMP-1) are not mutually exclusive.

From the above discussion it is evident that further studies are necessary in order to fully uncover the mechanisms by which TIMP-1 stimulates proliferation.

**TIMP-1 and inhibition of apoptosis**

It is well established that TIMP-1 exhibits an anti-apoptotic effect. Basically, two different mechanisms of TIMP-1 suppression of apoptosis have been proposed. One pathway is linked to the anti-MMP activity of TIMP-1 and the other is MMP-independent. The two mechanisms will be discussed below.

The first mechanism suggested for TIMP-1 is dependent on its ability to inhibit the activity of MMPs. In this regard, an anti-apoptotic effect of TIMP-1 has been demonstrated on human and rat hepatic stellate cells. This effect was shown to be dependent on the MMP inhibitory function of TIMP-1 since a mutated form of this protein possessing no MMP-inhibitory capacity was not able to exert the anti-apoptotic effect (Murphy et al. 2002). So how does TIMP-1 suppress apoptosis by inhibiting MMPs? Studies have shown that integrity of the ECM and BMs as well as cell–matrix interactions suppress apoptosis and maintain the differentiated state of anchorage-dependent mammalian cells. In addition, disruption of these cells from their association with the matrix by MMP-mediated degradation of the ECM results in induction of molecular effectors of apoptosis such as the caspases. This leads to loss of the differentiated state and apoptotic cell death both in culture and in vivo (Boudreau et al. 1995). This shows that an intact matrix plays a key role in regulation of apoptosis, probably by providing a direct cell survival signal or by preventing the release of matrix-bound pro-apoptotic factors. Induction of apoptosis may then occur as a consequence of proteolytic activity of MMPs, removing this cell survival signal and/or liberating/activating pro-apoptotic factors from the matrix. Keeping this in mind it is obvious to speculate that TIMP-1 inhibition of apoptosis may result from its ability to preserve integrity of the ECM and stabilize cell–ECM interactions through inhibition of MMPs. In support of this model, Alexander et al. (1996) demonstrated that...
mammary epithelial cells derived from mice over-expressing MMP-3 underwent excessive apoptosis. When these mice were crossed with mice over-expressing TIMP-1, apoptosis was significantly reduced (Alexander et al. 1996).

TIMP-1 may also exert its anti-apoptotic effect independently of its ability to inhibit MMPs. In a study conducted by Guedez et al. (1998) it was found that the level of TIMP-1 expression was positively correlated with resistance to apoptosis in Burkitt’s lymphoma cells and that TIMP-1 inhibited apoptosis in normal tonsillar B cells. This anti-apoptotic effect of TIMP-1 was not a result of inhibition of MMPs as reduced/alkylated TIMP-1 completely devoid of MMP inhibitory activity was still able to suppress apoptosis. In addition, TIMP-1 overexpression inhibits apoptosis even after the loss of cell adhesion in human breast epithelial MCF10A cells, suggesting that the anti-apoptotic effect of TIMP-1 does not depend on its ability to stabilize cell–matrix interactions (Li et al. 1999). The mechanism for this MMP independent anti-apoptotic effect is not understood at present but as was the case concerning the proliferative effect of TIMP-1 it has been speculated that inhibition of apoptosis occurs via binding of TIMP-1 to a cell-surface receptor. Cell-surface binding of TIMP-1 has indeed been demonstrated for cell types responding to the anti-apoptotic activity of this protein (Guedez et al. 1998). Thus, TIMP-1 may exert its anti-apoptotic effect by targeting unidentified TIMP-1-binding proteins on the cell surface, thereby initiating as-yet undefined signalling pathways. Suggestions have been made regarding the signalling pathways possibly regulated by TIMP-1. In the MCF10A breast epithelial cell line overexpression of TIMP-1 was shown to induce constitutive activation of focal adhesion kinase (FAK) through tyrosine phosphorylation (Li et al. 1999, Liu et al. 2003). FAK has previously been shown to be upstream regulator of the phosphatidylinositol-3 kinase (PI-3 kinase), leading to regulation of the Bel-2 family members, a well-characterized signalling pathway leading to cell survival. Phosphorylated FAK associates with and thereby activates the PI-3 kinase, which then in turn activates the Akt kinase. Akt phosphorylates the protein Bad, which as a result is sequestered in the cytoplasm by the capture protein 14-3-3 and can therefore no longer interact with and inhibit Bel-2 and Bel-XL. Bel-2 and Bel-XL are proteins situated in the mitochondria membrane and when activated these anti-apoptotic proteins inhibit Bax, thereby preventing the release of cytochrome c from the mitochondria. This in turn prevents activation of the caspase cascade and accordingly prevents apoptosis. Thus TIMP-1 may inhibit apoptosis by acting like a trophic factor initiating the survival pathway including FAK, PI-3 kinase, Akt and Bel-2 family members resulting in inhibition of caspase activation and thereby inhibition of apoptosis (Fig. 1).

In support of this model, several studies have demonstrated phosphorylation and activation induced by TIMP-1 of the PI-3 kinase, Akt and Bad as well as TIMP-1-induced upregulation of expression of Bel-XL and Bel-2 (Guedez et al. 1998, Lambert et al. 2003, Lee et al. 2003, Liu et al. 2003). For example, it has been demonstrated that TIMP-1 was able to induce phosphorylation of Akt in the breast carcinoma T-47D cell line; however, this phosphorylation was blocked by pertussis toxin, a selective inhibitor of heterotrimeric G proteins and inhibitors of PI-3 kinase and the Src family of tyrosine kinases (Lee et al. 2003). These results suggest that TIMP-1-induced Akt activation pathways involve a G protein, tyrosine kinases and PI-3 kinase. This group did not detect any change in FAK phosphorylation suggesting that TIMP-1 may initiate different survival pathways in different cell types. Taken together, several lines of evidence suggest that TIMP-1 inhibits apoptosis, at least in some cell types, by initiating the well-established cell-survival pathway including the PI-3 kinase/Akt/Bad/Bcl-2 molecules, thereby preventing activation of the caspase cascade.

**TIMP-1 and angiogenesis**

TIMP-1 has also been implicated in the process of angiogenesis. However, the exact role of TIMP-1 in this process is still a matter of controversy since both anti-angiogenic as well as pro-angiogenic effects have been demonstrated.

Firstly, TIMP-1 has been shown to inhibit angiogenesis. For example, bovine aortic endothelial cells overexpressing TIMP-1 display a significant reduction in migration through gelatin-coated membranes (a model-system representing the ECM and BM) compared with control cells (Fernandez et al. 1999). Since this reduction was seen only when gelatin-coated membranes and not uncoated membranes were used, it was speculated that TIMP-1-mediated inhibition of endothelial cell migration was dependent on the anti-proteolytic activities of this inhibitor. During angiogenesis endothelial cells locally degrade the vascular basal lamina and migrate into the ECM to form new capillaries (Ausprunk & Folkman 1977). This is accomplished using proteinases including MMPs. Realising the importance of proteolytic activity in new vessel formation the anti-angiogenic effect of TIMP-1 should come as no surprise. Another possibility is that by inhibiting MMPs TIMP-1 prevents the angiogenic
switch. MMP-9 has been shown to be able to convert normal non-angiogenic endothelial cells of pancreatic islets into highly angiogenic cells (i.e. the angiogenic switch; Bergers et al. 2000). This was shown to be a result of MMP-9-mediated mobilization of VEGF from the ECM, rendering VEGF more available to the endothelial cells. Furthermore, when exposing the endothelial cells to a synthetic MMP-9 inhibitor, the angiogenic potential of the cells was significantly reduced. Thus, it seems logical that the suppression of MMP activity by TIMP-1 may inhibit angiogenesis by preventing mobilization of VEGF.

Secondly, a stimulatory role of TIMP-1 in the process of angiogenesis has been proposed. Some MMPs play a critical role in generation of potent inhibitors of angiogenesis. For example, MMP-12 is most efficient in converting plasminogen into angiostatin, which is a well-known anti-angiogenic factor (Cornelius et al. 1998). In addition, the generation of endostatin, another inhibitor of angiogenesis, has been shown to be mediated by MMPs, which cleave and thereby release endostatin from the precursor molecule collagen (Wen et al. 1999). Therefore, by inhibiting these MMPs TIMP-1 may prevent angiostatin and endostatin production, thus playing a positive role in tumor angiogenesis. In this regard, it has been shown that tumors derived from rat breast carcinoma cells overexpressing TIMP-1 grew larger and became more vascularized compared with controls (Yoshiji et al. 1998). In addition, overexpression of TIMP-1 by the rat carcinoma cells was associated with a marked upregulation of VEGF expression, explaining the growth advantage for cells possessing high levels of TIMP-1. A close relationship between TIMP-1 and VEGF expression was also found in clones of human MCF-7 breast carcinoma cells lines. These findings indicate that by stimulating the expression of VEGF directly, TIMP-1 confers growth advantage on tumors overexpressing this inhibitor by promoting angiogenesis, thereby promoting vascularization of the tumor. The mechanisms involved in the induction of VEGF expression by TIMP-1 are not known at present. However, since, as mentioned previously, TIMP-1 has been found to accumulate in the nucleus (Zhao et al. 1998, Ritter et al. 1999). Therefore, by inhibiting these MMPs TIMP-1 may prevent angiostatin and endostatin production, thus playing a positive role in tumor angiogenesis. In this regard, it has been shown that tumors derived from rat breast carcinoma cells overexpressing TIMP-1 grew larger and became more vascularized compared with controls (Yoshiji et al. 1998). In addition, overexpression of TIMP-1 by the rat carcinoma cells was associated with a marked upregulation of VEGF expression, explaining the growth advantage for cells possessing high levels of TIMP-1. A close relationship between TIMP-1 and VEGF expression was also found in clones of human MCF-7 breast carcinoma cells lines. These findings indicate that by stimulating the expression of VEGF directly, TIMP-1 confers growth advantage on tumors overexpressing this inhibitor by promoting angiogenesis, thereby promoting vascularization of the tumor. The mechanisms involved in the induction of VEGF expression by TIMP-1 are not known at present. However, since, as mentioned previously, TIMP-1 has been found to accumulate in the nucleus (Zhao et al. 1998, Ritter et al.
1999) it is reasonable to speculate that TIMP-1 can influence transcriptional activation directly.

Expression and localization of TIMP-1 in breast cancer tissue

Several studies have demonstrated that the expression of TIMP-1 is enhanced in breast tumor tissue compared with benign or normal breast tissue (Yoshiji et al. 1996, Brummer et al. 1999, Nakopoulou et al. 2002a). Using Northern blotting, Yoshiji et al. (1996) demonstrated that TIMP-1 mRNA expression was significantly increased in malignant breast tissue compared with non-malignant tissue. The study included a total of 53 samples of which 23 were primary breast carcinomas, four were benign tumors and 26 were non-tumor breast tissue. The same group also compared the protein level of TIMP-1 in six of the malignant tumor samples and six non-neoplastic breast samples by Western blotting. As was the case concerning the mRNA levels, the protein level of TIMP-1 was elevated in the tumor tissue compared with the non-neoplastic tissues. Employing in situ hybridization Brummer et al. (1999) confirmed the above findings. This group demonstrated that the expression of TIMP-1 mRNA increased gradually with increased invasive potential of transformed cells; TIMP-1 was only weakly expressed in normal breast tissue, the expression was slightly increased in carcinomas in situ and high levels of expression were observed in invasive carcinomas. TIMP-1 was found mainly to be expressed in the peritumoral stromal cells and only weakly by tumor cells.

Using the same approach, Nakopoulou et al. (2002a) demonstrated selective expression of TIMP-1 mRNA in invasive breast carcinomas whereas non-neoplastic breast tissue did not contain any detectable levels of TIMP-1 mRNA. The study showed that TIMP-1 was not localized in tumor cells but in stromal cells, both in areas in the central part of the tumor as well as in the tumor margin. The stromal localization of TIMP-1 in breast carcinomas has been confirmed by immunohistochemistry (Clavel et al. 1992, Jones et al. 1999). In a study conducted by Jones and co-workers (1999) TIMP-1 protein staining was predominantly seen in fibroblasts and in the wall of small blood vessels but rarely in the tumor cells. In another immunohistochemical study TIMP-1 protein was localized in vessel walls and in the vascular endothelium and was only rarely observed in cancer cells (Clavel et al. 1992).

Taken together, these studies demonstrate that the expression of TIMP-1 mRNA is upregulated in breast cancer tissue compared with benign or normal tissue. In addition, they indicate that this upregulation does not only apply to the mRNA levels but also to the protein levels of TIMP-1. Furthermore, the data show that stromal cells of the tumors rather than the cancer cells themselves are responsible for the production of TIMP-1.

Current status on TIMP-1 and prognosis in primary breast cancer

As mentioned earlier, preliminary data have suggested that TIMP-1 carries prognostic information in patients with primary breast cancer (Ree et al. 1997, McCarthy et al. 1999, Nakopoulou et al. 2002a, Schrohl et al. 2003, 2004). The association between high tumor-tissue TIMP-1 levels and a poor patient prognosis applies both to mRNA levels (Ree et al. 1997, Nakopoulou et al. 2002a) and protein levels (McCarthy et al. 1999, Schrohl et al. 2003, 2004) of the inhibitor. So far, all published data come from retrospective studies and all except for one are of relatively small size. The studies performed so far will be described briefly in the following section.

In a study conducted by Ree et al. (1997) the expression of TIMP-1 mRNA was assessed by Northern blotting in human primary breast carcinomas from 34 patients and TIMP-1 mRNA levels were correlated with known clinicopathological prognostic parameters and with clinical outcome. It was demonstrated that the presence of high levels of TIMP-1 mRNA were associated strongly with early death. In addition, the level of TIMP-1 mRNA correlated significantly with nodal status and development of distant metastases, whereas no correlation was found to other prognostic markers such as age, tumor size, grade of malignancy or hormone receptor status, suggesting that TIMP-1 can provide independent prognostic information. In situ hybridization studies have supported the independent prognostic value of TIMP-1 in breast cancer by semi-quantitative estimation of the expression of inhibitor mRNA levels (Nakopoulou et al. 2002a). In 117 invasive breast carcinomas high levels of TIMP-1 mRNA correlated significantly with the presence of lymph node metastases and short survival but not with other known prognostic indicators such as menopausal status, tumor size and grade of malignancy (Nakopoulou et al. 2002a).

Prognostic studies of total TIMP-1 protein levels have supported the association between high tumor-tissue TIMP-1 levels and a poor prognosis in breast cancer. McCarthy et al. (1999) assessed the levels of TIMP-1 protein by ELISA in cytosol extracts from 139.
breast carcinomas, 15 fibroadenomas and 15 nodal metastases. The group found that the concentration of TIMP-1 was significantly higher in breast carcinoma and lymph node metastases compared with the benign fibroadenomas. Furthermore, it was demonstrated that patients with carcinomas containing high levels of TIMP-1 had shorter disease-free survival as well as shorter overall survival. Also by employing ELISA, Schrohl et al. (2003) investigated the level of TIMP-1 in 341 tumor tissue extracts from patients suffering from primary breast cancer. It was shown that high tumor tissue levels of TIMP-1 were significantly associated with shorter overall survival and shorter recurrence-free survival (Schrohl et al. 2003). In multivariate analysis including nodal status, tumor size, hormone receptor status and grade of malignancy as well as the strong prognostic factors uPA and PAI-1, TIMP-1 still significantly predicted prognosis (Schrohl et al. 2003). Survival curves based on the total TIMP-1 measurements from this study are shown in Fig. 2. It is evident that patients with the highest tumor levels of TIMP-1 have significantly shorter recurrence-free survival compared with patients with lower levels of TIMP-1.

The largest study performed on the protein level to date included 2984 cytosol preparations from tumor tissue obtained from patients suffering from primary breast cancer. In agreement with the studies mentioned above, high levels of TIMP-1 protein as determined by ELISA were shown to be significantly associated with both recurrence-free survival and overall survival (Schrohl et al. 2004). Moreover, this study supported the findings that TIMP-1 may add prognostic information to that obtained from the classical prognostic factors as well as from the information obtained from uPA and PAI-1 measurements. Taken together, these studies indicate that TIMP-1 carries independent prognostic information in primary breast cancer; high tumor-tissue levels of TIMP-1 mRNA and TIMP-1 protein are associated with poor prognosis of breast cancer patients. Therefore, TIMP-1 may be a new putative prognostic factor, which may improve the risk classification of patients with primary breast cancer in combination with other prognostic factors including the emerging strong prognosticators uPA and PAI-1; however, since all studies are retrospective and most are of relatively small sizes, future studies should be aimed at validating these findings.

All of the studies described above have dealt with the total level of TIMP-1 (uncomplexed TIMP-1 and TIMP-1–MMP complexes). Interestingly, it has recently been demonstrated that including measurements of the uncomplexed fraction of TIMP-1 significantly improved the prognostic stratification based on TIMP-1 measurements (Würtz et al. 2005). This study showed that a score including information on total TIMP-1 as well as uncomplexed TIMP-1, thereby reflecting the level of TIMP-1–MMP complexes in the tumor was a more precise estimate of prognosis compared with

![Survival curve](image)

**Figure 2** Survival curves showing the association between tumor-tissue TIMP-1 and recurrence-free survival. Patients were divided into two groups: the first group was patients with TIMP-1 tumor-tissue levels of <22 ng/mg protein, the second group was patients with TIMP-1 tumor-tissue levels of >22 ng/mg protein. The number of events in each group during the period and the number of patients at risk after each 24-month interval is shown. HR = Hazard Ratio.
total TIMP-1 alone. This study thus raises the hypothesis that it is the tumor level of MMPs that determines prognosis rather than TIMP-1 itself.

Conclusions and future directions

In conclusion, several studies have demonstrated that the expression of TIMP-1 is significantly increased in breast cancer tissue and, in spite of the MMP-inhibitory effect of TIMP-1, this increase has in several cases been demonstrated to be associated with a poor patient prognosis. This paradox could merely be explained by a co-upregulation of TIMP-1 and the MMPs. However, the discovery of TIMP-1 as a multifunctional protein possessing several tumor-stimulating functions as discussed in this review may also be a reasonable explanation. So far, most prognostic studies of TIMP-1 have been concerned with only the total level of the inhibitor (Ree et al. 1997, McCarthy et al. 1999, Nakopolou et al. 2002a, Schrohl et al. 2003, 2004). However, as mentioned above a recent study has shown that including information on the level of uncomplexed TIMP-1 in combination with information on total TIMP-1 levels significantly improves the prognostic value of TIMP-1 (Würtz et al. 2005). Thus, future studies are needed to validate these findings.

So far, the prognostic evaluation of TIMP-1 in breast cancer has been based primarily on measurements in tumor-tissue extracts. As a result of more sensitive diagnostic methods and thus earlier detection of the disease, tumors of still smaller size are obtained from the patients and this makes it increasingly difficult to acquire access to frozen tumor samples. In addition, the use of tumor tissue for tumor-marker measurements can be criticized because of the heterogeneity of this material. Therefore, future studies should be aimed at determining the prognostic value of TIMP-1 levels measured in blood, which is a more preferable type of specimen compared with tumor tissue. Sample collection is easier and less invasive and the need for histological evaluation of the tissue to be analysed does not apply to blood samples. Furthermore, the problem of tissue heterogeneity would be encompassed, as blood is a more homogenous material. Maybe most importantly, the possibility of taking blood samples during follow up of patients after primary surgery allows sequential measurements of TIMP-1.

Preliminary data have indicated that plasma TIMP-1 levels may carry important clinical information in breast cancer (Holten-Andersen et al. 1999). However, it should also be mentioned that other data have failed to demonstrate a difference in the plasma TIMP-1 levels between primary breast cancer patients and healthy controls (Holten-Andersen et al. 2002). Future studies should be directed at validating the potential prognostic value of TIMP-1 measurements in plasma.

Finally, the discovery of TIMP-1 as a multifunctional protein possessing several tumor-stimulating functions as discussed above suggests that TIMP-1 could represent a novel target for anticancer treatment.

Acknowledgements

This work was supported by the Danish Cancer Society, the Foundation of Clinical-Experimental Cancer Research, Especially Concerning Breast Cancer, the Danish Medical Research Counsel, Beckett Foundation, the Danish Cancer Research Foundation, Eva og Henry Frænkel Foundation, Grosserer Valdemar Foersom og hustru Tharya Foersoms Foundations, Ib Henrikss Foundation, Kathrine og Vigo Skovgaard Foundation, Knud og Dagny Gad Andresens Foundation, P.A. Messerschmidt og Hustrus Foundation, and IMK Foundation.

Funding

There is no conflict of interest that would prejudice impartiality or be a potential conflict of interest for the authors.

References


Chesler L, Golde DW, Bersch N & Johnson MD 1995 Metalloproteinase inhibition and erythroid potentiation are independent activities of tissue inhibitor of metalloproteinases-1. Blood 86 4506–4515.


Schrohl AS, Christensen IJ, Pedersen AN, Jensen V, Mouridsen H, Murphy G, Foekens JA, Brünner N & Holten-Andersen MN 2003 Tumor tissue concentrations of the protease inhibitors tissue inhibitor of metalloproteinases-1 (TIMP-1) and plasminogen activator inhibitor type 1 (PAI-1) are complementary in determining prognosis in primary breast cancer. *Molecular & Cellular Proteomics* **2**:164–172.


Wang T, Yamashita K, Iwata K & Hayakawa T 2002 Both tissue inhibitors of metalloproteinases-1 (TIMP-1) and TIMP-2 activate Ras but through different pathways. *Biochemical and Biophysical Research Communications* **296**:201–205.


