Published in final edited form as:
Cancer Metastasis Rev. 2008 March ; 27(1): 57–66.

The tumor microenvironment: regulation by MMP-independent effects of tissue inhibitor of metalloproteinases-2

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Abstract

Proteolytic remodeling of the extracellular matrix is an important component of disease progression in many chronic disease states and is the initiating event in the formation of the tumor microenvironment in cancer. It is the balance of extracellular matrix degrading enzymes, the matrix metalloproteinases (MMPs) and their endogenous inhibitors that determine the extent of tissue remodeling. Unchecked MMP activity can result in significant tissue damage, facilitate disease progression and is associated with host responses to pathologic injury such as angiogenesis and inflammation. The tissue inhibitors of metalloproteinases (TIMPs) have been shown to regulate MMP activity. However, recent findings demonstrate that the tissue inhibitor of metalloproteinases-2 (TIMP-2) inhibits the mitogenic response of human microvascular endothelial cells to growth factors, such as VEGF-A and FGF-2 *in vitro* and angiogenesis *in vivo*. The mechanism of this effect is independent of metalloproteinase inhibition. Our lab is the first to demonstrate a cell-surface signaling receptor for a member of the TIMP family and suggest that TIMP-2 functions to regulate cellular responses to growth factors. These new findings are discussed in terms of a model of TIMP-2 regulation of cellular functions in the tumor microenvironment.

Keywords

Tissue inhibitor of metalloproteinase; TIMP-2; Anti-angiogenic; Anti-tumorigenic; Cellular differentiation; Cancer therapy

1 Extracellular matrix remodeling and chronic disease

Many chronic disease states are characterized by an imbalance between tissue destruction and endogenous mechanisms of tissue repair, potentially resulting in a vicious cycle of continued and expanding cellular injury coupled with incomplete repair or resolution. These pathologic conditions frequently disrupt the function and structural organization of both the parenchymal (cellular elements) and connective tissue stroma, such that the injury cannot be repaired by simple regeneration of the parenchymal elements alone. The resulting tissue damage is further complicated by host responses elicited during the initial pathologic insult. In cancer progression, the initial proteolytic remodeling of the extracellular matrix signals the progression of tumor and host responses that results in the formation of a pathologic milieu often referred to as the tumor microenvironment.

This tumor microenvironment is composed of a variety of cell types that includes not only tumor cells and stromal fibroblasts but also cells derived from a variety of host responses. One such host response is the proliferation of new blood vessels, termed angiogenesis, and is frequently associated with chronic diseases such as psoriasis, rheumatoid arthritis and cancer.

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The initiation of new blood vessel formation is itself due to a shift in the local balance of pro-angiogenic factors and endogenous inhibitors of angiogenesis, i.e. the angiogenic switch [1, 2]. The angiogenic response and associated host responses that constitute the tumor microenvironment may exacerbate the underlying local pathology, such as in medullary (inflammatory) carcinoma of the breast or in disease progression, i.e. cancer metastasis.

Pro-angiogenic factors, such as angiogenic growth factors VEGF-A and FGF-2, induce the expression of matrix-degrading proteinases whose activity results in remodeling of the extracellular matrix to facilitate invasion of new blood vessels and formation of the tumor microenvironment [1,3]. Inhibition of proteinase activity can result in diminution of the angiogenic response, which in some disease states can result in resolution of the underlying pathology and/or arrest disease progression. This finding suggests that protease inhibitors could be a novel therapeutic approach in the treatment of chronic diseases such as cancer. However, the translation of this strategy to the treatment of human cancer has been disappointing [4]. The reasons for this failure remain unclear, but suggest that our understanding of the molecular and cellular events involved in tissue remodeling and host responses such as angiogenesis, are at best incomplete. Further understanding of the mechanisms of tissue homeostasis and repair should lead to novel therapeutic strategies for the treatment of both chronic inflammatory and malignant diseases.

2 TIMPs: MMP inhibitors and activators

Members of the matrix metalloproteinase (MMP) family have been shown to mediate both tissue development (organogenesis) and remodeling. Collectively, the 24 members of the mammalian MMP family can degrade all components of the extracellular matrix, and many of these protease activities have been specifically associated with pathologic tissue destruction in chronic diseases such as cancer and arthritis. The role of metalloproteinases in cancer, inflammation and other diseases have been reviewed elsewhere [3–7].

The *T*issue *I*nhibitors of *M*etallo*P*roteinases, or TIMPs, have been identified in species ranging from drosophila, zebra fish and *C. elegans* to humans, suggesting that these proteins are ancient eukaryotic proteins [8–10]. Furthermore, recent studies have shown developmental defects in TIMP-deficient organisms, in both non-mammalian and mammalian systems, suggesting the importance of these proteins during embryonic development, as well as possible functional redundancy of some TIMPs in mammalian development [11–14].

The mammalian TIMP family has four members, which share significant homology and structural identity at the protein level. The features of the TIMP family members are described in Table 1 and have been reviewed in detail elsewhere. [8–10]. TIMP-2 is unique as a member of the TIMP family in that in addition to inhibiting MMPs TIMP-2 selectively interacts with MT1-MMP to facilitate the cell-surface activation of pro-MMP-2 [15]. Thus, TIMP-2 functions both as an inhibitor of MMPs, and is required for the cellular mechanism of pro-MMP-2 activation.

TIMP-2 also has a distinct gene structure compared with the other three members of the TIMP family. An interesting relationship exists between the TIMPs and the synapsin gene family in that three members of the TIMP family are nested within the synapsin genes [16–18]. The synapsin 1 gene nests TIMP-1, synapsin 2 nests TIMP-4 and synapsin 3 nests TIMP-3. TIMP-2 is the only member of the TIMP family that is not nested within a gene of the synapsin family. The synapsin-TIMP gene nesting relationship began phylogenetically as far back as *Drosophila* [18]. A recent report describes a nested gene within the very large (~60 kb) first intron of the TIMP-2 gene [19], known as DDC8 [20]. Furthermore, these authors demonstrate that the brain of the TIMP-2 knock out mouse described by Wang et al. contains TIMP-2 mRNA

encoding exons 2–5 downstream of DDC8, suggesting alternative splicing between these two genes [20,21].

3 Identification of non-MMP-dependent TIMP functions

Sequencing of the cDNA clone for TIMP-1 revealed identity of this metalloproteinase inhibitor with erythroid-potentiating activity (EPA) [22]. EPA was identified as a T lymphoblastic factor present in serum that supports the growth of erythroid precursors *in vitro* by a mechanism involving direct cell surface binding [23]. EPA potentiates erythropoietin (EPO)-stimulated colony formation by early or late erythroid stem cells (BFU-E or CFU-E). Subsequently, TIMP-2 was also shown to have EPA, suggesting that this biological activity may be attributed to a common structural element, which remains to be identified [24]. However, some investigators felt that the dual EPA and MMP inhibitor functions for these proteins were "incongruous" [25]. The argument for this conclusion was principally based on the observation that TIMPs are ubiquitously expressed and that the *in vivo* plasma concentration of TIMP-1 at approximately 17 nM was well above the concentration of 80 pM required for the maximal physiologic effect of EPA. The physiologic significance of EPA and its relationship to other TIMP functions remain unresolved. Although TIMPs have been principally viewed as functioning exclusively as MMP inhibitors, there remains a significant body of data, starting with the original cloning of TIMP-1, which suggests TIMPs may have other biological functions.

Hayakawa and colleagues were the first to report that TIMP-1 present in serum acted as a growth factor to support proliferation *in vitro* of a variety of cell types that included both normal mesenchymal and epithelial cells, as well as several tumor cell lines [26]. In these experiments depletion of TIMP-1 from the bovine serum used in cell culture was necessary to observe the growth effects of TIMP-1. Interestingly, TIMP-2 did not stimulate cell growth in these experiments, suggesting that these effects are TIMP-1 specific, although the requirement for MMP inhibitory activity was not examined. We and others have clearly demonstrated that TIMP-1 can inhibit apoptosis in a variety of cell types from Burkitt lymphoma cells [27–30] to breast cancer cells[31–34]. However, the mechanism of this effect seems to be cell type specific.

TIMP-3 has been shown to promote apoptosis in several *in vitro* systems [8,9]. It remains unclear if this effect is mediated independent of MMP inhibition by TIMP-3. Recent findings in TIMP-3-null mice suggest that TIMP-3 can either promote or inhibit apoptosis depending on the model system examined [12,13]. *In vivo* data now show that TIMP-3 deficient mice have an increase in TNF-α converting enzyme (TACE) activity that in a liver regeneration model results in chronic hepatic inflammation and failure of the liver to regenerate [35]. These results suggest that the effects of TIMP-3 on cell fate are mediated by inhibition of metalloproteinase activity, in this case TACE, also known as ADAM17, and not a member of the MMP family. Interestingly, TIMP-3 also functions as a direct antagonist of the VEGFR2, resulting in inhibition of angiogenesis, a function that is clearly independent of MMP inhibition [36]. TIMP-4 reportedly enhances or inhibits the *in vivo* growth of tumor xenografts, however, the mechanism of these effects has not been described [37,38]. It has recently been demonstrated that although TIMP-4 does inhibit endothelial cell migration *in vitro* it does not inhibit FGF-2-induced angiogenesis in the chick chorioallantoic assay [39].

TIMP-3 is unique amongst the TIMP family in that it has been shown to specifically interact with sulfated glycosaminoglycans and as a result is sequestered in the extracellular matrix [40], the other TIMP family members remain soluble and diffusible. Although the biological significance of the matrix association of TIMP-3 has not been determined, it does suggest that its pericellular distribution and availability to interact with cell surface proteins may be more

restricted than other members of the TIMP family. Furthermore, many *in vitro* studies of TIMP-3 cellular functions have been conducted by addition of soluble, exogenous recombinant TIMP-3 and have not addressed the role of matrix binding which is a unique feature of this inhibitor.

The central issue regarding all of these potential biological activities of the TIMPs is: “Are they unique biological activities of these proteins or are they dependent on inhibition of metalloproteinase activity?”

4 TIMP-2: separation of MMP-inhibitory activity from growth regulatory activity

By virtue of their ability to inhibit metalloproteinase activity members of the TIMP family should function as inhibitors of angiogenesis. This general principal is supported by the demonstration that TIMP-1, TIMP-2 and TIMP-3 all demonstrate anti-angiogenic activity *in vitro* and *in vivo* [41,42]. However, this concept is confounded by the recent demonstration that TIMP-4 did not inhibit FGF-2 induced angiogenesis *in vivo* [39]. Some evidence suggests that the anti-angiogenic effects of TIMPs may be functionally distinct [43]. The synthetic MMP inhibitor (BB94) effectively blocked angiogenesis in a murine hemangioma model *in vivo* [44], suggesting that inhibition of MMP activity, either by TIMPs or synthetic MMP inhibitors, was sufficient to block angiogenesis *in vivo*. Combined with additional evidence that synthetic MMP inhibitors could block tumor cell invasion, tumor growth and reduce metastasis formation, these data provided strong support for the development of synthetic MMP inhibitors for the treatment of human cancer [44]. Unfortunately, the enormous industrial effort involved in the development and preclinical testing of synthetic MMP inhibitors has not produced significant results in clinical trials with cancer patients [4], although the reasons for this failure are not completely understood.

In 1990, Moses and colleagues isolated and characterized a novel anti-angiogenic agent from bovine cartilage, the cartilage-derived inhibitor (CDI) of angiogenesis [45]. CDI co-purified with MMP inhibitor activity, and was shown to inhibit angiogenesis *in vivo*. In addition CDI inhibited endothelial cell proliferation in response to FGF-2 stimulation *in vitro* and blocked endothelial cell migration. The N-terminal amino acid sequence of CDI suggested that it was TIMP-related, showing close identity with TIMP-2 [45].

Subsequently, both TIMP-1 and TIMP-2 were shown to inhibit polyamine-stimulated angiogenesis in the chick chorioallantoic membrane assay, but the effects on endothelial cell proliferation and/or migration were not examined [46]. It is interesting to note that in these experiments, both TIMP-1 and TIMP-2 inhibited stimulated angiogenesis (polyamine-dependent) but did not alter vascular development in non-stimulated 3 day-old chick chorioallantoic membrane assays. This suggests that the process defined as vasculogenesis (*de novo* development of an organized vascular system) is functionally distinct from angiogenesis (development of new vessels form existing vasculature), with respect to both requirements for MMP activity and sensitivity to TIMP inhibition. Together, the experiments of Moses and Hayakawa suggested that although both TIMP-1 and TIMP-2 inhibit angiogenesis, the mechanism of these effects might be different.

To address this issue Murphy et al. examined the ability of both TIMP-1 and TIMP-2 to inhibit endothelial cell proliferation and migration [47]. These experiments demonstrated that TIMP-2, but not TIMP-1 or the synthetic MMP inhibitor, BB-94, inhibited the FGF-2-stimulated proliferation of human endothelial cells. This inhibitory effect was not observed using a pro-MMP-2/TIMP-2 complex suggesting that only free TIMP-2 was capable of inhibiting endothelial cell growth in response to FGF-2 stimulation. Furthermore, TIMP-2

slightly inhibited endothelial cell migration in response to FGF-2 stimulation and appeared to promote endothelial cell adhesion. Comparison of these effects of TIMP-2 with the lack of effect of either TIMP-1 or BB-94 led us to conclude that the effects of TIMP-2 on endothelial cell proliferation were unique biological activities of this member of the TIMP family, independent of MMP inhibitory activity and possibly mediated by cell surface receptor mechanism.

Following these observations, investigations began to focus on interaction of TIMP-2 with the cell surface. However, with the demonstration of a cell surface mechanism for activation of pro-MMP-2 by MT1-MMP that is mediated by interaction of TIMP-2 with MT1-MMP [8–10], it became evident that MT-MMPs represent a cell surface-binding site for TIMP-2. This finding suggests that one possible mechanism through which TIMP-2 could influence cell growth was by binding to MT1-MMP that would act as a signaling receptor. However, no evidence has been forthcoming to demonstrate that MT1-MMP can act as a signaling receptor to suppress endothelial cell proliferation.

In 1999, Wingfield et al reported a novel TIMP-2 mutant devoid of MMP inhibitory activity [48]. For the first time this mutant allowed investigators to assess the requirement for MMP inhibitory activity in TIMP-2-mediated suppression of cell growth. This mutant also allowed us to ultimately identify potential TIMP-2 binding sites on the cell surface that did not contain an MMP active site, vide infra. Preparation of this mutant was accomplished through appending a single amino acid, alanine, to the amino-terminus of TIMP-2 to produce Ala+TIMP-2 [48]. Ala+TIMP-2 does not inhibit MMP-2 or MT1-MMP activity or mediate MT1-MMP activation of pro-MMP-2 [48,49]. Furthermore, the inability of Ala+TIMP-2 to inhibit MMP activity was reversible by treatment with aminopeptidase activity that removes the amino-terminal Ala residue [48]. This suggests that other than the single Ala residue at the amino-terminus, the remainder of the TIMP-2 is correctly folded and capable of inhibiting MMP activity once the Ala residue blocking the amino terminus is enzymatically removed.

Subsequently, this Ala+TIMP-2 mutant was used to explore the interaction of TIMP-2 with the cell surface. Both TIMP-2 and Ala+TIMP-2 bound to the surface of human A549 lung cancer cells with very high affinity (K_d=147 pM) and this binding was not competed by the synthetic MMP inhibitor BB94 or TIMP-1 [49]. Furthermore, the binding of Ala+TIMP-2 showed only partial competition by MT1-MMP blocking antibodies, and immunofluorescence co-localization studies demonstrated that TIMP-2 and Ala+TIMP-2 binding was, at least in part, independent of MT1-MMP. These findings have been confirmed by investigators who have shown two distinct cell surface binding sites for TIMP-2 [50], cell-surface binding of TIMP-2 is independent of the level of MT1-MMP expression [51], and not all cell surface-bound TIMP-2 can be competed by synthetic MMP inhibitors [50].

More recently, Moses and colleagues definitively demonstrated uncoupling of the MMP-inhibitory activity and anti-angiogenic activity of TIMP-2 [52]. This was accomplished using *Pichia pastoris* expression system to engineer and produce both the N-terminal and C-terminal domains of TIMP-2. These authors found that although both domains of TIMP-2 inhibited angiogenesis in the embryonic CAM assay, the c-terminal domain and wild type TIMP-2 were more effective inhibitors of angiogenesis in the mouse corneal pocket assay (in which angiogenesis is driven by addition of exogenous pro-angiogenic mitogens) than the N-terminal TIMP-2 domain. Furthermore, the ability of the N-terminal domain was dependent on MMP-inhibitory activity, as blocking the amino-terminus of the TIMP-2 amino-terminal fragment by appending glutamic acid (E) and alanine residues (A) reversed the MMP-inhibitory activity and *in vivo* anti-angiogenic activity of this TIMP-2 domain [52]. The activity of TIMP-2 that inhibits endothelial cell proliferation was localized to the carboxy-terminal domain of TIMP-2, specifically to the carboxy-terminal disulfide loop, referred to as loop 6 [52]. It is interesting

to note that this region is encoded by exon 5 of the TIMP-2 gene, and as noted above the TIMP-2 deficient mice may produce alternative splice variants of the DDC8 gene that could express proteins containing a TIMP-2 loop 6 structure [20]. This may in part explain the benign phenotype of this knock out mouse strain.

The binding of TIMP-2 to the endothelial cell surface and the ability to inhibit endothelial cell proliferation were shown to be independent of MMP inhibition as demonstrated by Ala +TIMP-2 [53]. The binding of TIMP-2 to the human microvascular endothelial cell surface was saturable and reversible with a dissociation constant on the order of 900 pM. The binding of TIMP-2 to the endothelial cell surface did not antagonize growth factor (FGF-2 or VEGF-A) binding, was not competed by growth factor receptor blocking antibodies (anti-VEGFR-2 or anti-FGFR-1) or MT1-MMP blocking antibodies. Competition binding studies demonstrated that TIMP-2 binding to the surface of human microvascular endothelial cells could be competed by anti-β1 and anti-α3 integrin blocking antibodies. The interaction of TIMP-2 with α3β1 cell surface integrin was confirmed by immunoprecipitation experiments in which anti-TIMP-2 antibodies resulted in selective co-immunoprecipitation of α3β1 integrin. The ability of TIMP-2 to inhibit growth in response to tyrosine kinase growth factor stimulation was dependent on cell surface expression of β1 integrin subunits, as demonstrated using β1-null fibroblasts.

Subsequent studies have demonstrated that TIMP-2 or Ala+TIMP-2 binding via α3β1 results in G1 growth arrest and enhanced *de novo* expression of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ [54]. Further studies revealed that TIMP-2 and Ala+TIMP-2 enhanced the expression of the reversion-enhancing-cysteine-rich protein with Kazal motifs, also known as RECK [55]. TIMP-2 induction of RECK expression was shown to be mediated by inhibition of Src kinase activity resulting in an altered pattern of paxillin phosphorylation at residues 31 and 118 [56]. This altered phosphorylation results in inactivation of the small G protein Rac1 and a reciprocal activation of the small G-protein Rap1, resulting in loss of a migratory phenotype. Collectively, these findings suggest that TIMP-2 inhibits angiogenesis by inducing endothelial cell differentiation to a quiescent state. These findings are summarized in Fig. 1.

5 TIMP tissue distribution: clues to new functions?

Few studies have examined the expression and localization of TIMPs in adult tissues. However, we do know from many *in vitro* studies that in many cells the transcriptional activation of TIMP expression is differentially regulated. In most cell types expression of TIMP-2 is constitutive, whereas TIMP-1 and TIMP-3 expression can be induced by a variety of growth factors and cytokines.

A recent study utilizing quantitative PCR demonstrated essentially ubiquitous and abundant expression of all four mammalian TIMPs in most mouse tissues [57]. TIMP-2 was constitutively expressed at high levels in all tissues of the adult mouse, with the expression of the other three TIMPs demonstrating more selective patterns of tissue distribution. These patterns for tissue expression of TIMPs are identical to previous studies and the constitutive high-level expression of at least one TIMP family member in each organ of the adult mouse suggests that TIMPs “provide a crucial checkpoint for tissue degradation” [57]. Although this study did not localize cellular expression of the TIMPs, previous studies of TIMP-2 expression by *in situ* hybridization suggest selective expression in the stromal compartment, with complete absence of TIMP-2 transcripts in epithelial cells [58]. It should be pointed out that although TIMP concentrations may be significant in some “normal” tissues, the expression of active MMP species in “normal tissues” is usually very low or nonexistent [57]. This raises the question: “What is the functional role of TIMPs in normal tissues lacking MMP activity or evidence of active extracellular matrix remodeling?” We propose that these observations are

consistent with TIMP-2 functioning in the absence of MMPs to maintain cellular differentiation and tissue homeostasis.

Consistent with this hypothesis is the recent work of Jaworski and colleagues who have demonstrated that TIMP-2 inhibits growth and promotes neurite differentiation *in vitro* via an α3β1 integrin dependent mechanism [59]. Interestingly, in this system cell cycle arrest also occurs in G1 but appears to be mediated by enhanced expression of the cyclin-dependent kinase inhibitor $p21^{Cip}$, not $p27^{Kip1}$ as we observed in the endothelial cell system. These authors extended this work to demonstrate that TIMP-2 expression correlates with the appearance of microfilament positive neurons and that live cell labeling experiments show TIMP-2 association only with α3 integrin positive cells [60]. These observations led the authors to suggest that up-regulation of TIMP-2 expression by proliferative stimuli implicates TIMP-2 expression in the transition from neuronal proliferation to promotion of terminal neuronal differentiation. This concept is further supported by their subsequent demonstration that TIMP-2 KO mice have abnormal motor deficits, and shows for the first time a significant phenotype for these TIMP-2 deficient mice [61].

6 Model for TIMP-2 in modulating the tumor microenvironment

It is now evident that the matrix metalloproteinase inhibitor TIMP-2 has multiple functions that include inhibition of MMP activity, mediating the cell surface activation of pro-MMP-2 by MT-1-MMP, as well as the metalloproteinase inhibitory independent function of promoting cellular differentiation that is mediated by binding to its cell surface receptor α3β1. The finding that TIMP-2 promotes cellular differentiation *in vivo* via a mechanism that is independent of MMPs implies a new function for this member of the TIMP family.

These observations suggest the following model for the bifunctional role of TIMP-2 in the tumor microenvironment presented in the context of the angiogenic response (Fig. 2). In quiescent normal tissues the levels of TIMP-2 are sufficient to allow free, uncomplexed TIMP-2 to accumulate in the pericellular milieu. The source of this TIMP-2 may be stromal fibroblasts, perivascular smooth muscle or endothelial cells, all of which have been shown to synthesize and secrete TIMP-2 *in vitro*. In these quiescent adult tissues the growth suppressing activity of TIMP-2 is functional through binding to available α3β1 integrin receptors on fibroblasts and endothelial cells. In this scenario TIMP-2 functions to suppress fibroblast and endothelial cell responses to transient or minor fluctuations in angiogenic growth factors, i.e. VEGF-A and/or FGF-2.

However, during tumor progression there is an increase in secretion and activation of MMPs produced by either the tumor cells themselves or tumor-associated fibroblasts. The secretion of these proteases initiates the formation of the tumor microenvironment. The local increase in MMP activity is initially counteracted by the MMP inhibitory activity of endogenous TIMP-2, at the expense of TIMP-2 cell differentiating activity. We speculate that this may facilitate activation of the tumor associated fibroblasts, which may also contribute to the evolving tumor microenvironment. As the tumor progresses, more MMPs are produced overwhelming the local TIMP-2 concentration and contributing to extensive remodeling of the ECM. Furthermore, continued tumor growth leads to tissue hypoxia and MMP-mediated release of matrix sequestered angiogenic factors. These events promote the tumor angiogenic response, which also requires MMP activity. As recently demonstrated, endothelial cells responding to angiogenic factors, such as VEGF-A, decrease the synthesis and secretion of TIMP-2 [62]. This further promotes the local decline in TIMP-2 concentrations and potentiates the proteolytic remodeling of the extracellular matrix. Thus, in this model TIMP-2 initially functions as a rheostat by limiting cellular responsiveness to stimuli leading to cellular proliferation, cellular activation and extracellular matrix remodeling. However, as the tumor

microenvironment evolves TIMP-2 function shifts to inhibition of MMP activity, and further depletion of TIMP-2 levels may even facilitate TIMP-2 function in the MT-1-MMP activation of pro-MMP-2.

The primary question that arises is if TIMP-2 functions to maintain tissue homeostasis, why do TIMP-2 deficient mice reproduce normally and show no overt vascular defects, only a motor deficit phenotype?[61] With respect to vascular development in mammals we do know that this process is regulated in a fashion distinct from angiogenesis in the adult. Vascular development occurs through the process of vasculogenesis, which is functionally distinct from angiogenesis [63]. We propose that either: (1) TIMP-2 plays no direct role in vasculogenesis; or (2) that other members of the TIMP family compensate for the loss of TIMP-2 MMP inhibitory activity during embryonic vascular development. Although no developmental defects are observed in TIMP-2 deficient mice [21,64], a recent report demonstrates that in zebra fish the single TIMP expressed during development is most homologous to TIMP-2 and that ablation of TIMP-2 expression results in abnormal zebra fish development [11]. This finding suggests that in mammalian systems other TIMPs may compensate for the loss of TIMP-2 function. Angiogenesis on the other hand is a process limited to adult tissues responding to a pathologic stimulus. Therefore, the question should be: “Do TIMP-2 deficient animals have a normal or abnormal angiogenic responses to tissue injury?” To our knowledge such experiments have not yet been reported.

The next question that arises is can TIMP-2 be used as a therapeutic in the treatment of cancer? If the concentration of TIMP-2 or even better Ala+TIMP-2, which would not be sequestered by MMP active sites, could the cellular activation of host responses contributing to the tumor microenvironment, such as fibroblast activation and tumor angiogenesis be suppressed by promoting cellular differentiation. That such a therapeutic approach is possible is supported by the recent report of a TIMP-2 transgenic murine model using the MMTV-Wnt-1 mammary carcinogenesis model [65]. Although exact tissue concentrations of TIMP-2 were not determined, the authors demonstrated that enhanced TIMP-2 expression in the mammary glands of the MMTV-Wnt-1 double transgenic mice resulted in increased tumor latency, ~26% reduction of tumor formation, 18% decrease in tumor cell proliferation and a 12% increase in tumor cell apoptotic rate. Although tumor-associated angiogenesis was reduced in the double transgenics, the authors’ primary focus was demonstrating a role for MMPs in the MMTV-Wnt-1 tumor model. Therefore, possible direct effects of TIMP-2 on cellular elements of the tumor microenvironment were not examined.

7 Summary

Although the concept of MMP-independent functions for TIMPs is not new, the demonstration that TIMP-2 promotes cellular differentiation of endothelial cells and neurons is mediated by cell surface receptors definitively demonstrates that TIMPs have functions other than inhibition of MMPs. Additionally, it has recently been reported that CD63 may function as a cell surface receptor for TIMP-1 and mediate the anti-apoptotic activity in breast cancer [31]. This finding has enormous implications for the role of TIMP-1 in modulating the cellular responses that are involved in formation and maintenance of the tumor microenvironment. Not only can TIMP-1 promote carcinogenesis by directly promoting tumor cell growth and tumorigenicity [31–33, 66], TIMP-1 has also been shown to modulate the immune function [28,30] as well as angiogenesis [67–71].

From their initial identification, TIMPs have been associated with regulation of cell growth and differentiation (i.e. erythroid-potentiating activity, EPA). However, we are now beginning to develop the experimental evidence to define the mechanisms of such activities. The question remains: Do all TIMPs also have cell surface receptors that may mediate regulation of cell

growth or differentiation? TIMP-3 has been shown to bind to VEGFR-2 and function as an antagonist of VEGF-A stimulation [36]. However, the significance of this mechanism in preventing cancer progression is unclear. Cell surface binding for TIMP-4 has been suggested, but identification of specific cell surface receptors has not been forthcoming. Characterization of these receptors and understanding the signaling mechanisms involved will identify new therapeutic targets not just for cancer but also for a variety of other chronic disease states.

8 Conclusions

Recent evidence suggests that TIMPs, particularly TIMP-1 and TIMP-2, may have unique biological properties, independent of their ability to inhibit MMPs. This is supported by the identification of cell surface receptors for these two members of the TIMP family. TIMP-2 impacts the tumor microenvironment by initially promoting cellular differentiation of endothelial cells and fibroblasts, and possibly cells in the epithelial compartment. However, subsequent progression and massive production and activation of MMPs by a variety of cell types that comprise the tumor microenvironment, including tumor cells, endothelial cells, immune cells and tumor-associated fibroblasts, shifts the function of TIMP-2 to metalloproteinase inhibitor. Finally, continued progression and further depletion of TIMP-2 levels could again change TIMP-2 function from MMP inhibitor to activator by facilitating MT-1-MMP activation of pro-MMP-2.

9 Key unanswered questions

Do all four members of the TIMP family have cell surface receptors and if so what unique biological activities do they support?

Can over expression of TIMP-2 within the tumor microenvironment overcome the MMP-mediated activation of the tumor microenvironment and promote cellular differentiation of host cells?

Can over expression of TIMP-2 promote differentiation of the malignant carcinoma cells leading to reversal of the epithelial to mesenchymal transition?

Can addition of TIMP-2 over expression enhance the tumoricidal activity of conventional cytotoxic agents?

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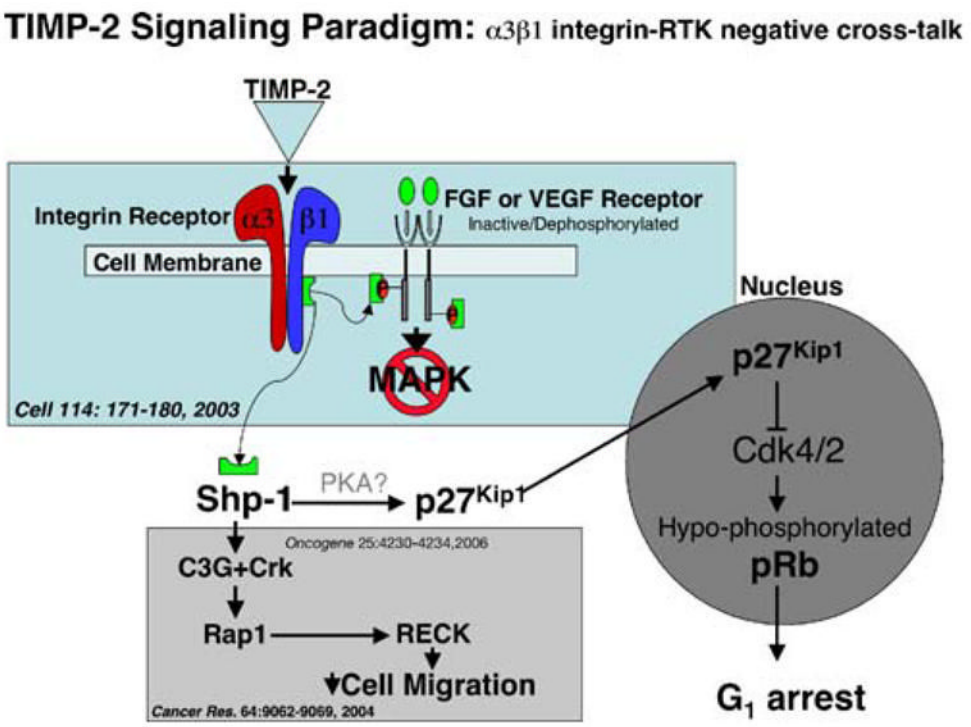

Fig. 1.

Multiple pathways of TIMP-2/α3β1 signaling. TIMP-2 binding to α3β1 initiates receptor tyrosine kinase inactivation via the action of the protein tyrosine phosphatase activity. Cell cycle arrest is mediated by *de novo* synthesis of $p27^{Kip1}$, that down-regulate the cyclin-dependent kinases 4 and 2, resulting in hypophosphorylation of pRb and cell cycle arrest in G1. TIMP-2 also mediates activation of the small G protein Rap1 via a mechanism involving altered association of paxillin scaffolding proteins, guanidine exchange factors and ultimately results in enhanced expression of RECK. This suggests that in addition to arresting cellular proliferation, TIMP-2 also seems to promote expression of cellular differentiation markers

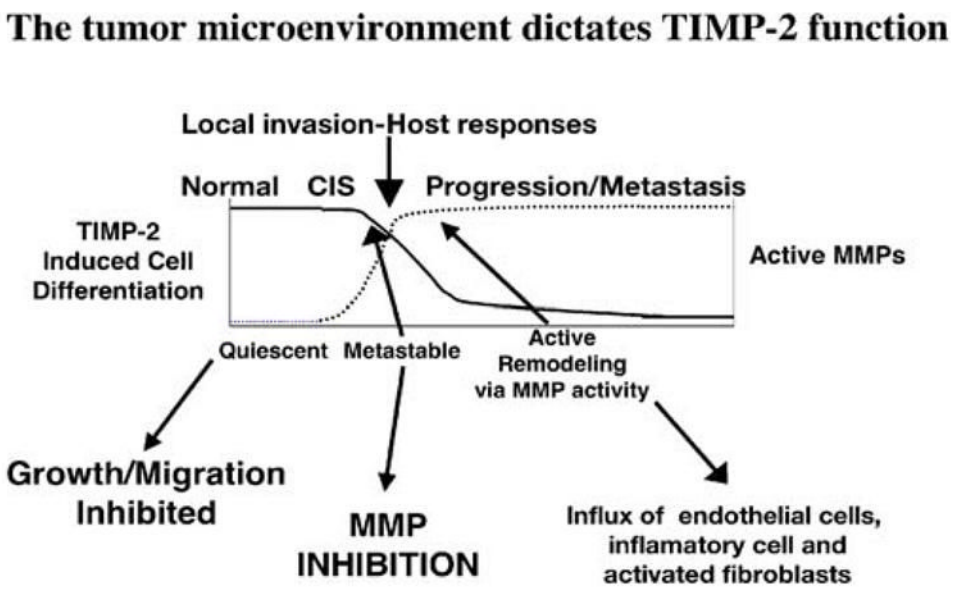

Fig. 2.

TIMP-2 controls cell behavior directly through α3β1 integrin receptors and indirectly by modulating the activity of MMPs. In physiological quiescent states high levels of free TIMP-2 promotes cellular quiescence and maintenance of the differentiated state. This occurs independently of MMP inhibitory action. As local concentrations of angiogenic factors increase, endothelial cells respond by increasing MMP production and limiting TIMP-2 expression. Increasing concentrations of activated MMPs acts as a sink to reduce free TIMP-2 concentrations, limiting interaction of TIMP-2 with α3β1, thus reducing the growth inhibitory effects. As active MMP concentrations continue to increase, TIMP-2 concentrations may be insufficient to completely inhibit MMP activity. At low concentrations TIMP-2 is insufficient to inhibit MMP activity, and actually enhances MMP-2 activation (via MT1-MMP-dependent mechanism) resulting in remodeling of extracellular matrix, facilitating angio-invasion

Table 1

Properties of mammalian TIMPs

Property	TIMP-1	TIMP-2	TIMP-3	TIMP-4	References
Mass (mature protein)	28.5	21	22/27	22	[7–10]
Core protein size (aa residues)	195	194	188	184	[7–10]
Protein expression	Inducible	Constitutive	Inducible	Inducible	[7–10]
N-glycosylation	Yes	No	Yes	No	[8–10,40]
Diffusible/soluble	Diffusible	Diffusible	ECM bound	Diffusible	[8–10,40]
Chromosome location	Xp11.23–11.4	17q23–25	22q12.1–13.2	3p25	[8–10]
Nested genes	Nested in synapsin 1	Contains nested DDC8	Nested in synapsin 3	Nested in synapsin 2	[16–18]
mRNA (kbp)	0.9	1.0/3.5	5.0	1.4	[8–10]
Weak inhibitor of MMP	MT-MMPs	None identified	None identified	None identified	[7–10]
ADAM Inhibition	10	None reported	12,17,10, TS-4, TS-5	None reported	[8–10]
Growth/apoptosis	Inhibits apoptosis	Inhibits growth	Promotes apoptosis	Inhibits growth & promotes tumor growth	[24–26,35–38,43]
Cell surface binding	Nanomolar	Low-nanomolar	Nanomolar	Not reported	[24,26,35–38,43,47,49,53]
Receptor	CD63	α3β1	VEGFR-2, TACE	Not identified	[3135,38,53,59]
Signaling	FAK, PI3K, $p27^{Kip1}$	PTP, Shp-1, $p27^{Kip1}$, $p21^{Cip1}$, Rap1	Antagonist, Inhibit TNF release	Not reported	[31,34,35,38,53,55,56,59]

Summarized in this table are a number of properties of the mammalian TIMP family. Notable distinguishing features for TIMP family members include the ECM binding of TIMP-3 and that TIMP-2 is the only member whose gene is not nested within a synapsin family gene.