Anti-tumoral Properties of Endogenous Angiogenesis Inhibitors: A Case for Continued TIMP-2 Preclinical Development

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Abstract: Dr. Judah Folkman first introduced the concept of anti-angiogenic therapy almost four decades ago. This novel idea has subsequently been supported by extensive research in the multistep process of tumor-associated angiogenesis. As new blood vessels are formed, angiogenesis is switched on within the tumor microenvironment and the physiological balance between endogenous pro-angiogenic and anti-angiogenic factors is disrupted. Since then, angiogenesis inhibitors have been developed as drugs and administered in the clinic as part of anti-cancer therapy. Tissue inhibitor of metalloproteinase 2 (TIMP-2) is an endogenous inhibitor of angiogenesis that was initially discovered through the ability to inhibit matrix metalloproteinase (MMP) activity. Subsequently, TIMP-2 was shown to suppress endothelial cell proliferation and migration through MMP dependent and independent mechanisms. Moreover, recent studies indicate that TIMP-2-mediated inhibition of tumor growth may occur, at least in part, via mechanisms that are distinct from its ability to inhibit MMP activity. TIMP-2 is an essential element of the normal tissue microenvironment in the presence of low levels of MMP expression. However, in tumor tissue TIMP-2 levels are reduced. Recent experiments demonstrate that reconstitution of TIMP-2 expression in tumors not only inhibits tumor angiogenesis, but also acts directly on tumor cells to modulate interactions between the tumor cells and the microenvironment. These recent research findings support the idea that TIMP-2 is an excellent candidate for preclinical development as a novel biological agent for cancer therapy.

Keywords: Anti-angiogenic therapy, endogenous angiogenesis inhibitor, tumor growth inhibition.

INTRODUCTION

Angiogenesis plays an essential role during development and participates in homeostatic events in adulthood such as wound repair and reproduction [1]. Angiogenesis is deregulated in many pathologic conditions described as 'angiogenesis-dependent diseases' including macular degeneration, ischemic heart disease, rheumatoid arthritis, benign vascular neoplasias (hemangiomas) and cancer [2]. It is well documented that tumor growth depends on the formation and recruitment of new blood vessels. This process of tumor angiogenesis results from an increase of proangiogenic growth factors and cytokines released by tumor cells and/or tumor associated inflammatory cells. Proangiogenic factors, such as vascular endothelial growth factor-A (VEGF-A), may also be released from sequestration in the extracellular matrix within the tumor microenvironment by proteolytic activity, as has been reported for matrix metalloproteinase 9 (MMP-9). The increase in proangiogenic influences in the tumor microenvironment is usually accompanied by a decrease in endogenous angiogenic inhibitors, with thrombospondin-1 as the prototypic example, and others such as endostatin, interferon alpha and tissue inhibitor of metalloproteinases (TIMPs). This change in the tumor microenvironment from a dormant avascularized cellular hyperplasia to a vascularized, invasive neoplasia with metastatic potential, is referred to as the "angiogenic switch" [3]. Interestingly, the degree of tumor angiogenesis can vary significantly between tumor types. Most tumors such as glioblastoma, renal, lung, colorectal and various neuroendocrine tumors are highly vascularized and as such are excellent targets for antiangiogenic therapies. In contrast, other cancers such as highly aggressive pancreatic adenocarcinomas are hypovascularized compared to most tumor types. These observations suggest that a series of distinct cellular and extracellular components within the developing tumor microenvironment influence the extent to which the angiogenic response develops following the initial angiogenic switch. Therefore, unraveling the complex biological interactions involved in the angiogenic response specific to individual tumor types may help to develop therapies that more effectively modulate the eventual extent of the angiogenic response.

In the majority of tumors studied, VEGF-A is the most common angiogenic inductive signal, although basic fibroblast growth factor (FGF-2), and interleukin-8 (IL-8) have also been demonstrated as potent tumor angiogenic factors in tumors such as angioblastoma and giant cell tumors in bone [4]. The discovery of proangiogenic molecules led to the development of synthetic angiogenesis inhibitors that target the tumor endothelium and inhibit tumor angiogenesis. In 2004, the Food and Drug Administration (FDA) approved bevacisumab (Avastin), the first antiangiogenic drug for metastatic colon cancer that targets and neutralizes VEGF-A, thereby inhibiting proliferation, migration and survival of vascular endothelial cells within the tumor microenvironment. The FDA has also approved several drugs including sorafenib (Nexavar), sunitinib (Sutent) and pazopanib (Vot-

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rient) that directly target cellular tyrosine kinase (RTK) receptors with proangiogenic activity, or the mammalian target of rapamycin (mTOR) pathway such as everolimus (Afinitor). Studies have shown that these angiogenesis inhibitors are less toxic than many cytotoxic chemotherapeutic drugs, but are not completely without side effects. Since they target normal endothelium and not tumor cells, they also have a lower risk of introducing drug resistance, although tumors may use genetic instability to switch or escape dependence on VEGF-A as a primary angiogenic stimulus [5]. In addition, angiogenesis inhibitors targeting tumor cell RTKs may disrupt tumor cell production of angiogenic factors (referred to as indirect angiogenesis inhibitors) and include drugs such as Tarceva, Ireesa, Herceptin and Erbitux [4, 5]. However, due to the genetic instability characteristic of malignant tumors, further oncogenic mutations in tumor cells are frequently activated following treatment with many current approved antiangiogenic therapeutics leading to drug resistance, resumption of tumor angiogenesis and tumor growth/recurrence [5].

ENDOGENOUS INHIBITORS OF ANGIOGENESIS

The unanticipated development of drug resistance to and/or escape from existing synthetic angiogenesis inhibitors makes it apparent that new antiangiogenic therapies need to be developed for this strategy to become clinically effective over the long term. In fact, over the past 30 years a number of laboratories have identified and reported the existence of several endogenous angiogenesis inhibitors. These inhibitors are intact proteins, protein fragments or steroids naturally produced by various cell types and found in the blood circulation (plasma) or in the extracellular matrix of a variety of normal tissues making them excellent therapeutic candidates with little or no side effects [6].

Dr. Judah Folkman is credited for posing the question: 'Do endogenous angiogenesis inhibitors suppress angiogenesis-dependent disease?' [6]. Indeed, a myriad of studies have demonstrated that endogenous angiogenesis inhibitors are capable of suppressing pathologic angiogenesis in the context of a variety of disease processes (e.g. rheumatoid arthritis, hemangiomas, cancer) [7, 8]. The first endogenous angiogenesis inhibitor to be discovered, interferon alpha, was shown to suppress Fibroblast Growth Factor-2 (FGF-2) overexpression and has been successfully used to inhibit tumor growth in such tumors as malignant melanoma, pulmonary hemagiomatosis, angioblastomas, giant cell tumors of the bone and hemangiomas in newborns and infants [6, 9]. Several other potent angiogenesis inhibitors derive from the proteolytic cleavage of larger proteins within the extracellular matrix or circulation [2]. The first of these to be described was Angiostatin, a 38 kDa fragment of plasminogen that was purified from the urine of tumor bearing mice and demonstrated to be an angiogenesis inhibitor by O'Reilly in the Folkman lab in 1994 [10]. Angiostatin binds to ATP synthetase, angiomotin and annexin II resulting in the inhibition of endothelial cell proliferation and migration. Angiostatin underwent early phase clinical trials but is no longer in use as more potent angiogenesis inhibitors have superseded it. Endostatin, a protein fragment derived from type XVIII collagen was also discovered by O'Reilly in the Folkman laboratory in 1997 and shown to completely suppress tumor-induced angiogenesis and *in vivo* tumor growth of Lewis lung carcinoma and B16F10 melanoma [7]. Significantly, the use of endostatin failed to induce acquired drug resistance. Moreover, the cyclic discontinuation of endostatin, followed by tumor regrowth and reinstitution of endostatin therapy resulted in apparent cures after a few rounds of therapy in several mouse tumor models including Lewis lung and B16 melanoma [11]. Endostatin targets integrin $\alpha5\beta1$ to inhibit endothelial cell proliferation and migration while inducing apoptosis of proliferating endothelial cells. Treatment with endostatin has been tested in Phase I and Phase II clinical trials in the U.S., and is currently approved for clinical use in China [12]. A final example of a protein fragment used as an endogenous angiogenesis inhibitor is tumstatin, first reported by the Kalluri laboratory in 2002 [13]. Tumstatin, a proteolytic product of type IV basement membrane collagen, is generated by the action of MMP-9. This is interesting in that MMP-9 has also been shown to be pro-angiogenic by processing extracellular matrix to release bound VEGF-A, suggesting that MMP-9 has both pro- and anti-angiogenic activities. Additional temporal and kinetic studies are necessary to help delineate when MMP-9 inhibitors would be most effective in disrupting tumor growth during the course of tumor development. Genetic studies by the Kalluri group have also shown that tumors grow 3-4 times faster in mice with a deletion of the alpha chain of type IV collagen that results in a deficiency of tumstatin levels [14]. Conversely, when tumstatin was administered to these type IV collagen deficient animals to produce physiologic levels found in wild type mice, tumor growth slowed dramatically. A reduction in tumor growth was also observed when the tumstatin receptor, integrin $\beta3$, was deleted [15]. Accordingly, measurement of circulating plasma levels of tumstatin have been tested as a biomarker in clinical trials for the efficiency of other therapeutic agents (such as Phase I trials using AZD2171 (Cediranib) in patients with glioblastoma, [16]) but not as a direct antiangiogenic agent alone.

In addition to protein fragments, several intact, full-length extracellular matrix proteins demonstrate antiangiogenic activity leading to inhibition of tumor growth and progression. These include proteins such as fibulin-5, platelet factor 4, pigment epithelial derived factor and thrombospondins (1 and 2) [6, 17]. For example, thrombospondin-1 null mice showed significantly faster tumor growth compared to wild type mice [17], while transgenic expression of thrombospondin-1 or -2 resulted in decreased tumor angiogenesis and reduced tumor growth in mice with squamous cell carcinoma xenografts [8].

The relationship between loss of endogenously expressed angiogenesis inhibitors and proangiogenic agents in terms of their relative contributions to the initiation of the 'angiogenic switch' is poorly understood. To address this issue Kalluri and colleagues examined the role of three endogenous angiogenesis inhibitors in regulating the induction of the angiogenesis switch, as well as maintenance of established tumor angiogenesis [18]. Exogenous administration of endostatin and thrombospondin-1 peptides, but not tumstatin, early in tumor development prevented activation of the "angiogenic switch", in a pancreatic islet cell tumorigenesis mouse model (Rip1/Tag2 model) by counteracting the inductive activity of VEGF-A [19]. These results suggest that endostatin and

thrombospondin peptides would be effective as preventive agents in cancer therapy. In addition, all three peptides, endostatin, tumstatin and thrombospondin peptide, were effective in inhibiting tumor progression at the intervention stage. Moreover, in loss of function studies, it was shown that all three were important in controlling tumor progression and survival. Taken together these experiments demonstrate the potential role of some endogenous angiogenesis inhibitors in controlling the "angiogenic switch" early on in tumorigenesis, and show that these endogenous inhibitors, as well as others, also function to counterbalance the post angiogenic switch proangiogenic stimuli of the developing tumor microenvironment that is necessary to further promote tumor growth, progression and metastasis.

TISSUE INHIBITORS OF METALLOPROTEINASES, CANCER AND ANGIOGENESIS

There are four members in the TIMP family that share significant homology and structure [20, 21]. TIMPs 1-4 are relatively small proteins that by definition inhibit the endoproteolytic activity of MMPs and some members of the adamalysin (ADAMs) family. Collectively these protease activities can degrade most extracellular matrix components, as well as a variety of cell surface receptors [20-22]. Within the context of physiological and pathological conditions, TIMP functioning to suppress tissue remodeling and/or disease progression is primarily attributed to the ability to inhibit MMPs. However, within the last decade several novel biological functions for TIMP family members as well as evidence that some members, such as TIMP-1, may promote disease progression have emerged. The details of many of these mechanisms are not dependent on MMP inhibition, but rather through newly identified TIMP-dependent pathways that have yet to be fully characterized [23, 24]. Indeed, studies have revealed that TIMPs regulate several biological activities including cell growth, migration, invasion, angiogenesis and apoptosis, and that these cellular effects are not mediated via MMP inhibitory activity but instead through newly identified cell surface receptors (reviewed in [21, 24]).

In cancer, MMPs have been associated with disruption of the basement membrane and sub-endothelial extracellular matrix resulting in enhanced tumor invasion, metastasis and angiogenesis [25-27]. TIMP family members can prevent tumor cell migration and invasion (reviewed in [23]). Although there is no evidence that TIMP-4 inhibits endothelial cell proliferation and angiogenesis *in vivo*, TIMPs 1-3 have been shown to be potent endogenous angiogenesis inhibitors *in vitro* and *in vivo* [28]. In a TIMP-1 transgenic mouse model, TIMP-1 suppressed Ehrlich tumor cell growth and neovascularization [29, 30]. Forced expression of TIMP-1 in Burkitt's lymphoma xenografts resulted in an initial increase and subsequent suppression of tumor growth [31]. While tumor regression was associated with a failure of these tumors to develop tumor angiogenesis, the initial enhancement of tumor growth was consistent with the demonstrated ability of TIMP-1 to inhibit tumor cell apoptosis [32, 33]. TIMP-3 has been shown to act as a VEGF-A antagonist by interacting with vascular endothelial growth factor receptor-2, the primary VEGF-A receptor expressed on endothelial cells [34]. This interaction prevents VEGF-A binding to its receptor and therefore inhibits endothelial cell proliferation and survival. A possible interaction between TIMP-3 and angiotensin II type 2 receptor, as identified with yeast two-hybrid screening, may also additively inhibit angiogenesis [35]. In addition, specific TIMP-3 mutations are associated with Sorsby Fundus Dystrophy, an inherited genetic disease characterized by submacular neovascularization and loss of vision [36, 37].

TIMP-2 is a unique member of the TIMP family member in that, in addition to inhibiting MMPs, interaction with MMP-14 (or MT1-MMP) on the cell surface facilitates the activation of pro-MMP-2 [38, 39]. TIMP-2 is also distinct in that it is ubiquitously expressed in most normal tissues and contains a promoter similar to many "housekeeping" genes, suggesting that TIMP-2 may function in normal tissue homeostasis [40]. TIMP-2 was first isolated as a complex with pro-MMP-2 from the conditioned media of human A2058 melanoma [41]. Shortly thereafter, Moses and colleagues reported that cartilage derived inhibitor of angiogenesis (CDI) isolated from bovine cartilage had both MMP inhibitory activity and anti-angiogenic activity [42]. The amino acid sequence of CDI was very similar to that previously published for TIMP-2 and led our laboratory to test the ability of TIMP-1 and TIMP-2 to inhibit FGF-2-induced endothelial cell proliferation. The results showed that TIMP-2, but not TIMP-1 or the synthetic MMP inhibitor BB-94, inhibited endothelial proliferation in response to FGF-2 stimulation and that this activity appeared to be independent of MMP inhibitory activity [43]. It is now well established that TIMP-2 is the only member of the TIMP family that has been shown to directly inhibit endothelial cell growth. More recently, TIMP-2 mediated inhibition of endothelial cell proliferation was uncoupled from its MMP inhibitory function [44]. Our laboratory showed that pre-treating endothelial cells with either TIMP-2 or Ala+TIMP-2, a TIMP-2 mutant with an alanine appended at the N-terminus that lacks MMP inhibitory activity, suppressed VEGF-A or FGF-2 stimulation of endothelial cell growth. The mechanism involved reduction of the cognate tyrosine kinase growth factor receptor phosphorylation and activation, and translocation of the protein tyrosine phosphatase, SHP-1. More specifically, TIMP-2 was shown to bind to the integrin $\alpha 3\beta 1$ receptor on the endothelial surface and competition experiments with blocking antibodies to each integrin subunit led to loss of TIMP-2 growth suppressive activity. This was the first study to demonstrate that TIMP-2 binds to the endothelial cell surface via a receptor, and that the growth inhibitory activity is dependent on this interaction. Subsequent experiments with exogenous treatment of TIMP-2 or Ala+TIMP-2 demonstrated induction of *de novo* expression of cyclin-dependent kinase inhibitor p27 in microvascular endothelial cells and hypophosphorylation of pRb leading to G1 cell cycle arrest [45]. Endorepellin, another anti-angiogenic agent, shares similar biological properties with TIMP-2 in that they both bind beta1-containing integrin, induce SHP-1 to dephosphorylate VEGFR-2, as well as inhibit endothelial cell migration and tumor growth *in vivo* [46, 47]. TIMP-2 also regulates endothelial cell migration via up regulation of the reversion-enhancing, cysteine-rich protein with Kazal motifs (RECK); a membrane bound protein that inhibits MMPs [48, 49]. Enhanced RECK expression and inhibition of cell migration occur as a result of Src tyrosine kinase inactivation

and modification of paxillin phosphorylation pattern. In turn, this causes inactivation of the small guanosine triphosphatase (GTPase) Rac1 and subsequent enhanced Rap1 activation leading to RECK up regulation and decreased motility. These findings have been reviewed in detail elsewhere [24].

Recent studies identified two separate regions of TIMP-2 involved in its antiangiogenic activity, and confirm that the two functions of TIMP-2, the metalloproteinase inhibition and the antiangiogenic activities, are distinct. In the first study, the antiangiogenic function was localized to a 24-mer region of the N-terminus of TIMP-2, within the B-C loop [50]. Synthetic peptides from that region were shown to contain the α3β1 binding domain that interacts with the endothelial cell surface to suppress VEGF-A-stimulated endothelial mitogenesis. Using a murine Kaposi's sarcoma model, a tumor derived from the endothelium, the α3β1 binding peptides demonstrated significant anti-tumor activity *in vivo*. More recently, we have used luciferase labeled Lewis lung carcinoma cells in a modified *in vivo* angiogenesis assay (directed *in vivo* angiogenesis assay (DIVA assay [51]) to examine the effects of exogenous TIMP-2 B-C loop peptide (10-mer, Peptide 8-9) on tumor growth and angiogenesis [50]. The results show that TIMP-2 B-C loop peptide reduced tumor growth by ~90% at day 23 and significantly reduced tumor associated angiogenesis as revealed by angiosense (αVβ3) and CD31 immunofluorescence staining, Fig. (**1 A** and **B**).

In the second study, the mechanism involved in the antiangiogenic activity implicates a 24-mer at the C-terminal domain of TIMP-2 known as loop 6 [52]. Since the MMP inhibitory activity is located at the N-terminus of TIMP-2, inhibition of angiogenesis due to TIMP-2 mediated MMP inhibition is not questioned [53, 54]. Furthermore, TIMP-2 loop 6 was reported to directly bind to the insulin-like growth factor receptor I (IGF-IR) on endothelial cells, a tyrosine kinase receptor known to regulate tumor growth and angiogenesis [55, 56]. Thus, the effects of TIMP-2 loop 6 peptide appear completely independent of integrin α3β1-binding. Upon this interaction, both AKT and MAPK pathways were analyzed for Akt and/or Erk phosphorylation *in vitro*. Both pathways were inhibited when cells were pretreated with loop 6 suggesting that the downstream IGF-IR signaling was disrupted. Moreover, *in vivo* administration of exogenous loop 6 in SCID mice transplanted with PC-3 tumor cells resulted in inhibition of tumor volume and reduced tumor angiogenesis. This antiangiogenic mechanism occurs

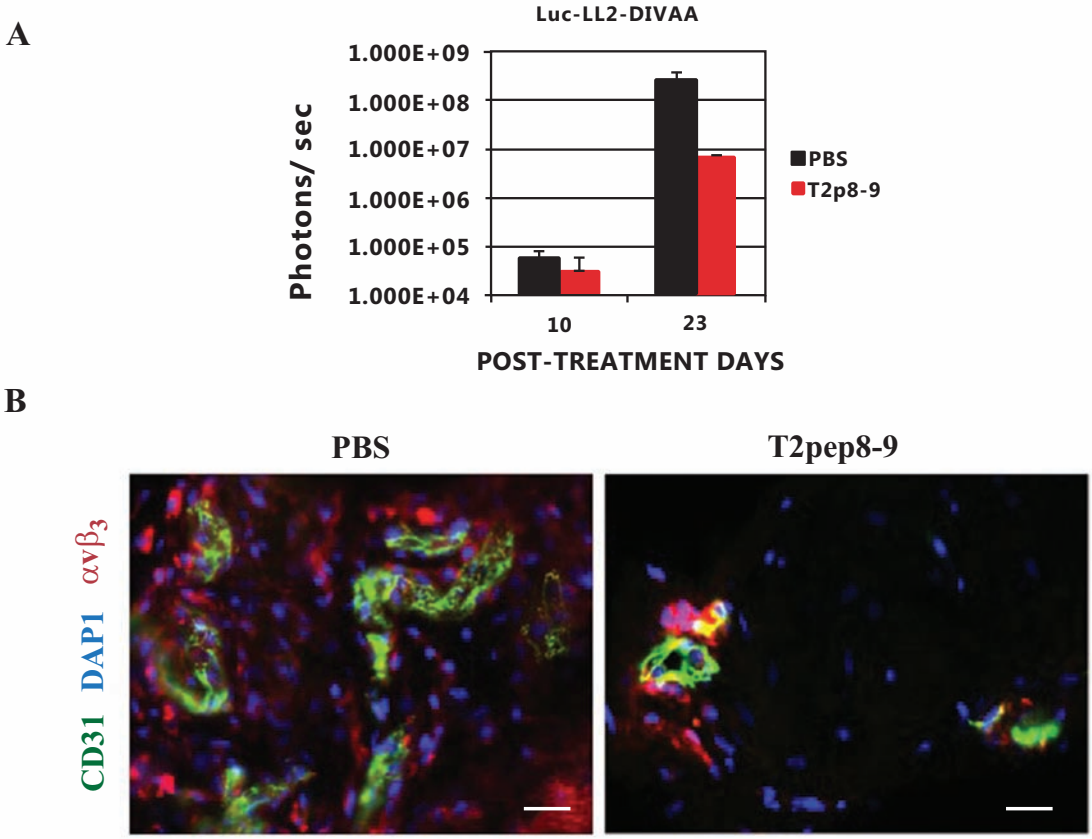

Fig. (1). A. TIMP-2 B-C loop peptide (T2pep8-9) effect on tumor growth was determined using a modified *in vivo* angiogenesis assay by luminescence using IVIS™ bioluminescence detector and expressed in photons per second at 10 and 23 days post implantation. The B-C loop peptide significantly reduced tumor bioluminescence by two orders of magnitude (>90%) (50, 51), suggesting that tumor growth is significantly reduced at day 23 post implantation. **B.** Anti-angiogenic effects of TIMP-2 B-C loop peptide (peptide 8-9). Confocal microscopy (z-stack) of Lewis Lung carcinoma in angioreactors treated with TIMP-2 B-C loop peptide (peptide 8-9 demonstrated a marked reduction in the expression of angiogenic markers alphaV-beta 3 and CD31 in blood vessels as well as in the number of tumor cells as compared with tumor cells in PBS treated group (bar = 50 μm).

independently of TIMP-2 interaction to its receptor α3β1or to MT1-MMP. These results suggest that loop 6 inhibits endothelial cell proliferation and angiogenesis by interacting with IGF-IR and disrupting its downstream signaling through Akt and Erk.

The levels of free angiogenesis inhibitors, such as TIMP-2, are frequently down regulated in many types of cancer due to both genetic and epigenetic mechanisms. Furthermore, recent studies have clearly demonstrated that hypoxia common in many solid tumors can down regulate TIMP-2 expression in macrophages and endothelial cells, further reducing this "barrier" to angiogenesis and tumor cell invasion by cellular elements of the tumor microenvironment [57]. Despite potent angiogenesis inhibitory activity of TIMP-2 that has been shown to be independent of MMP inhibition, some investigators remain skeptical regarding the contribution of these MMP-independent activities to the observed anti-tumor activity of TIMP-2 *in vivo*. To address these concerns we overexpressed TIMP-2 and the mutant Ala+TIMP-2 in A549 lung cancer cells [58]. Our goal was to determine if the apparent MMP-independent antiangiogenic activity of TIMP-2 (Ala+TIMP-2) was sufficient to suppress *in vivo* tumor growth. *In vitro*, TIMP-2 or Ala+TIMP-2 did not alter A549 basal cell growth (in the presence of serum, not growth factor stimulated). However, in a chemoattractant migration assay both TIMP-2 and Ala+TIMP-2 inhibited the migration of A549 tumor cells. The inhibitory effects were further demonstrated in a chemoinvasive assay where both cell lines showed significantly lower invasive properties. These experiments suggest that TIMP-2 directly regulates A549 tumor cell motility and invasion independent of MMP-2 inhibition, without altering the growth *in vitro*. Ala+TIMP-2, and thus the MMP-independent activity of TIMP-2, was shown to inhibit the migration and invasion of breast cancer cell lines such as MCF-7, T47D and MDA-MB-231, which implies that MMP-independent effects of TIMP-2 may be observed in a variety of tumor types [59].

In order to investigate the impact of elevated TIMP-2 expression on the tumor microenvironment and tumor growth, we subcutaneously implanted the cells into both nude and NOD-SCID mice [58]. TIMP-2 was shown to inhibit tumor growth >90 % in both mouse models, while Ala+TIMP-2 inhibited growth > 70% in the nude mouse model and >90 % in the NOD-SCID murine background. These findings suggest that the TIMP-2 anti-tumoral effects may involve TIMP-2 interaction with cellular or protein elements of the tumor microenvironment and/or tumor cells directly. In addition, the fact that Ala+TIMP-2 was also able to inhibit tumor growth suggests that both MMP inhibition and MMP-independent mechanisms contribute to suppression of tumor growth by TIMP-2. The somewhat surprising effectiveness of Ala+TIMP-2 (> 70-90 % inhibition) suggests that the MMP-independent activities of TIMP-2 contribute significantly to the tumor growth suppressive activity.

A detailed immunohistochemical analysis of tumor sections showed decreased tumor angiogenesis and increased tumor cell apoptosis in both TIMP-2 and Ala+TIMP-2 A549 xenografts [58]. These data indicate that TIMP-2 controls tumor growth through its interaction with the microenvironment (i.e. inhibits tumor angiogenesis) and as a result induces tumor cell apoptosis, although a direct regulation of apoptosis by TIMP-2 cannot be excluded at this time. However, direct immunohistochemical staining for activated (cleaved) Caspase 3 showed enhanced staining in both TIMP-2 and Ala+TIMP-2 A549 xenografts grown in nude mice when compared with wild type controls, Fig. (**2**).

In order to examine cellular signaling pathways that might be impacted by TIMP-2 during tumor progression, we analyzed the levels and activities of Focal Adhesion Kinase (FAK) and AKT proteins in tumor sections by western blot and immunohistochemistry [58]. Since TIMP-2 was able to inhibit A549 migration and invasion we looked at the FAK levels and activation. We showed that FAK levels were reduced and its activation was impaired in the tumor cells. Similarly, total and activated AKT levels were also reduced. Given that the AKT signaling pathway occurs downstream of FAK this may explain the reduced AKT activity. However, as it was previously shown, AKT phosphorylation was also decreased in endothelial cells upon TIMP-2 loop 6 interactions with IGF-IR [52]. Recent studies have demonstrated that in human pancreatic adenocarcinoma cells, FAK and IGF-IR physically interact and co-localize on the focal adhesions [60]. Decreased phosphorylation status for both molecules resulted in reduced cell viability and increased apoptosis [61]. It is therefore possible that TIMP-2 loop 6 acts as an antagonist and disrupts this complex, however, further experiments are necessary to answer these questions.

The TIMP-2 regions and specific peptides/fragments recently identified as responsible for its antiangiogenic and antitumorigenic effects further support the view of developing TIMP-2 as a therapeutic. The potential use of endogenous angiogenesis inhibitors as prophylactic or adjuvant regimens for cancer therapy should be further investigated. Cocktails of certain antiangiogenic fragments derived from different angiogenesis inhibitors could synergistically and effectively target not only different proangiogenic or protumorigenic molecules, but also prevent the initiation of tumor growth.

It is well known that the MMP/TIMP ratio may determine whether inhibition or activation of proteolysis occurs within a given tissue. Furthermore, elevated MMP/TIMP protein levels are associated not only with cancer but many other chronic diseases such as rheumatoid arthritis, cardiovascular disease and chronic inflammatory disease and more recently a variety of central nervous system (CNS) pathologic conditions such as multiple sclerosis, Alzheimer's disease and Parkinson's disease [59, 62]. Interestingly, an increased MMP/TIMP ratio is also observed in diseases of the CNS. In immune-mediated models of CNS demyelination, activated T lymphocytes produce both MMPs and TIMPs. Elevation of the MMP/TIMP ratio is often viewed as definitive evidence of an increase in protease activity resulting in extracellular matrix remodeling and disease progression. Such assumptions have led to the long-standing ascendant hypothesis that TIMPs primarily function to regulate protease activity (MMPs and ADAMS). However, the observation that many normal cells synthesize and secrete TIMP-2 in the absence of MMP secretion, as well identification of cells that

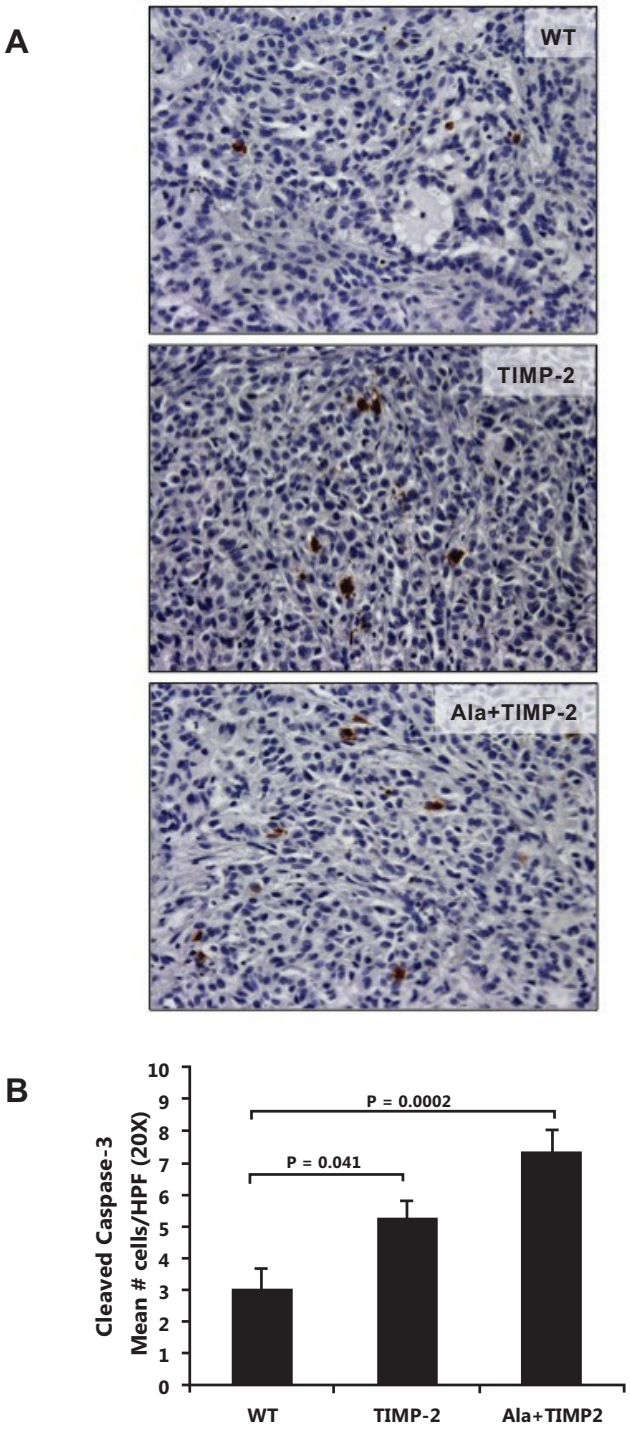

Fig. (2). A. Routine immunostaining of paraffin-embedded tissues was performed following standard antigen retrieval in 0.01M Citrate buffer, pH 6.0. with the rabbit polyclonal antibody to cleaved caspase-3 to detect endogenous levels of activated caspase-3 (Cell Signaling 3992) using the avidin-biotin peroxidase method (Vector, Burlingame, CA). **B.** Quantification of staining was assessed on 5 mice per group (A549WT, A549T2, A549AT2) by calculating the mean number of positive cells within 10 high power (20X) fields. Mean values were compared with control values using the Mann–Whitney Rank sum test. Statistical significance for all comparisons was acknowledged when *P*-values were <0.05.

simultaneously secrete both TIMPs and MMPs has led to the novel concept that MMPs may in some cases function to regulate TIMP mediated functions by controlling availability of 'free TIMP' as recently posited by Moore and Crocker [62].

As more and more studies demonstrate the MMP independent effects of TIMP-2 on different normal cell types (e.g. endothelial, neuronal, fibroblasts) as well as tumor cells, our laboratory is currently exploring additional ways to determine how TIMP-2 works and influences cellular behavior. Could it be possible that TIMP-2 initiates some of its functions from inside the cell? We hypothesized that TIMP-2 overexpression in A549 tumor cells may affect the secretion of growth or angiogenic factors that promote tumor growth or progression of the tumor cells or cells within the microenvironment (e.g. endothelial cells). Indeed, unpublished data from our laboratory have demonstrated that TIMP-2 down regulates the expression of angiogenic factors such as VEGF-A and bFGF-2 in an MMP-independent manner (Guedez, Bourboulia and Stetler-Stevenson, unpublished observations). These findings led us to examine the effects of TIMP-2 and Ala+TIMP-2 on other cytokines through antibody protein array analysis. For example, we can demonstrate that both TIMP-2 and Ala+TIMP-2 expression in A549 cells suppressed IL-6 expression, Fig. (**3**), a cytokine that is essential to tumor growth, angiogenesis and inflammation, as well as bone metastasis [63]. How TIMP-2 controls expression of these growth factors and cytokines via an MMP-independent mechanism is the subject of our current laboratory investigations.

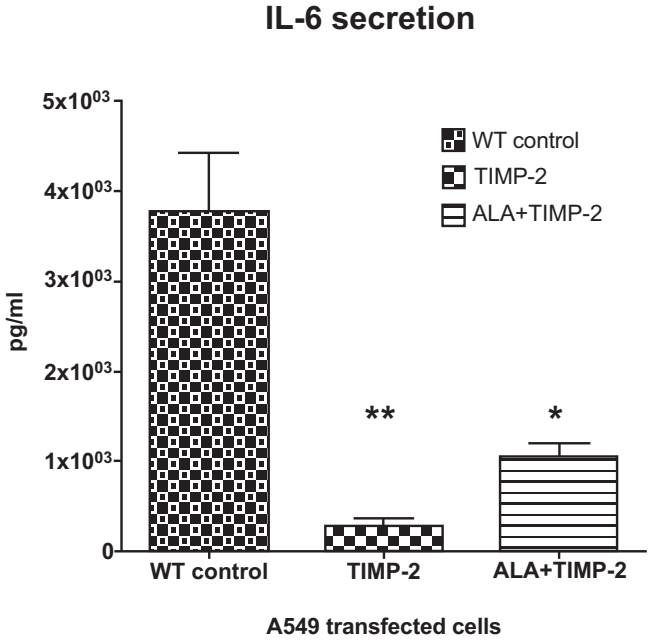

Fig. (3). TIMP-2 overexpression in A549 cells influences IL-6 protein levels secreted in serum free conditioned media (CM). Using a custom-made human antibody-based angiogenic/cytokine protein array (Capital Biosciences, Rockville, MD), we analyzed the CM collected from A549 cells overexpressing TIMP-2 and Ala+TIMP-2. IL-6 levels were significantly reduced in CM from TIMP-2 and Ala+TIMP-2 overexpressing A549 cells (**p=0.006 and *p=0.01 respectively) compared to control cells.

SUMMARY AND FUTURE DIRECTIONS

Work over the past two decades, summarized briefly in the current review and in Fig. (**4**), reveals that TIMP-2 is a unique member of the tissue inhibitor of matrix metalloproteinases family. TIMP-2 has been shown to be present in most normal tissues in the absence of significant proteolytic remodeling and is essential for normal development in the CNS [64]. TIMP-2 inhibits angiogenesis via multiple, possibly inter-related cell signaling pathways initiated by two distinct receptors, $\alpha 3\beta 1$ and IGFR-1. The potential functional inter-relationship between these receptors has not been examined. TIMP-2 also demonstrates potent anti-tumor activity *in vivo* that involves a distinct contribution from MMP-independent signaling pathways.

Based on the studies presented here, we posit that TIMP-2 is an excellent candidate for further preclinical development as a biological cancer therapeutic agent. Several advantages that TIMP-2 would present include the fact that it is a ubiquitously expressed, endogenous protein present in the circulation and most normal tissues [40], and at low micromolar concentrations [65]. These findings would suggest that modest increases in the TIMP-2 concentration within the tumor microenvironment may result in effective suppression of tumor growth with minimal side effects. However, prior to testing in early clinical trials we face significant issues. These include the scale up of biologically active TIMP-2 production using good laboratory practice (GLP) environment, understanding the pharmacokinetic and pharmacodynamics of TIMP-2 administration, and formulation issues. We have begun to work on these questions using 6X His-tagged human recombinant TIMP-2 which we are now able to produce at levels that are relevant for preclinical/clinical trials (60 mg/L; Wei and Stetler-Stevenson, unpublished) and a simplified bioprocess purification scheme using two step liquid chromatography. We have also developed assays for capture and quantification of exogenous human recombinant TIMP-2 in murine tumor models. We plan to utilize these new tools to explore the therapuetic effectiveness, pharmacokinetics/pharmacodynamics of exogenously administered TIMP-2 in murine tumor models, and report these findings in the near future.

CONFLICT OF INTEREST

The authors have no conflicts, financial or otherwise to declare.

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Fig. (4). Mechanisms of TIMP-2 signaling and effects on endothelial cells. (**A**) Binding to $\alpha_3\beta_1$ receptor: (1) Protein tyrosine phosphatase (Shp-1) mediated inactivation of receptor tyrosine kinase (RTK) activity by de-phosphorylation of the kinase domain leading to inhibition of cell proliferation and angiogenesis, (2) cell cycle arrest mediated by nuclear translocation of p27 leading to inhibition of Cdk2/4 resulting in G1 cell cycle arrest, and (3) C-terminal Src kinase (Csk) mediated inactivation of Src and altered paxillin (PAX) phosphorylation resulting in Rap1 mediated upregulation of RECK expression and decreased cell migration. (**B**) TIMP-2 (Loop 6) binding to IGF-1R leads to inhibition of cell proliferation and angiogenesis via decreased phosphorylation of pERK and pAKT (4).

W.G. Stetler-Stevenson supervised the research and prepared the manuscript. D. Bourboulia, S. Jensen-Taubman and L. Guedez performed original research/studies, collected and analyzed data and participated in the preparation of the manuscript.

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