Localization of the Death Domain of Tissue Inhibitor of Metalloproteinase-3 to the N Terminus

METALLOPROTEINASE INHIBITION IS ASSOCIATED WITH PROAPOPTOTIC ACTIVITY*

The tissue inhibitors of metalloproteinases (TIMPs) are a family of four secreted inhibitors of matrix metalloproteinases (MMPs). Recently, additional functions have been attributed to the TIMPs, including cell growth and inhibition of angiogenesis. In particular, we demonstrated that TIMP-3 overexpression using gene transfer induces apoptosis in a variety of cell types and can inhibit vascular neointima formation in vivo. However, little is known about the mechanisms underlying TIMP-3-mediated apoptosis. Here, using both purified recombinant proteins and novel adenoviral vectors we demonstrate that the prodeath domain of TIMP-3 is located within the N-terminal three loops of TIMP-3. Although both wild type and N-terminal TIMP-3 proteins promoted apoptosis, a T-2/T-3 chimera, in which the N-terminal three loops of TIMP-3 are replaced by those of TIMP-2, failed to induce cell death. Furthermore, a point mutation at residue 1 of TIMP-3 totally abolished MMP-inhibitory activity of TIMP-3 and also failed to promote apoptosis. This study demonstrates, using multiple apoptosis assays, that the prodeath function of TIMP-3 is located within the N-terminal three loops and the presence of functional metalloproteinase-inhibitory activity is associated with the induction of apoptosis.

The tissue inhibitors of matrix metalloproteinases (TIMPs) are a family of secreted inhibitors that block the activity of the matrix metalloproteinases (MMPs). The family currently consists of four members (TIMPs 1 through 4) that show a high degree of homology at the amino acid sequence level (1–4). The TIMPs typically consist of two-domain structure in which each domain being folded into three loops constrained by three disulfide bonds. The N-terminal domain contains the highly conserved CXC motif that is responsible for metalloproteinase inhibition, whereas the C-terminal domain confers specific functions such as the ability of TIMP-1 and TIMP-2 to bind pro-MMP-9 and pro-MMP-2, respectively, and the ability of TIMP-3 to bind the extracellular matrix (5, 6, 7–12). All TIMP family members inhibit the activity of the MMPs that regulate the composition of basement membranes and extracellular matrix. Thus, TIMPs play essential roles in regulating matrix composition, cell growth, invasion, and migration. Recently, several additional biochemical and physiological functions have been attributed to certain TIMP family members, including regulation of proliferation, pro-MMP-2 activation, inhibition of angiogenesis, transformation, and apoptosis (5, 13–18). We recently characterized the roles of TIMP-1, -2, and -3 on vascular smooth muscle cells growth and proliferation in vitro and in vivo using adenovirus-mediated gene transfer (19, 20).

We discovered that overexpression of TIMP-3, but not TIMP-1 or -2, promoted apoptotic death of smooth muscle cells, which contributed to reduced neointima formation in vitro and in vivo. The fact that TIMP-3 could prevent neointima formation was attributed to its activity as a classical MMP inhibitor and the promotion of apoptosis (20). TIMP-3 has also been reported to promote apoptosis in a number of cancer cell lines (21–23). For example, Smith et al. (23) suggested that TIMP-3 promotes apoptosis of colon carcinoma cells by blocking proteolytic shedding of tumor necrosis factor alpha (TNF-α) receptors.

TIMP-3 has also been implicated in disease progression. Imbalances between TIMPs and metalloproteinases have been observed in numerous disease processes, including atherogenesis, rheumatoid arthritis, and tumor metastasis (24, 25). This is largely due to altered matrix remodeling that occurs in these pathologies. However, the additional functions of TIMPs such as promotion of cell growth may contribute to disease progression. With reference to TIMP-3, mutations in the TIMP-3 gene are associated with Sorsby’s fundus dystrophy (SFD), a degenerative disease affecting the retinal epithelial cells (26, 27). Interestingly, all known mutations of TIMP-3 associated with SFD, which cause the loss or gain of a cysteine residue, retain their ability to inhibit metalloproteinases and bind the extracellular matrix. Rather, it is thought that these mutations cause mutant TIMP-3 to accumulate in the retina and promote disease. Whether TIMP-3 is directly responsible for increased apoptosis associated with SFD is unclear.

Therefore, to evaluate the domain(s) of TIMP-3 required for induction of apoptosis and fully define the need for functional MMP-inhibitory activity in this induction, we generated a series of TIMP-3 mutant proteins and adenoviral constructs. Our
results demonstrate that the N terminus of TIMP-3 harbors the death domain and functional metalloproteinase-inhibitory activity is a requirement for induction of apoptosis.

**EXPERIMENTAL PROCEDURES**

**Materials**

Human kidney embryonic 293 cells were purchased from Microbiex (Toronto, Canada) and HeLa cells from the European Collection of Animal Cell Cultures (Salisbury, UK). All chemicals, unless otherwise stated, were obtained from Sigma Chemical Co. (Poole, UK) and were of the highest grade available. Culture media and additives were obtained from Life Technologies, Inc. (Paisley, Scotland). Rabbit polyclonal anti-TIMP-3 antibody was purchased from Chemicon International (Harrow, UK).

**Methods**

**Cell Culture**—293 cells were maintained in minimal essential medium supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin, and 10% v/v fetal calf serum. Rat smooth muscle cells were prepared from the thoracic aortas as described previously (28). Rat SMC and HeLa were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (v/v) 100 units/ml penicillin and 100 μg/ml streptomycin. All cells were maintained at 37 °C in an atmosphere of 95% air and 5% carbondioxide.

**Generation and Purification of Recombinant TIMP-3 Proteins**—Recombinant human TIMP-3 was expressed from myeloma cells and purified as described previously (29). The N-terminal three loops of TIMP-3 (N-TIMP-3) was constructed in a prokaryotic expression system as follows: the residues encoding N-terminal TIMP (residues 1–121) were amplified by PCR using the primer (5′-GCC TAT GTG GCA CAT GCT GCC CCA GCC ACC CCC AG-3′), which encodes an NdeI site (underlined) prior to Cys1 of the TIMP-3 cDNA sequence and was used in a PCR reaction with the reverse primer, to introduce a stop codon following the coding sequences for Asn121 and a unique EcoRI site (underlined) included (5′-GGA ATT CAG TTA CAA CCC AGG TGA TAC CGA TAG TT-3′). The PCR fragment was ligated into EcoRV-cleaved Bluescript, and a positive clone for each was identified by restriction analysis. The N-TIMP-3 vector was generated by ligating the NdeI fragment into pRSET A previously cleaved in pET23d. The expression vector was used to transform BL21(DE3)pLysS with these restriction enzymes. The resulting expression vector was used to transform E. coli with TIMP-3 (5′-GCC TAT GTG GCA CAT GCC ACC CCC AG-3′), which encodes an NdeI site (underlined) and overlap-extended by PCR using the coding primer upstream of the template. The two PCR fragments were isolated from a 1% agarose gel using the pET 23d TIMP-2 cDNA as the template. The -GAT CTT GCA CTC GCA GCC CAT CTG GTA CCT GTG (TIMP-2) primers (underlined) and a reverse primer (TIMP-3 (5′-GTC ACG TTA CAA CCC AGG TGA TAC CGA TAG TT-3′)). An Nhel restriction site (underlined) and cloned was synthesized at the 5′-ends of each primer to create unique Nhel cloning sites. 0.1 ng of TIMP-3 cDNA template was amplified using a Thermocycler (PerkinElmer, Emeryville, CA). PCR products were purified using Wizard PCR prep (Promega, Southampton, UK) and fragments cloned into the Nhel site of pAL119 (32) to create pAL119.TIMP-3. The entire insert was sequenced to confirm the presence of the mutation and ensure that no PCR-induced mutations were present. Sequencing primers were located in the cytomembranolvirus immediate early promoter (5′-GCG CAT CCA CGC TGT TTT GA-3′) and the polyadenylation region (5′-TGG AGT AGG AAA ATG TAG TCA-3′).

cDNA representing the N-terminal domain (residues 1–121) of TIMP-3 was generated using the antisense primer 5′-CCG GCT AGG CTA GGT ACA ACC CAG GAT GTA G-3′ (clamp and Nhel restriction site underlined) and cloned into the Nhel site of pAL119 (32). Cotransfection of pAL119.TIMP-3cDNA with pAL119.NITIMP3 with the adenovirus genome vector pM17 into 293 cells resulted in formation of recombinant adenovirus carrying the TIMP-3 gene. Recombinant TIMP-3 transgenes. Recombinant adenovirus was purified through two rounds of plaque purification then propagated, purified, and tested using standard methods (33). Lack of replication competence was confirmed by infection of non-permissive cell lines.

**Adenovirus Infection**—Rat SMC and HeLa cells were cultured in six-well plates or on sterile coverslips until 80% confluent. An accurate cell number was assessed preinfection by trypanization of three wells and counting using a Neubauer hemocytometer. Remaining wells were infected at 300 plaque-forming units (pfu/cell) (rat SMC) and 100 pfu/cell (HeLa) in 1 ml of fresh complete media for 18 h. Media were then replaced with 2 ml of fresh complete media and left for the required length of time prior to analysis.

**Reverse Zymography for TIMP-3**—To allow analysis of TIMP-3 expression, cells were non-enzymatically removed from the extracellular matrix by washing in ice-cold 1× citric saline (132 mM KCl, 17 mM sodium citrate, pH 7.6). Conditioned media were washed and extracted in 1× non-reducing sample buffer (30 mM Tris-HCl, pH 6.8, 5% glycerol, 2% SDS, 0.0012% bromphenol blue). Protein extracts were electrophoresed through 12% polyacrylamide gels under reducing conditions and blotted onto a polyvinylidene difluoride membrane (Bio-Rad). Membranes were blocked in 5% fat-free milk and incubated with 1:5000 dilution antibody in 5% fetal bovine serum/PBS for 3 h. Detection was performed using enhanced chemiluminescence (ECL, Amer sham Pharmacia Biotech).

**Reverse Zymography**—TIMP-3 metalloproteinase-inhibitory activity in extracellular matrix extracts was evaluated by reversed zymography essentially as described (34, 35). Briefly, 12% polyacrylamide gels containing 0.8 mg/ml gelatin and 8% baby hamster kidney cell-conditioned media as a source of activated metalloproteinase. Following electrophoresis, SDS was removed by washing in 2.5% Triton X-100 and gels were incubated at 37 °C for 18 h in zymogram incubation buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10 mM CaCl2, and 0.05% Brij35). Gels were then stained in 0.1% Coomassie Brilliant Blue. TIMP activity was visualized as dark zones of gelatinase inhibition against a pale partially digested background.

**Cell Death Assay**—Cell death in response to purified recombinant TIMP-3 proteins was assessed using the 2-(4-i dodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt (WST-1) assay (Roche Molecular Biochemicals, Lewes, UK). Briefly, cells were seeded in 96-well plates (100% confluence) with increasing nanomolar concentrations of purified recombinant TIMP-3 proteins. After 72 h, cells were incubated at 37 °C with 200 μl of WST-1 reagent, and optical density was measured using a Multiscan spectrophotometer (Labsystems) at 450 nm.

**In Situ End Labeling of DNA**—Rat SMC were grown on glass coverslips and fixed in ice-cold methanol 72 h after addition of virus.
**RESULTS**

The N-terminal Domain of TIMP-3 Is Responsible for Induction of Apoptosis—We have previously demonstrated that TIMP-3 has the unique ability among TIMPs to induce apoptosis in vascular smooth muscle cells and various cancer cell lines. To determine the structural features of TIMP-3 responsible for its apoptotic activity, we used several recombinant proteins representing the isolated N-terminal domain or C-terminal domain of TIMP-3. These are: wild type (wt) TIMP-3, proteins representing the isolated N-terminal domain or C-terminal domain of TIMP-3, with a metalloproteinase-inhibitory activity without affecting protein conformation (36). We used a recombinant adenovirus to over-express the TIMP-3Cys1-Ser mutant in rat SMC and HeLa and compared its proapoptotic activity with a previously characterized recombinant adenovirus carrying the wild type TIMP-3 gene (RAd wt TIMP-3) or a control adenovirus (RAd 66) (19, 20). Rat SMC and HeLa cells were infected with recombinant adenovirus (RAd TIMP-3Cys1-Ser) carrying TIMP-3Cys1-Ser transgene at 300 and 100 pfu/cell, respectively, to obtain 100%...
infection. Cell matrix extracts were analyzed for expression 72 h post-infection (Fig. 2, A and B). Infection with RAd:TIMP-3Cys1-Ser resulted in expression of high levels of matrix-bound TIMP-3 immunoreactive protein in both rat SMC and HeLa cells (Fig. 2, A and B). Expression levels produced by RAd:TIMP-3Cys1-Ser were similar to those produced by RAd:wtTIMP-3. Reverse zymography of matrix extracts demonstrated, as expected, that recombinant TIMP-3Cys1-Ser lacked metalloproteinase-inhibitory activity (Fig. 2C).

Comparison of Apoptosis Induced by Wild Type TIMP-3 and TIMP-3Cys1-Ser Using Adenovirus-mediated Gene Transfer—In agreement with our previous data, infection of rat SMC and HeLa cells with RAd:wtTIMP-3 induced apoptotic cell death similarly to exogenously added recombinant wild type TIMP-3 (19). Apoptotic cell death was evident 2–3 days post-infection and was detected and quantified using in situ end labeling (ISEL) (Fig. 3B). Cells infected with RAd:wtTIMP-3 became increasingly rounded and detached from the substratum and exhibited higher refractive index under phase contrast microscopy (Fig. 3E). These cells also exhibited extensive membrane blebbing and showed Annexin V staining (data not shown) for externalized phosphatidyl serine, which is characteristic of an apoptotic mode of death. Infection with RAd:wtTIMP-3 resulted in significantly greater ISEL staining (34.7 ± 8.4% compared with 0.3 ± 0.2% for control virus-infected cells), indicative of DNA fragmentation, and highlighted the condensed and fragmented nuclei present in these cells (Fig. 3B).

In contrast, cells infected with control virus (RAd66) or RAd:TIMP-3Cys1-Ser exhibited no morphological features associated with apoptotic death and showed very few apoptotic cells with condensed ISEL labeled nuclei (0.3 ± 0.2% and 0.8 ± 0.8%, respectively). These data demonstrate that the ability of TIMP-3 to induce apoptosis is associated with its metalloproteinase-inhibitory activity.

Flow cytometry of propidium iodide-stained cells was employed as a second method to quantify cell death and cell cycle parameters of cells infected with either RAd:wtTIMP-3 or RAd:TIMP-3Cys1-Ser. Infection of rat SMC or HeLa with RAd:wtTIMP-3, but not RAd:TIMP-3Cys1-Ser, resulted in an 8- and 3.5-fold increase, respectively, in the number of cells in the pre-G1/G0 phase of the cell cycle, characteristic of apoptosis (Fig. 4A). No increase in pre-G1/G0 cells was observed in cells infected with RAd:wtTIMP-3Cys1-Ser. As described previously, wild type TIMP-3 also induced an increase of cells in S and G2/M phase cell cycles with a reduction of cells in G1 phase (Fig. 4, B and C). It is not known if this occurs because of a block in M phase progression or promotion of S phase entry. However, no changes in cell cycle profile occurred after infection with RAd:TIMP-3Cys1-Ser suggesting that similar mechanisms account for induction of apoptosis and promotion of S phase entry. Consistent with the data for cell counts (Fig. 1D), incubation of HeLa cells with the synthetic metalloproteinase inhibitor BB-94 did not cause an increase in apoptosis measured by flow cytometry (control cells 1.9 ± 0.28% apoptosis; 10 μM BB-94, 1.83 ± 0.24% apoptosis) or cause any change in the cell cycle profile (data not shown).

DISCUSSION

The major biological role of TIMPs identified so far is to inhibit the matrix metalloproteinases in a 1:1 stoichiometric ratio. In addition to this role, numerous studies have attributed additional functions to the TIMPs, including inhibition of tumorigenesis, angiogenesis, invasion, and metastasis (5, 14, 16, 18, 21). For example, TIMP-1 and TIMP-2 have cell growth-and erythroid-potentiating activities (14, 37). Some of these functions may be at least partially dependent on metalloproteinases inhibition. For example, the inhibition of cell migration by the TIMPs is due to their metalloproteinase-inhibitory activities and is mimicked by synthetic MMP inhibitors (15, 20, 38). However, the cell growth-promoting activity of TIMP-2 has been shown to be independent of metalloproteinase inhibition (16). The erythroid-potentiating activity of TIMP-1 has also been shown to be independent of metalloproteinase inhibition (17). This suggests that the multiple biological functions of the TIMPs are due to discrete structural features within the protein.

We previously demonstrated that TIMP-3 had the unique ability, compared with TIMPs-1 and -2, to promote apoptosis of vascular smooth muscle cells and various cancer cell lines (19–21). Furthermore, these effects were not mimicked by synthetic MMP inhibitors. Two major hypotheses can be distinguished from these findings. Either TIMP-3 inhibits a protease that is not inhibited by TIMPs-1 and -2 or synthetic inhibitors, or the apoptotic effect is mediated by a function independent of metalloproteinase inhibition. As one approach to resolving this
uncertainty, we sought to determine the structural features of TIMP-3 responsible for its apoptotic activity. We demonstrate that the N-terminal domain of TIMP-3 alone is sufficient to induce apoptosis of HeLa and rat vascular smooth muscle cells, whereas an N-terminal TIMP-2:C-terminal TIMP-3 chimera, representing the isolated C-terminal domain of TIMP-3, is not. Previous studies have shown that the three loops comprising the N-terminal domain of TIMP-1 and TIMP-2 are sufficient for metalloproteinase inhibition, and we can confirm that the N-terminal domain of TIMP-3 is inhibitory to MMP-2 gelatinase A. The reduced binding constant reflects the role of the C-subdomain in binding, as was seen for other TIMPs in their interactions with MMP-2 (39, 40). It has been shown that the three C-terminal loops of TIMP-3 contain a component of the ability to bind the matrix (41), but N-TIMP-3 also plays a role (7). Expression of N-TIMP-3 in mammalian cells using the adenoviral system confirmed that a substantial proportion of the N-TIMP-3 secreted from the cells became incorporated into their extracellular matrix (data not shown). This mutant, therefore, did not allow us to dissociate either the MMP or the matrix binding properties of TIMP-3 as potential mechanisms of apoptosis.

Because the N-terminal domain of TIMP-3 is responsible for metalloproteinase inhibition, we investigated whether induction of apoptosis by TIMP-3 is dependent on an intact metalloproteinase-inhibitory domain. To this end, we constructed a mutant of TIMP-3 devoid of metalloproteinase-inhibitory activity by mutating the N-terminal cysteine residue to a serine. In wild type TIMP-3 the N-terminal cysteine residue is a key to the TIMP-3-inhibitory mechanism, because it bidentally coordinates the Zn\textsuperscript{2+} ion in the active site of metalloproteinases through the $\alpha$-amino group and the peptide carbonyl group (41). An analogous TIMP-1 mutant completely lacks inhibitory activity toward MMP-1 and MMP-2, while maintaining structural integrity (36). Likewise, we demonstrated that the Cys$^1$ to Ser mutation of TIMP-3 results in loss of inhibitory activity toward MMP-2. Because the Cys$^1$ to Ser mutation of TIMP-3 also resulted in loss of ability to induce apoptosis in both rat SMC and HeLa, the simplest conclusion is that inhibition of a metalloproteinase underlies the mechanism of TIMP-3-induced apoptosis. Consistent with our previous data (19), incubation with the synthetic metalloproteinase inhibitor BB-94 failed to induce apoptosis. This is in agreement with our earlier observations where Ro-9790, a hydroxamate-based inhibitor with similar broad specificity for MMPs, had no effect on apoptosis of rat vascular smooth muscle cells (19). This indicates that TIMP-3 is a more potent and selective inhibitor of the metalloproteinase involved in this apoptotic mechanism than these synthetic MMP inhibitors. This conclusion is in agreement with Smith and colleagues (23), who suggested that TIMP-3 promotes apoptosis of DLD carcinoma cells by blocking metalloproteinase-mediated shedding of TNF-$\alpha$ receptors, an effect that was not mimicked by BB-94. Our previous observations also show that overexpression of TIMP-1 and TIMP-2 in vascular smooth muscle cells and melanoma cells potently inhibits
invasion and MMP activity but does not result in increased apoptotic cell death (19, 21). Because TIMP-1 and TIMP-2 are both effective MMP inhibitors, this suggests that the enzyme targeted by TIMP-3 is not an MMP but a related metalloproteinase that is preferentially inhibited by TIMP-3. For example, TIMP-3 has recently been shown to have the unique ability of TIMPs to inhibit members of the adamalysin (a disintegrin and metalloproteinase) family such as TNF-\( \alpha \)-converting enzyme (TACE (31)). TACE is involved in the processing of TNF-\( \alpha \), a proapoptotic cytokine. It is possible that inhibition of TACE would lead to an accumulation of proapoptotic TNF-\( \alpha \) on the cell surface and autocrine or juxtacrine death signaling. On the other hand, the ability of BB-94 to inhibit TACE (31) implies that this is not the only or even the principle mechanism of TIMP-3-induced apoptosis in our experiments. Other adamasulins are involved in the proteolytic shedding of numerous plasma membrane-anchored proteins, including growth factors, cytokines, receptors, and adhesion molecules. Inhibition of these shedding events by TIMP-3 could have profound effects on cell viability.

In summary, this study has highlighted that functional metalloproteinase-inhibitory activity is associated with TIMP-3-induced death and that the N-terminal domain is responsible for the proapoptotic effect of TIMP-3. These observations have potential implications for TIMP-3-related pathologies, such as SFD, and the future development of TIMP-3 variants for use in gene therapy.

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