TIMP-3 Binds to Sulfated Glycosaminoglycans of the Extracellular Matrix*

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Of the four known tissue inhibitors of metalloproteinases (TIMPs), TIMP-3 is distinguished by its tighter binding to the extracellular matrix. The present results show that glycosaminoglycans such as heparin, heparan sulfate, chondroitin sulfates A, B, and C, and sulfated compounds such as suramin and pentosan efficiently extract TIMP-3 from the postpartum rat uterus. Enzymatic treatment by heparinase III or chondroitinase ABC also releases TIMP-3, but neither one alone gives complete release. Confocal microscopy shows colocalization of heparan sulfate and TIMP-3 in the endometrium subjacent to the lumen of the uterus. Immunostaining of TIMP-3 is lost upon digestion of tissue sections with heparinase III and chondroitinase ABC. The N-terminal domain of human TIMP-3 was expressed and found to bind to heparin with affinity similar to that of full-length mouse TIMP-3. The A and B β-strands of the N-terminal domain of TIMP-3 contain two potential heparin-binding sequences rich in lysine and arginine; these strands should form a double track on the outer surface of TIMP-3. Synthetic peptides corresponding to segments of these two strands compete for heparin in the DNase II binding assay. TIMP-3 binding may be important for the cellular regulation of activity of the matrix metalloproteinases.

The extracellular matrix (ECM)† provides mechanical support to cells and regulates signals reaching the cell that govern cell localization, differentiation, proliferation, and apoptosis. Components of the ECM, particularly the glycosaminoglycans (GAGs), are able to sequester bioactive molecules such as growth factors (1), proteases (2), and inhibitors. Turnover of the ECM is a highly regulated process necessary for movement of cells and for release of growth factors. Matrix metalloproteinases (MMPs) are believed to be key participants in this remodeling; there are at least 20 MMPs, all able to digest various ECM components (3, 4).

The MMPs, in turn, are regulated by tissue inhibitors of metalloproteinases or TIMPs. The major function of the TIMPs is to inhibit MMPs; any imbalance in which the activities of MMPs outweigh the TIMP levels will favor tissue destruction and pathological processes (5, 6). The TIMPs also possess growth stimulatory and regulatory activities (7, 8). The four members of the TIMP family all have similar secondary structures of six loops stabilized by six highly conserved disulfide bonds. The TIMPs all bind tightly, albeit with widely varying affinity, to the various MMPs. The x-ray structure (9) shows that the N-terminal cysteine chelates the active site zinc. TIMPs have N- and C-terminal domains, each with three loops. The N-terminal domain of TIMP-1 folds readily and displays full inhibitory activity (10).

TIMP-3 has several features that distinguish it from the other TIMPs. First, it is the only TIMP to bind tightly to the ECM: it was first observed as a transformation-sensitive protein bound to the ECM of chick embryo fibroblasts (11) and extractable with SDS or guanidine. This protein was subsequently shown to be TIMP-3 (12). Second, TIMP-3 is the only TIMP to inhibit members of the ADAM (a disintegrin and metalloprotease) family such as tumor necrosis factor-α-converting enzyme (13); this may account for its ability to induce apoptosis (14). It is the only TIMP to inhibit shedding of L-selectin (15) and interleukin-6 receptors (16). Third, TIMP-3 is the only TIMP directly implicated in a disease process: Ser-Cys mutants of TIMP-3 accumulate in Bruch’s membrane of the eye and cause Sorsby’s fundus dystrophy (17). TIMP-3 also promotes the detachment of transformed cells from the ECM (18) and is involved in the formation, branching, and expansion of epithelial tubes in and regulating trophoblast invasion of the uterus (19).

The present study is concerned with the mechanism of binding of TIMP-3 to the extracellular matrix. We recently reported that matrilysin could bind to heparan sulfate in rat uterine tissues (2). In this work, it was noted that heparin not only extracted matrilysin from the tissue, but also solubilized TIMP-3. The present results indicate that heparan sulfate and other sulfated glycosaminoglycans may be responsible for the binding of TIMP-3 to the ECM.

EXPERIMENTAL PROCEDURES

Extraction of TIMP-3—Uteri were collected 1 day postpartum from Harlan Sprague-Dawley rats (Harlan), weighed (2 g), washed 3× with cold 50 mM Tris, pH 7.5, 0.03% sodium azide, and homogenized in 20 ml of this buffer containing 0.1% Triton X-100 with a Polytron for 6 min at 4 °C. The mixture was centrifuged at 11,000 rpm for 20 min. The pellet was washed 2× and resuspended in the same volume of cold 50 mM Tris, pH 7.5, 0.03% sodium azide, 50 μM Z-Phe-chloromethyl ketone, 50 μM aminoethyl-benzensulfonyl fluoride. The suspension was divided at 0.5 ml per tube. Extractants (50 μl, Sigma) were added to a final
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concentration of 0.2 mg/ml of the various GAGs and 2 mg/ml of pento-
san and suramin. Extraction at 4 °C for 30 min was followed by cen-
trifugation at 14,000 rpm for 10 min. For the heat extraction procedure, see Ref. 20. To destroy TIMPs, control extracts were reduced with 5 mM dithiothreitol for 25 min, 24 °C, and then boiled in the heating block (Fisher Scientific) and then treated with the Escherichia coli (Sin-ga) and chondroitinase ABC (Sigma) digestion, pel-
lets were resuspended in 50 mM Tris, pH 7.5, 0.03% sodium azide, 5 mM CaCl2, 10 μM ZnCl2, 50 μM Z-Phe-chloromethyl ketone, and 50 μM aminothiolbenzenesulfonyl fluoride and incubated with 0.2 unit of enzyme/ml at 37 °C for 18 h. Controls were incubated without added enzyme to check for endogenous activity.

Reverse Zymography—For 12.5% SDS-polyacrylamide gels were mixed with gelatin (final concentration 1 mg/ml) and a propri-
estory mixture of gelatines (University Technology International, Cal-
gary) and cast as gels. Extracts containing TIMPs were electrophore-
sed, and the gels were then washed 3 × with 2.5% Triton X-100/50 mM Tris/5 mM CaCl2/0.03% azide and 3 × with 50 mM Tris, pH 7.5, 5 mM CaCl2, 50 μM Z-Phe-chloromethyl ketone, and 100 μM phenylmethylsulfonyl fluoride. The gels were incubated in this latter mixture at 37 °C for 18 h and stained with Coomassie Blue. The blue gelatin staining was cleaved by gelatinease action except where TIMPs bands blocked this activity. Marker TIMPs (mouse) were also obtained from the University Technology International, Calgary.

Frozen tissue sections (5 μm) from 26- to 32-h postpartum rat uterus were air-dried and soaked in 95% ethanol for 10 min and then exposed to goat polyclonal antibody against human TIMP-3 (Santa 

Human TIMP-3 cDNA from a placental cDNA library was kindly provided by Dr. H. Nagase, Univer-
sity of Kansas Medical Center. A set of primers, 5′-AGCTCATATGG-
CACATGCGTCG3′ (forward) and 5′-GGCCCGCGTCTACAACACCAC-
GGTG-3′ (reverse), was used in a one-step polymerase chain reaction to amplify the cDNA insert encoding N-terminal TIMP-3 (residues Cys4′-
Asn32) (20). The amplified insert was digested by NdeI and NofI and ligated into the EcoR1-BamHI site of the heat shock protein (HSP70) expression vector pET3a-1 (20, 21). The recombinant pET3a-1 was used to transform the E. coli BL21(DE3). Cells containing pET3a-N-TIMP-3 were grown in 3 ml of LB/ampicillin medium at 37 °C overnight then inoculated into 500 ml of fresh LB/TMP/ampicillin (1 μg/ml) and enriched with isopropyl-β-D-thiogalactopyranoside (0.4 mM) for protein expression. Cells were grown for another 3 h and harvested by centrifugation.

Purification of N-TIMP-3—N-TIMP-3 was expressed as a fusion pro-
tein with a C-terminal His tag. Inclusion bodies were dissolved in 50 ml of loading buffer (0.5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.8, 6 M guandine) and centrifuged at 10,000 rpm for 40 min. The supernatant was loaded onto a Ni-NTA column (7 × 80 mm, Qiagen) equi-
ibrated with loading buffer. The column was washed at room temper-
ature with 60 mM imidazole, 0.5 M NaCl, 8 mM urea, 20 mM Tris-HCl, pH 7.9, then eluted with 500 mM imidazole, 0.25 M NaCl, 8 mM urea, 10 mM Tris-HCl, pH 7.9. Fractions from the Ni-NTA column were analyzed by SDS-polyacrylamide gel electrophoresis.

Purification of N-TIMP-3—Recombinant N-TIMP-3 in 8 mM urea, 0.15 M NaCl, 50 mM Tris, pH 7.5, 0.05% Triton X-100, and 5 mM dithiothreitol was diluted 1:10 in loading buffer A (20 mM acetate buffer, pH 5.6, 0.15 M NaCl, 5 mM dithiothreitol, and 0.05% Triton X-100). Heparin-agarose beads (Sigma) were suspended in 50 mM Tris, 0.15 mM NaCl, pH 7.8. A mixture of 1 ml of TIMP-3 plus 4 ml beads (0.5 mg) was transferred into 6-kDa cut-off dialysis tubing and dialyzed against loading buffer B (50 mM Tris, 0.15 M NaCl, pH 7.8, 0.05% Triton, 0.03% sodium azide, and 10 mM cysteinate), 50 ml, with three changes. In the final dialysis, folding buffer C (50 mM Tris, 0.15 M NaCl, pH 7.8, 0.05% Triton, 0.03% sodium azide) was used. The mixture was poured into a small column then washed with 50 mM Tris, pH 7.5, plus 0.2 M NaCl. Elution of folded N-TIMP-3 was then carried out using increasing amounts of NaCl or heparin in Tris buffer.

DNase II-based Homogeneous Heparin Binding Assay—In this assay, binding of heparin to DNase II was assayed (21). Competitive test compounds were added at 4 °C for 20 min in pH 4.8 acetate buffer + 5 mM dithiothreitol; then substrate was added for digestion at 37 °C. Heparin concentration was adjusted to inhibit DNase II activity by 90%; compounds binding heparin reversed this inhibition. The percent-
age inhibition observed in the presence of heparin and added test 

sulfated compounds such as pentosan polysulfate and suramin also liberated TIMP-3, but higher concentrations (2 mg/ml) were required (Fig. 1). Heat extraction, effective for matrilysin (2), was much less so for TIMP-3. Two bands of TIMP-3 (27 and 22 kDa, corresponding to glycosylated and nonglycosylated forms) (22), appeared in the reverse zymogram. Most of the TIMP-1 and TIMP-2 appeared in the initial Triton extract (not shown). A band corresponding in position to TIMP-2 was also extracted by GAGs, suramin, and pentoxifylline (Fig. 1); this may be TIMP-2 that was bound to gelatinase A, but positive identifi-
cation of the inhibitor has not been made. Heparin and sura-
min also extracted a small inhibitory protein of 16 kDa that was not sensitive to reduction/alkylation and has not been identified. Optimal extraction of TIMP-3 was achieved at 2–4 mg of heparin/ml or 1–2 mg of suramin/ml (not shown). A
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Extract aliquots (10 µl) were analyzed by reverse zymography as described under “Experimental Procedures.” The lanes contain: Mw, Marker 12 protein standards (Novex); T-3, authentic TIMP-3 marker from University Technology International, Calgary. T3+, glycosylated TIMP-3; R/A, reduced and alkylated to destroy TIMP; CS-A, CS-B, CS-C, chondroitin sulfates A, B, and C; All GAGs, extraction with heparin and CS-A, -B, and -C combined. Left axis, Mw values × 10^-2. The gel to the right serves as a control: samples were electrophoresed in a gel without added gelatinase, held for 18 h at 4 °C and stained together with the reverse zymogram. Coomassie Blue shows intensity of the protein bands at each position.

Parallel gel was run on SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue (right portion of Fig. 1) to show that the dark bands interpreted as TIMPs in the reverse zymograms were not due to nonspecific protein bands. The intensity of the glycosylated TIMP-3 (T3+) band appears to be less in the heparan sulfate/heparin lanes than in the CS lanes. This is attributed to interaction with a comigrating band of matrilysin extracted by heparin but not by chondroitin sulfate; this protease band can be directly visualized in the heat extract lane.

TIMP-3 was identified by its position on the reverse zymogram and by its sensitivity to destruction by reduction and alkylation (Fig. 2). A polyclonal antibody to human TIMP-3 (Santa Cruz) was used to show that heparin removed the factor from the uterus (see Fig. 4 below), but the antibody proved unsuitable for Western blotting of rat TIMP-3. Therefore, the identification of TIMP-3 depended on size, sensitivity to reduction and alkylation, and binding to matrix as shown by loss from tissue sections following treatment that increases TIMP-3 in reverse zymograms. TIMP-3 is the only TIMP that binds to the extracellular matrix. (5).

Fig. 3 illustrates further extraction studies; reverse zymography is not quantitative and permits only rough qualitative comparisons. It was also affected by MMPs that migrated to the same region and reacted with the TIMPs. The initial tissue extract in Triton X-100 (normally discarded) contained some TIMP-3. This might reflect binding of TIMP-3 to proteoglycans of the cell membranes, which were disrupted by Triton, or TIMP-3 in complex with gelatinase A and B (29). SDS should have released the bulk of the inhibitor (based on its ability to release the more tightly binding MMP-7 (2)), although some might remain bound to MMP-2 through its hemopexin domain (23). The highly positively charged heparin antagonist, polylysine, extracted TIMP-3; it is suggested that it competed with TIMP-3 for binding sites on the GAG chains. Digestion with chondroitinase ABC released less TIMP-3 than did heparinase III digestion.

Confocal Microscopy—The postpartum rat uterus contained both heparan sulfate (Fig. 4A) and TIMP-3 (Fig. 4B), which were largely localized near the uterine lumen, in the epithelial cells, and in their underlying basement membrane. There was little of either molecule in the deep stroma (right side of Fig. 4C). Superimposition of images indicates colocalization of the two proteins with some small patches of green remaining, perhaps on chondroitin sulfate. Washing with heparin (Fig. 4E) completely eliminated the TIMP-3 staining. Digestion with chondroitinase ABC gave some reduction in TIMP staining (Fig. 4F); but a better estimate of the chondroitinase-sensitive binding is probably provided by the residual staining in Fig. 4I. Digestion with heparinase III gave extensive losses of both heparan sulfate and TIMP-3 (Fig. 4, H and I). Both components were completely removed by digestion with the two enzymes together (Fig. 4, J and K). Incubation of sections without added enzyme did not lead to significant losses of either component (not shown).

Properties of Full-length and C-terminally Truncated TIMP-3—In reverse zymography (Fig. 5A), mouse TIMP-3 activity...
could be detected in BHK-TIMP-3 cells but not in the mock-transfected cell line. Two forms of TIMP-3 were found in the medium: a 27-kDa form, corresponding to the glycosylated form (24) and a 22-kDa nonglycosylated form. To see TIMP-3 in the medium, it was necessary to culture for several days, presumably until binding sites in the ECM are first filled. The denatured truncated human N-TIMP-3 (14 kDa) was prepared from *E. coli* and folded ("Experimental Procedures"). Similar amounts of protein were found before and after folding (Fig. 5B) but only the folded form was inhibitory (Fig. 5C).

Culture medium containing recombinant mouse TIMP-3 was mixed with heparin-agarose beads and packed in a small column (see "Experimental Procedures"). The initial concentration of NaCl was 0.15 M and the wash was with 0.2 M. Stepwise increases in salt concentration eluted TIMP-3 between 0.3 and 0.8 M NaCl, with the peak at about 0.5 M (Fig. 6A). Both the glycosylated and nonglycosylated forms emerged at the same position. Further washing with 2% SDS removed a small amount of residual TIMP (about 5%). The purified and folded human N-TIMP-3 bound in similar fashion (Fig. 6B); both monomeric and dimeric forms were eluted with a peak also around 0.5 M NaCl but with somewhat longer tailing. This tailing, with a distinct band at 0.9 M NaCl was attributed to the propensity of the truncated TIMP to aggregate. Such aggregates may be eluted at higher salt, but the aggregates did not appear on the gel because of dissociation by SDS in the gel. The elution of full-length and truncated TIMP-3 around 0.5 M NaCl indicated that the major heparin binding site was located in the N-terminal part of the protein.

Three peptides were synthesized based on the A and B strand sequences of rat TIMP-3 (25): Pep1 = residues 19–32 (IRAKVVGKLVKFG); Pep2 = residues 41–52 (IKQMKMRYEGFSKM); and the spanning peptide Pep3 = residues 19–52 (IRAKVVGKLVKFGPFTLVYTIKQMKMRYFHSKM). This last peptide contained 9 basic residues potentially involved in heparin binding. It can be seen from Fig. 7 that the two shorter peptides have similar affinity for heparin and that this is about 10-fold less than the binding affinity of the long peptide (IC_{50} =

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**Fig. 4.** Confocal microscopy of postpartum rat uterus. Microscopy and antibody staining are detailed under "Experimental Procedures." A, section stained with antibody to heparan sulfate; the uterine lumen is to the left. B, same section stained with antibody to TIMP-3. C, superimposed staining showing colocalization of heparan sulfate and TIMP-3. D, section stained with heparan sulfate antibody following a heparin wash. E, same section stained for TIMP-3. F, section stained for heparan sulfate following treatment with chondroitinase ABC. G, same section stained for TIMP-3. H, section stained for heparan sulfate following heparinase III treatment. I, same section stained for TIMP-3. J, section (similar to that in H) stained for heparan sulfate following combined treatment with heparinase III and chondroitinase ABC. K, same section stained for TIMP-3.

**Fig. 5.** Expression of recombinant mouse TIMP-3 in mammalian BHK cells and C-terminally truncated human N-TIMP-3 in *E. coli* BL21 (DE3) cells. A, conditioned media (10 µl) from TIMP-3 BHK cells and mock-transfected cells at 3 days culture were subjected to reverse zymography. The expected molecular weights (22 and 27 kDa) of TIMP-3 activities appeared only in the TIMP-3-transfected BHK cells. The 27-kDa band is the glycosylated form. B, silver staining demonstrating the purity of N-terminal TIMP-3. R, folded N-TIMP-3; D, denatured, prior to folding. C, reverse zymography showing the inhibitory activity of folded versus denatured N-TIMP-3, together with full-length markers. 10 ng of N-TIMP-3 was applied to each lane in B and C.
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3.5 μM). All three bound more firmly than the RHAMM 401–410 peptide (KKKHKIV)K. N-TIMP-3 was unstable under the reducing conditions of this assay and could not be measured.

DISCUSSION

There has been no systematic study of the extraction of TIMP-3 from the ECM. It was earlier noted that guanidine and SDS are effective extractants (26). Here we show that negatively charged molecules (heparin, various GAGs, and polysulfated compounds) gave extensive extraction comparable to SDS, but positively charged compounds such as polylysine were also extractants. However, enzymatic treatment with heparinase III and chondroitinase ABC gave extensive extraction, pointing to the negatively charged GAG molecules as binding sites, so positively charged compounds such as polylysine probably competed with TIMP-3 for binding to heparin. A preponderance of basic over acidic residues (26:13) in TIMP-3 also supports this interpretation. It is interesting to note that cultured mouse mesangial cells produce TIMP-3 in the medium only after pentosan polysulfate or heparin treatment (27); we suggest this might be due to the release of TIMP-3 bound to the ECM of the cultures.

The N-terminal domain of TIMP-3 contains 17 positive and 8 negative charges (25) and exhibits the OB (oligosaccharide/oligonucleotide binding) fold (9). Because GAGs are similar in charge and linearity to oligosaccharides/nucleotide polymers, the N-terminal domain of TIMP-3 could be a binding site for GAGs. The binding of TIMP-3 is strong, but not nearly as strong as the binding of matrilysin: TIMP-3 is eluted from a heparin affinity column with 0.5 M NaCl, whereas matrilysin is not eluted at 2 M NaCl (2). Matrilysin also has a great many more positive residues that might participate in binding. The fact that chondroitinase ABC appeared to release TIMP-3 from tissue but not as efficiently as heparinase III (Figs. 3 and 4) suggests that the binding is not highly specific. Several types of sulfated GAGs may serve as binding sites, but heparan sulfate chains are probably the major sites. Full-length TIMP-3 eluted from a heparin affinity column at 0.5 M NaCl. This matches exactly the results of Butler et al. (23); they also showed that TIMP-1 and TIMP-2 did not bind to heparin at 0.15 M NaCl, supporting our hypothesis that TIMP-3 is distinctive in binding to heparin. They further showed that glycosylation at the site Asn164 near the C terminus has no effect on binding and elution, comparable to the results in Fig. 6. We found that the N-domain of TIMP-3 bound to heparin with affinity similar to that of full-length TIMP-3, indicating that most of the free energy of binding is contributed by the N terminus. It must be mentioned that our results are not in agreement with those of Langton et al. (22) who expressed the N-domain of human TIMP-3 in COS-7 cells. In that case, full-length TIMP-3 bound to the ECM produced by the COS cells, whereas the N-domain did not and adding the N-domain of TIMP-2 to the C-domain of TIMP-3 gave partial but not full binding. We cannot explain this contradictory finding except to suggest that COS cells may make a different ECM than other cells.

The three-dimensional structure of TIMP-3 is not known but is assumed to be similar to that of TIMP-1 and TIMP-2; for both cases we know the complete x-ray crystallographic structure (9, 28) and the NMR structure of the N-domain (29, 30). In these two structures there are two β-strands, A and B, within the first disulfide-bonded loop. These strands lie across the protein surface and remain exposed when TIMP binds to MMPs. Fig. 8 shows models of the A and B strands (plus 5 further residues) of human TIMP-3 based on human TIMP-1 and -2. We have focused on this region, because its sequence is quite distinct from that of the A and B strands of the more readily soluble TIMP-1 and TIMP-2. This region contains 9 basic residues and only 1 acidic residue (Glu30), which is directed downward and interacts with Lys49 (not shown). TIMP-1 has only 6 basic residues and 3 acidic, and TIMP-1, although it also has 9 basic residues, has 7 acidic residues. So, only TIMP-3 has a large excess of positive charge in this region, which might explain its unique matrix-binding property. In both models (Fig. 8) 6 basic residues are directed upward from the surface and 3 are lying...
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none involving the basic and acidic residues. The data from rat uterus indicate that TIMP-3 is produced in large amount in the postpartum uterus and that it is colocalized to a great extent with heparan sulfate. Both compounds occur in the epithelium and upper stroma and not in the deeper stroma of the endometrium. At the same time in involution, the uterus is also producing a high level of matrixlys in the epithelial cells around the lumen. One role of TIMP-3 might be to regulate the matrixlys activity so that it does not attack the immediately underlying stroma. In this respect, it is interesting that matrixlys plays a role in protection against bacterial infection. In the intestine, this takes the form of activating precursors of α-defensin peptides with bactericidal activity (31). It is possible that a similar defense role is played in the postpartum uterus, which would be quite susceptible to infection, and that TIMP-3 would be an important regulator of this process.

Why would TIMP-3 be firmly bound to the ECM, whereas the other TIMPs are relatively soluble? Anand-Apte et al. (32) have suggested several reasons, based on the ability of TIMP-3 to suppress growth of tumors when melanoma cells are transfected with TIMP-3. First, the deposition of TIMP-3 in the surrounding matrix may prevent local expansion of the tumor by blocking MMP activity. Second, TIMP-3 may retard the release of sequestered growth factors, needed for tumor growth, from the ECM. Third, the protein may inhibit angiogenesis, preventing adequate blood supply to the tumor.

The anti-adhesive property of TIMP-3 has been observed in fibroblast cells (33), and this might sensitize the anoikis processes. Interestingly, whereas TIMP-1 and -2 are known for their growth effects (5), overexpression of TIMP-3 can induce apoptosis in melanoma cells (34) and smooth muscle cells (35).

In certain cell types such as melanoma, smooth muscle, MCF-7, and HT109 cells overexpression of TIMP-3, but not of TIMP-1 or TIMP-2, promotes entry into the cell cycle, which could lead to induction of apoptosis (36), and stabilizes pro-apoptotic surface molecules such as tumor necrosis factor-α receptor (37). It has been shown that TIMP-3 added exogenously to cell culture can induce apoptosis by extracellular action and that this effect is not due to inhibition of matrix metalloproteinases as shown by the lack of effect of the inhibitor BB-94 (36). Finally, many GAG chains are part of proteoglycan molecules attached to the cell surface; TIMP-3 bound in this location would be well-positioned to inhibit the shedding of tumor necrosis factor-α (10) and the syndecans 1 and 4 (38).

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