Background: Tissue inhibitor of metalloproteinases-3 (TIMP-3) is endocytosed, but its regulatory mechanism is not well understood.

Result: TIMP-3 endocytosis occurs mainly through low-density lipoprotein receptor-related protein-1 (LRP-1), but shed sLRP-1 binds TIMP-3.

Conclusion: TIMP-3-sLRP-1 complexes are retained extracellularly with metalloproteinase inhibitory activity.

Significance: LRP-1 is the master regulator of extracellular levels of TIMP-3.

SUMMARY

Tissue inhibitor of metalloproteinases-3 (TIMP-3) plays a key role in regulating extracellular matrix turnover by inhibiting matrix metalloproteinases (MMPs), adamalysins (ADAMs) and adamalysins with thrombospondin motifs (ADAMTSs). We demonstrate that levels of this physiologically important inhibitor can be post-translationally regulated by endocytosis. TIMP-3 was endocytosed and degraded by a number of cell types including chondrocytes, fibroblasts, and monocytes, and we found that the endocytic receptor low-density lipoprotein receptor-related protein-1 (LRP-1) plays a major role in TIMP-3 internalization. However, the cellular uptake of TIMP-3 significantly slowed down after 10 h due to shedding of LRP-1 from the cell surface and formation of soluble LRP-1 (sLRP-1)–TIMP-3 complexes. Addition of TIMP-3 to HTB94 human chondrosarcoma cells increased the release of sLRP-1 fragments of 500, 215, 160 and 110 kDa into the medium in a concentration dependent manner, and all these fragments were able to bind to TIMP-3. TIMP-3 bound to sLRP-1, which was resistant to endocytosis, retained its inhibitory activity against metalloproteinases. Extracellular levels of sLRP-1 can thus increase the half-life of TIMP-3 in the extracellular space, controlling the bioavailability of TIMP-3 to inhibit metalloproteinases.

INTRODUCTION

The tissue inhibitors of metalloproteinases (TIMPs) are a family of proteins that regulate the activity of metalloproteinases through formation of tight, non-covalent complexes in a 1:1 stoichiometry (1). Among the four mammalian TIMPs, TIMP-3 is unique in a number of respects. It has a broader inhibitory profile than the other TIMPs, being able to inhibit matrix metalloproteinases (MMPs) and the related metalloproteinases of the ADAM (a disintegrin and metalloproteinase) and the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) families (1). TIMP-3 is the only member of the family that can bind to the extracellular matrix (ECM) (2,3), while the other TIMPs are readily extracted from the tissue.
indicated that it is the major regulator of metalloproteinase activities in vivo. Ablation of the Timp3 gene leads to a number of defects in mice, including impaired lung development (4), dilated cardiomyopathy (5), increased apoptosis during mammary gland involution (6), and accelerated development of osteoarthritis upon aging (7). These are due to dysregulation of ECM turnover. In addition, uncontrolled activity of ADAMs, particularly ADAM17, resulted in elevated levels of TNFα and increased inflammation in murine models of liver regeneration (8), sepsis (9), and antigen-induced arthritis (7), and the phenotypes of the Timp3-null mouse are reflected in a number of human pathologies (see ref. (1) for review). Thus, there is considerable interest in understanding factors regulating TIMP-3 levels, and it has been shown that transcription of TIMP-3 can be increased by the histone deacetylase SirT1 (10) and growth factors such as TGFβ (11) and oncostatin M (12) and reduced by promoter methylation (13).

We have recently shown that TIMP-3 levels can be post-translationally regulated by endocytosis and intracellular degradation and suggested that this is an important factor determining extracellular levels of TIMP-3 (18). In the previous studies we used endocytic blockers to demonstrate the cellular uptake of TIMP-3. For example, HTB94 chondrosarcoma cells transfected with an expression plasmid for TIMP-3 produced high levels of TIMP3 mRNA, but TIMP-3 protein was not detected in the conditioned medium. When heparin, pentosan polysulfate (PPS), or receptor-associated protein (RAP), an antagonist of ligand binding to low-density lipoprotein (LDL) receptor and related receptors, was added to the cells, TIMP-3 accumulated in the medium. These studies suggested that the receptor responsible for TIMP-3 endocytosis is a member of the LDL receptor-related protein (LRP) family (18). There are 13 members in the mammalian LDL receptor family that endocytose a wide variety of ligands and deliver them to endosomes for degradation (19). Among them, LDL receptor-related protein 1 (LRP-1) endocytoses more than 30 ligands, including proteinase-inhibitor complexes and ECM proteins, making it an important regulator of ECM composition and turnover (20). LRP-1 is comprised of 2 chains, a 515 kDa α-chain that interacts with ligands via its 4 clusters of LDL receptor type A repeats, and a non-covalently associated 85 kDa β-chain that tethers the receptor in the membrane and interacts with intracellular adaptors to direct endocytosis via clathrin-coated pits (20). LRP-1 can be shed from cell membranes, releasing a soluble form of the receptor, sLRP-1, that can act as a competitive inhibitor of ligand endocytosis (21,22).

In this report, we used metabolically radiolabelled, isolated recombinant [35S]TIMP-3 to investigate the endocytic pathways for TIMP-3 in more detail and analyzed the distribution of radioactivity in different cell fractions over time. Using LRP-1-deficient cells and sulfated proteoglycan mutant cells, we examined the contribution of LRP-1-dependent and LRP-1-independent pathways of TIMP-3 endocytosis. More importantly, our study has revealed that LRP-1 shed from the cell surface (sLRP-1) also binds to extracellular TIMP-3 and sequesters TIMP-3 in the medium and that TIMP-3 bound to sLRP-1 retained metalloproteinase inhibitory activity. This suggests that LRP-1 is a master regulator of extracellular levels of TIMP-3 and regulates ECM catabolism.

EXPERIMENTAL PROCEDURES

Materials — Heparin, de-N-sulfated heparin, chondroitin sulfate, hyaluronan and pronase were from Sigma-Aldrich (Dorset, UK); dermatan sulfate from Calbiochem (Nottingham, UK); PPS from Arthropharm (Sydney, Australia) and GM6001 from Elastin Products Co (Owensville, MOS, USA). Dulbecco’s modified Eagle’s medium (DMEM), DMEM without L-glutamine or phenol red, L-glutamine, penicillin/streptomycin, fetal calf serum (FCS), amphotericin B and trypsin-EDTA from PAA Laboratories (Somerset, UK); DMEM without L-glutamine, cysteine, methionine or cystine from MP Biomedicals (Solon, OH, USA). The catalytic domain of human MMP-1 (24); recombinant His-tagged RAP (25), ADAMTS-4 lacking the C-terminal spacer domain (26) and non-radiolabelled FLAG-tagged TIMP-3 (27) were prepared as previously described.

Cell culture — HTB94 human chondrosarcoma and THP-1 human monocyte acute leukaemia cell lines were from American Culture Type Collection (Manassas, VA, USA). Human uterine cervical fibroblasts were kindly provided by Akira Ito (Tokyo University of Pharmacy and Life Sciences, Japan). CHO-K1
and CHO-745 (28) were generously provided by Jeffrey Esko (Department of Cellular and Molecular Medicine, University of California San Diego, La Jolla, USA). MEF-1 and PEA-13 (homozygous LRP-1-deficient) (29) cells were generated as described previously. Syndecan-4-deficient and wild-type MEF (30) were kindly provided by John R. Couchman (Department of Biomedical Sciences and Biotech Research and Innovation Centre, University of Copenhagen, Copenhagen, Denmark). All cells were maintained in DMEM with 10 % FCS, 100 U/ml penicillin and 100 U/ml streptomycin at 37 ºC in 5 % CO2.

Porcine articular cartilage chondrocytes were isolated from metacarpophalangeal joints within 24 h of slaughter (31). Cartilage was incubated with 1 mg/ml Clostridium histolyticum collagenase (Sigma-Aldrich, Dorset, UK) in 10 ml DMEM with 10 % FCS for 4 h at 37 ºC. The digested material was passed through a cell strainer, washed and cells cultured in DMEM containing 10 % FCS, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mg/ml amphotericin B and 10 mM HEPES at 37 ºC in 5 % CO2.

Expression and purification of \( ^{35}S \)TIMP-3 and \( ^{35}S \)N-TIMP-3 — \( ^{35}S \)TIMP-3 and \( ^{35}S \)N-terminal domain of TIMP-3 \((^{35}S\)N-TIMP-3) were prepared by metabolic labelling. HEK-293/EBNA cells transfected with a pCEP4-based expression vector for C-terminally FLAG-tagged TIMP-3 (27) were grown to confluence in a 150 cm² flask, washed and cells cultured in DMEM containing 10 % FCS, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mg/ml amphotericin B and 10 mM HEPES at 37 ºC in 5 % CO2.

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Expression and purification of \( ^{35}S \)TIMP-3 and \( ^{35}S \)N-TIMP-3 — \( ^{35}S \)TIMP-3 and \( ^{35}S \)N-terminal domain of TIMP-3 \((^{35}S\)N-TIMP-3) were prepared by metabolic labelling. HEK-293/EBNA cells transfected with a pCEP4-based expression vector for C-terminally FLAG-tagged TIMP-3 (27) were grown to confluence in a 150 cm² flask, washed and cells cultured in DMEM containing 10 % FCS, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mg/ml amphotericin B and 10 mM HEPES at 37 ºC in 5 % CO2.

Endocytosis of \( ^{35}S \)TIMP-3 — Cells were plated at a density of 1x10⁶ cells/well and washed three times with serum-free DMEM. For some experiments, cells were then incubated for 1 h at 37 ºC with GM6001 (10 µM), heparin, de-N-sulfated heparin, PPS, chondroitin sulfate, dermatan sulfate, hyaluronan (all at 200 µg/ml) or RAP (500 nM) in DMEM with 0.1 % FCS before addition of \( ^{35}S \)TIMP-3. As a control for agents diluted in DMSO, the equivalent volume of DMSO was added to separate wells. \( ^{35}S \)TIMP-3 (1 nM) was added to cells in 2 ml of DMEM with 0.1 % FCS. After 0-24 h incubation, the conditioned medium was removed and 5 % (v/v) trichloroacetic acid (TCA) added (4 ºC, overnight). The TCA-soluble fraction (2 ml) was separated from the TCA-insoluble fraction by centrifugation (13 000 rpm, 15 min, 4 ºC). The TCA-insoluble pellet was dissolved in 1 N NaOH (500 µl). The cell layer was washed three times with ice-cold PBS and solubilized in 1 N NaOH [1 ml, 1 h, room-temperature (RT)]. Each fraction was mixed with 3 ml of scintillant (Goldstar Multipurpose Liquid Scintillation Cocktail, Meridian, Surrey, UK) and radioactivity counted. TCA-soluble radioactivity present in the \( ^{35}S \)TIMP-3 preparation prior to incubation with cells (5-15% of total radioactivity) was subtracted from the amount of TCA-soluble radioactivity at each time point. The amount of radioactivity in the different fractions was calculated as a percentage of the total amount of \( ^{35}S \)TIMP-3 radioactivity added to the cells.

Endocytosis of surface-bound \( ^{35}S \)TIMP-3 — \( ^{35}S \)TIMP-3 (40 nM) in ice cold TBS was added to cells and incubated for 2 h at 4 ºC. To quantify cell surface binding of \( ^{35}S \)TIMP-3, cells were washed twice in TBS, solubilized in 1 N NaOH (1 ml) and radioactivity quantified. In parallel experiments, endocytosis of cell surface-bound \( ^{35}S \)TIMP-3 was quantified by washing cells in TBS and then incubating them for 2 h at 37 ºC in pre-warmed DMEM with 0.1 % FCS. After 2 h, conditioned media were harvested and TCA-precipitated to quantify degraded \( ^{35}S \)TIMP-3 (TCA-soluble counts in medium) and the amount of intact \( ^{35}S \)TIMP-3 released from the cell surface (TCA-insoluble counts in medium). Cell layers were treated with 0.1 % pronase in serum-free DMEM on ice for 10 min, and then centrifuged (5000 rpm, 5 min, 4 ºC) to allow quantification of cell surface-bound \( ^{35}S \)TIMP-3 (pronase-sensitive radioactivity)
and intracellular [35S]TIMP-3 (pronase-resistant radioactivity, solubilized in 1 ml of 1 N NaOH).

Confocal microscopy analysis of TIMP-3 endocytosis — HTB94, MEF-1 and PEA-13 cells were cultured on 18 mm glass cover slips coated with gelatin (0.1 % w/v). Non-radio-labelled TIMP-3 (40 nM) was added to cells either alone or in combination with heparin (200 µg/ml) or RAP (500 nM) in phenol-red free DMEM with 0.1% FCS (2 h, 37 ºC). Cells were then fixed with 3% paraformaldehyde in PBS (10 min, RT) and blocked with 5% (v/v) goat serum, 3% (w/v) bovine serum albumin in PBS (1 h, RT). Cells were permeabilized with PBS containing 0.1% (v/v) Triton X-100 (15 min, RT) and incubated with anti-FLAG M2 antibody (Sigma-Aldrich, UK, F1804, 5 µg/ml, 1 h, RT). Alexa Fluor 488–conjugated goat anti-mouse IgG (Molecular Probes, Cambridge, UK) was used to visualize the antigen signal (5 µg/ml, 1 h, RT). Actin was stained with Alexa Fluor 568–conjugated phalloidin (Molecular Probes, Cambridge, UK). The signals were analyzed using a CCD camera-equipped microscope (Nikon TE-2000) with a 60 × objective lens.

siRNA targeting of syndecan-1 expression — HTB94 cells (1-1.5x10⁵ cells) were transiently transfected with 50 nM of two siRNAs targeting syndecan-1 (AM12432 and AM142557, Ambion) or scrambled (Ambion) using Lipofectamine 2000 for 3 h. After 24 and 48 h, expression of syndecan-1 was reduced by about 90% compared to the housekeeping gene GAPDH.

Isolation of sLRP-1 from plasma — sLRP-1 was isolated from fresh-frozen human plasma by a modification of previously published methods (32). Plasma was diluted 5-fold with equilibration buffer (50 mM citrate, pH 6.0, 75 mM NaCl, 0.02 % NaN₃) supplemented with proteinase inhibitor cocktail (P8340, Sigma-Aldrich, Dorset, UK) and applied to a MacroPrep S ion exchange resin (Bio-Rad, Hemel Hempstead, UK) equilibrated in the same buffer. The resin was extensively washed in equilibration buffer to remove unbound material and bound proteins then eluted in a gradient of 75 to 500 mM NaCl in equilibration buffer over 10 column volumes. Eluted fractions were analyzed by SDS-PAGE with silver staining and immunoblotting for LRP-1 using the 8G1 antibody (Abcam, Cambridge, UK).

ELISA detection of TIMP-3 binding to sLRP-1 — High-binding microtitre plates (Corning, NY, USA) were coated with 0.4 nmol sLRP-1 in 50 mM citrate, pH 6.0, 75 mM NaCl overnight. Wells were blocked using 3 % BSA-PBS (1 h, 37 ºC) and washed in PBS containing 0.1 % (v/v) Tween 20 (Sigma-Aldrich, Dorset, UK) after this and each subsequent step. Wells were then incubated with recombinant TIMP-3 in blocking solution (0.15 - 20 nM, 3 h, 37 ºC). Bound TIMP-3 was detected by incubation of wells with murine M2 anti-FLAG antibody (3 h, 37 ºC) and then with an anti-mouse secondary antibody coupled to horseradish peroxidase (1 h, 37 ºC) (DAKO, Ely, UK). Hydrolysis of tetramethylbenzidine substrate (KPL, Gaithersburg, MA, USA) was measured at 450 nm using a BioTek EL-808 absorbance microplate reader (BioTek, Winooski, VT, USA).

Inhibitory activity of TIMP-3 remnants — HTB94 cells were treated with or without TIMP-3 (1 nM) for 0, 24 or 48 h in serum-free, phenol-red free DMEM. Conditioned media were collected and centrifuged (13000 rpm, 15 min, 4 ºC) to remove cell debris. Recombinant ADAMTS-4 lacking the C-terminal spacer domain (1 nM) was incubated with dilutions of the media or with purified recombinant TIMP-3 in assay buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM CaCl₂, 0.05 % Brij, 0.02 % NaN₃) for 1 h at 37 ºC. Residual activity against the fluorescent peptide substrate carboxyfluorescein-Ala-Glu~Leu-Asn-Gly-Arg-Pro-Ile-Ser-Ile-Ala-Lys-N,N,N',N'-tetramethyl-6-carboxyrhodamine (FAM-AE~LQGRPISIAK-TAMRA, custom synthesized by Bachem, Switzerland) was determined (0.5 µM, excitation wavelength 485 nm, emission wavelength 538 nm with 495 nm cut-off) (27).

Co-immunoprecipitation of sLRP-1 with TIMP-3 — HTB94 cells were incubated in serum-free DMEM for 24 h, the conditioned media collected and centrifuged (13000 rpm, 15 min, 4 ºC) to remove cell debris. TIMP-3-FLAG (1 nM) was added to the conditioned medium (10 ml) and incubated overnight at RT with mixing. The solution was then applied to an anti-FLAG M2-agarose affinity resin (1 ml) equilibrated in TBS and the resin extensively washed with 10 volumes of TBS. Bound proteins were sequentially eluted with 50 mM Tris-HCl, pH 7.5, 10 mM CaCl₂, 0.2 % NaN₃ containing 1 M NaCl (2 x 0.5 ml) and then with the same buffer containing 150 mM NaCl and 200 µg/ml FLAG peptide (2 x 0.5 ml). Eluted samples were analysed by SDS-PAGE and immunoblotting using M2 anti-FLAG and 8G1.
anti-LRP-1 antibodies. A band corresponding to the LRP-1 immunoreactive band was excised from the silver stained gel, digested with trypsin and analyzed by mass spectrometry.

RESULTS

TIMP-3 is endocytosed by HTB94 cells — Metabolically labelled [35S]TIMP-3 was purified and added to HTB94 cells in culture. The radioactivity in the TCA-insoluble fraction (intact [35S]TIMP-3) of the conditioned medium decreased over 24 h, while that in the TCA-soluble fraction (degraded [35S]TIMP-3 fragments) increased (Fig. 1A). Radioactivity was also detected in the cell and ECM layer (the cell-associated fraction). Treatment of the cell-associated layer with pronase indicated that about half of the [35S]TIMP-3 in this fraction was present within cells, with the remainder was present on the cell surface and in the ECM. These results indicate that [35S]TIMP-3 bound to the cell surface, was endocytosed and degraded by the cells, and peptide fragments were released back into the medium. [35S]N-TIMP-3 was also endocytosed by HTB94 cells with indistinguishable kinetics, suggesting that the N-terminal domain contains the minimal structure required for endocytosis.

We previously reported that TIMP-3 accumulated in the conditioned medium of HTB94 cells in the presence of RAP, an antagonist of ligand binding to the LRP family of endocytic receptors (18). In line with this finding, RAP (500 nM) reduced endocytosis of [35S]TIMP-3 by HTB94 cells, i.e., an increase in TCA-insoluble radioactivity and a decrease in TCA-soluble and cell-associated radioactivity (Fig. 1A). These results indicate that [35S]TIMP-3 bound to the cell surface, was endocytosed and degraded by the cells, and peptide fragments were released back into the medium. [35S]N-TIMP-3 was also endocytosed by HTB94 cells with indistinguishable kinetics, suggesting that the N-terminal domain contains the minimal structure required for endocytosis.

TIMP-3 endocytosis is reduced in LRP-1-deficient cells — Since TIMP-3 endocytosis was inhibited by RAP, we postulated that a member of the LRP family of receptors was involved in TIMP-3 endocytosis. We thus compared [35S]TIMP-3 endocytosis by LRP-1-deficient mouse embryonic fibroblasts (PEA-13 cells) and wild-type cells (MEF-1). As shown in Fig. 2A, [35S]TIMP-3 was readily endocytosed by MEF-1 cells with similar kinetics to that observed in HTB94 cells, but [35S]TIMP-3 endocytosis was impaired in PEA-13 cells, with the rate of endocytosis approximately half of that observed in HTB94 and MEF-1 cells (Fig. 2B). This indicates that LRP-1 mediates a major pathway for TIMP-3 endocytosis, but that an LRP-1-independent pathway also operates in these cells. Similar results were obtained using confocal microscopy, with endocytosed TIMP-3-FLAG visible in vesicles within MEF-1 cells, but lower intracellular staining observed in PEA-13 cells (Fig. 2C). RAP inhibited [35S]TIMP-3 endocytosis and degradation by MEF-1 cells to the level observed in PEA-13 cells (Fig. 2A and B). In contrast, RAP had no effect on [35S]TIMP-3 endocytosis by PEA-13 cells. Based on these observations we propose that LRP-1 is the only member within the LRP family that mediates [35S]TIMP-3 endocytosis in these cells.

On the other hand, heparin strongly inhibited [35S]TIMP-3 endocytosis by both MEF-1 and PEA-13 cells (Fig. 2A and B). In this case, only a minimal decrease in TCA-insoluble radioactivity and little increase in TCA-soluble radioactivity in the medium were observed, and [35S]TIMP-3 was barely detectable in the cell-associated layer.

We further investigated the role of LRP-1 in [35S]TIMP-3 surface binding by adding 1 nM [35S]TIMP-3 to cells at 4 ºC. Comparable binding of [35S]TIMP-3 to MEF-1 and PEA-13 cells was observed (Fig. 2D). Upon warming
the cells to 37 °C, higher radioactivity was observed in MEF-1 cells, confirming their greater ability to endocytose \(^{35}\text{S}\)TIMP-3. Interestingly, both MEF-1 and PEA-13 cells released about a third of the surface-bound radioactivity back into the medium, indicating there was a notable dissociation of \(^{35}\text{S}\)TIMP-3 from the cell surface under these experimental conditions. The release of TCA-soluble radioactivity by MEF-1 and PEA-13 cells was measured following cell surface binding of \(^{35}\text{S}\)TIMP-3 and warming of the cells to 37 °C. MEF-1 cells released approximately twice as much TCA-soluble radioactivity as PEA-13 cells, confirming the existence of LRP-1-dependent and -independent endocytic pathways for TIMP-3.

**Cell surface sulfated GAGs are not involved in TIMP-3 endocytosis** — Since highly sulfated polysaccharides (e.g. heparin and PPS) blocked TIMP-3 endocytosis \((18)\), we postulated that HSPGs or CSPGs may be involved in TIMP-3 endocytosis. To test this possibility we compared \(^{35}\text{S}\)TIMP-3 endocytosis by wild-type (CHO-K1) and mutant CHO-745 cells which lack HS and CS chains due to a mutation in xylosyltransferase \((28)\). We observed no significant differences in the rate of \(^{35}\text{S}\)TIMP-3 endocytosis by CHO-K1 and CHO-745 cells \(\text{Fig. 3A}\), indicating that neither HSPG nor CSPG may be required for the internalization of TIMP-3. \(^{35}\text{S}\)TIMP-3 endocytosis by both CHO-K1 and CHO-745 cells was partially inhibited by RAP \((500 \text{ nM})\) \(\text{(Data not shown)}\). Heparin \((200 \mu\text{g/ml})\) inhibited \(^{35}\text{S}\)TIMP-3 endocytosis by CHO-745 cells \(\text{(Figure 3B)}\) indicating that the blocking effect of heparin is independent from the presence of HSPGs or CSPGs on the cell surface. Similarly, heparin blocked \(^{35}\text{S}\)TIMP-3 endocytosis by CHO-K1 cells. Interestingly, both CHO-745 and CHO-K1 exhibited a higher amount of cell-associated radioactivity than HTB94 or MEF cells. \(^{35}\text{S}\)TIMP-3 levels in this fraction were not affected by RAP, but were strongly inhibited by heparin.

We further investigated possible involvement of the syndecan family in TIMP-3 endocytosis, since some protein ligands have been shown to bind to the core protein of these proteoglycans \((33)\). Comparable endocytosis of \(^{35}\text{S}\)TIMP-3 was observed in syndecan-4-deficient and wild-type MEF, and siRNA targeting of syndecan-1 \(\text{(leading to a 90% reduction in expression)}\), had no effect on \(^{35}\text{S}\)TIMP-3 endocytosis.

Since heparin inhibits TIMP-3 endocytosis by cells lacking HSPG and CSPG, we tested whether heparin can directly interfere the binding of TIMP-3 to LRP-1. We isolated sLRP-1 from human plasma and measured binding of TIMP-3 and TIMP-3-heparin complexes to immobilized sLRP-1 by ELISA. TIMP-3 bound readily to LRP-1, but upon pre-incubation with 100 \(\mu\text{g/ml}\) heparin, binding of TIMP-3 to LRP-1 was completely abolished \(\text{Fig. 3C)}\).

**sLRP-1 inhibits TIMP-3 endocytosis** — During the course of this study we noticed that for all the cell lines we tested, TIMP-3 endocytosis was initially fast, but decreased over time and little endocytosis was observed after 10 h. \(^{35}\text{S}\)TIMP-3 remaining in the medium at 24 h \((^{35}\text{S}\)TIMP-3 remnants) appeared intact by reducing SDS-PAGE and autoradiography, with no fragmentation evident. The ability of TIMP-3 remnants to inhibit metalloproteinase activity was assessed by adding 1 nM TIMP-3 to HTB94 cells for 0, 24 or 48 h, after which time TIMP-3 remnants in the media were harvested and titrated against 1 nM ADAMTS-4 \textit{in vitro}. TIMP-3 collected after 0 h incubation on HTB94 cells completely inhibited 1 nM ADAMTS-4 \(\text{(Fig. 4A)}\). TIMP-3 harvested after 24 and 48 h incubation on HTB94 cells inhibited ADAMTS-4 activity by 60%, indicating that while the concentration of TIMP-3 in the medium had been reduced by endocytosis, the TIMP-3 remnants remaining in the medium retained their full inhibitory activity against metalloproteinases. These data indicate that TIMP-3 remnants had not undergone any substantial structural modification or inactivation.

We therefore considered whether the reduced rate of \(^{35}\text{S}\)TIMP-3 endocytosis was due to a change in the endocytic capacity of the cells. Although TIMP-3 has been shown to induce apoptosis in a variety of cell types \((34)\), we found that incubation of HTB94 cells with 1 nM \(^{35}\text{S}\)TIMP-3 for 24 h had no effect on cell viability as measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Furthermore, pre-treatment of HTB94 cells with non-radiolabelled TIMP-3 for 24 h did not alter the rate of endocytosis of subsequently added \(^{35}\text{S}\)TIMP-3.

We then investigated the possibility that TIMP-3 remnants in the medium were resistant...
to being taken up by the cells. To test this, $^{[35S]}$TIMP-3 remnants were added to naïve cells that had not previously been exposed to TIMP-3. Remnants collected from HTB94 cells after 24 h of incubation were largely resistant to endocytosis by naïve HTB94 cells, indicating that the $^{[35S]}$TIMP-3 remnants had become refractory to endocytosis (Fig. 4B). This phenomenon was further studied by incubating PEA-13 cells, which lack LRP-1, with $^{[35S]}$TIMP-3 for 24 h, and transferring the conditioned medium containing $^{[35S]}$TIMP-3 remnants to naïve MEF-1 cells. $^{[35S]}$TIMP-3 remnants from PEA-13 cells were readily endocytosed by naïve MEF-1 (Fig. 4C), whereas $^{[35S]}$TIMP-3 remnants from MEF-1 cells were resistant to endocytosis by naïve MEF-1 cells (Fig. 4D). Based on these results, we postulated that TIMP-3 became resistant to endocytosis as a result of association with sLRP-1 shed from cells into the conditioned medium during the culturing period.

sLRP-1 accumulated over time in the conditioned medium harvested from HTB94 cells grown in serum-free medium in the absence of TIMP-3 (Fig. 4E). Binding of TIMP-3 to sLRP-1 was investigated first by incubating TIMP-3-FLAG (1 nM) in vitro with the conditioned medium from HTB94 cells. After overnight incubation at ambient temperature, the mixture was applied to an M2 anti-FLAG resin, and bound proteins were eluted with FLAG peptide. Analysis of the eluted samples with the 8G1 anti-LRP-1 antibody indicated that LRP-1 was co-eluted with TIMP-3-FLAG by FLAG-peptide (Fig. 4F). The identity of the eluted LRP-1 band was further confirmed by mass spectrometry, confirming that TIMP-3 can interact with sLRP-1 shed from cells into the conditioned medium of cells.

We also found that addition of TIMP-3 to HTB94 cells increases the release of sLRP-1 fragments of 500, 215, 160 and 110 kDa into the conditioned medium in a concentration-dependent manner, suggesting that the inhibitor can induce shedding and fragmentation of LRP-1 (Fig. 4G). When TIMP-3-FLAG (1 nM) was incubated with HTB94 cells for 24 h and conditioned medium was applied to an M2 anti-FLAG resin column, bound TIMP-3-FLAG co-eluted with the 500 kDa sLRP-1 and with LRP-1 fragments of 215, 160 and 110 kDa (Fig. 4H). This indicates that these fragments generated upon addition of TIMP-3 to the cells are capable of binding to the inhibitor.

**DISCUSSION**

Our study has revealed a new mechanism for regulating levels of TIMP-3 in the extracellular space. We previously reported that levels of this physiologically and pathologically important inhibitor can be post-translationally regulated by endocytosis (18). TIMP-3 accumulates in the medium of HTB94 chondrosarcoma cells and porcine articular chondrocytes treated with receptor-associated protein (RAP), an antagonist of ligand binding to the LRP family of endocytic receptors (18). Using radiolabelled TIMP-3 we investigated the kinetics of TIMP-3 endocytosis and degradation by LRP-1-deficient PEA-13 mouse embryonic fibroblasts and their wild-type counterpart. TIMP-3 endocytosis was substantially reduced in LRP-1-deficient PEA-13 cells compared to wild-type cells, indicating that LRP-1 is an important component of the TIMP-3 endocytic machinery. RAP, which inhibits ligand binding to all LRPs, had no effect on the residual endocytosis of TIMP-3 by PEA-13 cells, indicating that LRP-1 is the only member of the LRP family that mediates TIMP-3 endocytosis in mouse embryonic fibroblasts.

We compared the endocytosis of TIMP-3 by several cell types, including chondrosarcoma cells, porcine articular chondrocytes, fibroblasts and monocyte-like THP-1 cells. All cell types showed similar kinetics for TIMP-3 endocytosis. The rate of TIMP-3 endocytosis was not constant over 24 h. After addition of $^{[35S]}$TIMP-3 to cells, endocytosis was initially rapid, but then slowed, so that only minimal endocytosis was observed after 10 h. We demonstrated that this reduction in endocytosis is due to interaction of TIMP-3 with a soluble form of LRP-1 shed from the cell surface into the medium. Different LRP-1 ‘sheddases’ have been described, including ADAM10, ADAM12, ADAM17, MT1-MMP and β-secretase (23,35-38). Although the specific site(s) at which these enzymes cleave LRP-1 have not been characterized, shedding is known to involve cleavage of the β-chain of LRP-1, releasing a fragment of the β-chain in complex with the entire ligand-binding α-chain from the cell membrane (39). Inflammation is a key stimulus for LRP-1 shedding. For example, interferon γ and LPS stimulate LRP-1 shedding by ADAM17 (35). Increased levels of sLRP-1 are detectable in the plasma of patients with rheumatoid arthritis, systemic lupus erythematosus (35) and
liver disease (32). sLRP-1 and lower molecular weight fragments of LRP-1 are found in brain and in cerebral spinal fluids, and are increased in older people (23). Cholesterol depletion has also been found to stimulate LRP-1 shedding by MT1-MMP and ADAM12 (36). We observed LRP-1 shedding in the absence of any pro-inflammatory stimuli, and addition of IL-1 had no effect on the rate of TIMP-3 endocytosis by primary chondrocytes (S. Scilabra and H. Nagase, unpublished observations). Since a number of the proposed LRP-1 ‘sheddases’ are metalloproteinases, production of sLRP-1 should be inhibited by metalloproteinase inhibitors such as TIMP-3 and GM6001. However, we found that TIMP-3 could induce shedding of LRP-1 and generation of LRP-1 fragments. A previous report showed that N-TIMP-3 inhibited MT1-MMP- and ADAM12-mediated shedding of LRP-1 (36). We found that, unlike TIMP-3, N-TIMP-3 did not induce shedding and fragmentation of LRP-1 (S. Scilabra and H. Nagase, unpublished observations), therefore we hypothesize that the C-terminal domain of TIMP-3 can play a crucial role in this process. GM6001 has also been shown to inhibit LRP-1 shedding from macrophages and lung fibroblasts (21,35), but we found that GM6001 had no effect on the rate of [35S]TIMP-3 endocytosis by HTB94 cells. This suggests that a GM6001-insensitive sheddase may mediate LRP-1 shedding in HTB94 cells. The stimulus and sheddases responsible for LRP-1 constitutive shedding and TIMP-3 induced shedding in HTB94 cells and chondrocytes are currently under investigation in our laboratory.

Shedding of LRP-1 has numerous consequences. Firstly, it can reduce endocytosis of LRP-1 ligands in a loss-of-function manner by reducing the number of endocytic receptors on the cell surface (21,36,38). Secondly, sLRP-1 can bind to and inhibit endocytosis of its ligands, as we found for TIMP-3, and as has been reported for tPA (32). By antagonizing LRP-1-mediated endocytosis, sLRP-1 can increase the half-life of TIMP-3 in the extracellular space. More importantly, we found that the TIMP-3-sLRP-1 complex is still able to inhibit metalloproteinases. We found that the ratio between cell surface LRP-1 and sLRP-1 is an important factor that regulates the bioavailability of TIMP-3 in the extracellular space, and hence controls ECM proteolysis and proteolysis of cell surface molecules.

In addition to the LRP-1-mediated pathway, we observed LRP-1-independent endocytosis of TIMP-3 in PEA-13 cells and RAP-treated HTB94, MEFs and CHO cells. Although this is a slower process compared with the LRP-1-mediated endocytosis, it is likely to be receptor-mediated. Members belonging to the macrophage mannose receptor protein family may be possible candidates as they have been shown to internalize some LRP-1 ligands and play a crucial role in ECM turnover. For instance, urokinase plasminogen activator receptor-associated protein/endocytic recycling protein Endo180 is expressed in chondrocytes and fibroblasts and is involved in collagen internalization (40,41). TIMP-1 and TIMP-2 are also endocytosed by LRP-1 (42,43). While TIMP-1 is internalized only in complex with MMP-9, with the binding determinants thought to reside on the MMP-9 (42). TIMP-2 can be endocytosed by LRP-1 both as a free inhibitor and in complex with MMP-2 or proMMP-2 (43). Interestingly, binding of TIMP-2 to HT1080 cells was insensitive to RAP, suggesting that TIMP-2 initially binds to a cell surface receptor other than LRP-1 prior to endocytosis (43). Furthermore, endocytosis and degradation of TIMP-2 was only partially inhibited by RAP, indicating that an LRP-1-independent endocytic pathway also occurs (43). These studies thus suggest that both TIMP-2 and TIMP-3 can be endocytosed by LRP-1-dependent and LRP-1-independent pathways. It would be interesting to determine whether TIMP-2 and TIMP-3 share the same mechanism of LRP-1-independent endocytosis.

Endocytosis of TIMP-3 in all tested cell types was almost completely inhibited by heparin. This suggested that an HSPG or CSPG might participate in TIMP-3 endocytosis, either as a direct endocytic receptor or as a co-receptor of LRP-1. HSPG have been demonstrated to mediate endocytosis of ligands including lipoproteins (44). Alternatively, HSPG might act as co-receptors of LRP-1-mediated endocytosis as proposed for thrombospondin 1 (45), tissue factor pathway inhibitor (46), amyloid-β (47) and factor VIII (48). However, we found that TIMP-3 endocytosis was not affected in xylosyltransferase-deficient CHO-745 cells, indicating that sulfated cell surface proteoglycans are not required for TIMP-3 endocytosis. In line with this finding, TIMP-3 endocytosis was also unimpaired in syndecan-4-null and syndecan-1-silenced cells. We therefore
conclude that cell surface HSPG or CSPG do not mediate the LRP-1-independent pathway of TIMP-3 endocytosis.

The unimpaired endocytosis of TIMP-3 in CHO-745 cells nullified our hypothesis that heparin inhibits TIMP-3 endocytosis by blocking interaction with a sulfated cell surface proteoglycan component of the endocytic pathway. Interestingly, heparin has been shown to directly inhibit interaction of other ligands, including factor IXa (49), apolipoprotein A-V (50), C4b-binding protein (51) and PAI-1 (52), to LRP-1. In the case of PAI-1, the heparin-binding and LRP-1-binding regions have been found to overlap (53,54). Binding of proteins to heparin is commonly mediated by clusters of positively charged residues (55). Similarly, binding of protein ligands to LRP-1 and related LDL receptors is often mediated by positive residues, as has been demonstrated for RAP (56,57) and α2M (58). In the case of RAP, crystallography and mutagenesis studies have confirmed that K256 and K270 mediate binding to acidic pockets in the ligand binding motifs of the receptor (56,57). Inhibition of TIMP-3 binding to sLRP-1 by heparin suggests that the heparin-binding and LRP-1-binding sites of TIMP-3 may also overlap. The three-dimensional structure of full-length TIMP-3 is currently unavailable, but the protein is predicted to contain an extended patch of basic residues, located on the opposite face of the protein to the inhibitory ridge that interacts with metalloproteinase. This may explain why TIMP-3-sLRP-1 complexes retain metalloproteinase inhibition activity.

The phenotypes of the Timp3−/− mouse reveal the central role of TIMP-3 in regulating activity of MMPs, ADAMs and ADAMTSs. Our study has shown that LRP-1 plays an important role in controlling the extracellular levels of TIMP-3. It is well established that TIMP-3 binds to ECM (2,3), but we have shown here that secreted TIMP-3 can be readily internalized via LRP-1, and this process competes with TIMP-3 binding to the ECM. On the other hand, shed sLRP-1 sequesters TIMP-3 from the above two reactions and increases its availability to extracellular MMPs, ADAMs and ADAMTs. It is notable that shedding of LRP-1 is increased under inflammatory conditions. We therefore propose that LPR-1 is a master regulator of extracellular trafficking of TIMP-3 and the ratio of cell surface LPR-1 and sLRP-1 dictates TIMP-3 availability.

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References


FOOTNOTES
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Abbreviations used are: ADAM, a disintegrin and metalloproteinase; ADAMTS, adamalysin with thrombospondin motifs; CSPG, chondroitin sulfate proteoglycan; ECM, extracellular matrix; GAG, glycosaminoglycan; HSPG, heparan sulfate proteoglycan; LDL, low-density lipoprotein; LRP-1, low-density lipoprotein receptor-related protein-1; MMP, matrix metalloproteinase; N-TIMP, N-terminal domain of TIMP; PPS, pentosan polysulfate; RAP, receptor associated protein; sLRP-1, soluble LRP-1; TCA, trichloroacetic acid; TIMP, tissue inhibitor of metalloproteinase.

FIGURE LEGENDS

Figure 1: TIMP-3 is endocytosed by HTB94 chondrosarcoma cells. A, [³⁵S]TIMP-3 (1 nM) was added to HTB94 chondrosarcoma cells in the absence or presence of RAP (500 nM) or heparin (200 µg/ml), and radioactivity in different cell fractions monitored over time (n=3). TIMP-3, black symbols; TIMP-3 plus 500 nM RAP, open symbols; TIMP-3 plus heparin, grey symbols. B, Confocal microscopy analysis of TIMP-3 endocytosis by HTB94 cells. Cells were incubated for 2 h at 37 °C with TIMP-3-FLAG (40 nM), either alone or with heparin (200 µg/ml) or RAP (500 nM). Control cells were incubated without TIMP-3-FLAG. Cells were washed and permeabilized, and endocytosed TIMP-3-FLAG visualised using an M2 anti-FLAG antibody and an Alexa 488-labelled secondary antibody (green channel). The cytoskeleton was visualized using Alexa 568-labelled phalloidin (red channel). Images were gathered using a 60× objective lens. Scale bar: 41 µm.

Figure 2: LRP-1 contributes to [³⁵S]TIMP-3 endocytosis. A-B, [³⁵S]TIMP-3 (1 nM) was added to MEF-1 (A) or PEA-13 (B) cells for 0-24 h and radioactivity in the cell-associated fraction (triangles), as well as in the TCA-insoluble (squares) and TCA-soluble (circles) fractions of the conditioned media were quantified by scintillation counting (n=3). TIMP-3, black symbols; TIMP-3 plus 500 nM RAP, open symbols; TIMP-3 plus 200 µg/ml heparin, grey symbols. C, Confocal microscopy analysis of TIMP-3 endocytosis by MEF-1 and PEA-13 cells. Cells were incubated for 2 h at 37 °C with TIMP-3-FLAG (40 nM), washed and permeabilized, and endocytosed TIMP-3-FLAG visualised using an M2 anti-FLAG antibody and an Alexa 488-labelled secondary antibody (green channel). The cytoskeleton was visualized using Alexa 568-labelled phalloidin (red channel). Images were gathered using a 60× objective lens. Scale bar: 41 µm.

Figure 3: Cell surface HSPGs and CSPGs are not required for TIMP-3 endocytosis. A-B, [³⁵S]TIMP-3 (1 nM) was added to CHO-K1 (A, B, solid lines and symbols), CHO-745 (A, dashed lines and open symbols) or heparin treated CHO-745 (B, dashed lines and open symbols) cells for 0-24 h and radioactivity in the cell-associated fraction (triangles), as well as in the TCA-insoluble (squares) and TCA-soluble (circles) fractions of the conditioned media were quantified by scintillation counting (n=3). TIMP-3, black symbols; TIMP-3 plus 500 nM RAP, open symbols; TIMP-3 plus 200 µg/ml heparin, grey symbols. C, Shed LRP purified from human plasma was coated onto microtitre plates and binding of TIMP-3 (0.1 – 40 nM) in the absence (◼) and presence (◻) of 100 µg/ml heparin was measured using an M2 anti-FLAG antibody and a horseradish peroxidase-conjugate secondary antibody. Control wells (○) were not coated with sLRP.

Figure 4: sLRP-1 interacts with TIMP-3 and inhibits its endocytosis. A, ADAMTS-4 (1 nM) was incubated (1 h, 37 °C) with dilutions of recombinant TIMP-3 in TNC buffer (from 1 nM, ◻) or with dilutions of TIMP-3 (1 nM) incubated on HTB94 cells for 0 h (◼), 24 h (▲) or 48 h (●). As a control, ADAMTS-4 was incubated with conditioned medium from HTB94 cells incubated in serum-free medium without TIMP-3 for 24 h (○) or 48 h (△). Residual activity against FAM-AE~LQGRPISIAK-TAMRA was determined. B, [³⁵S]TIMP-3 (1 nM) was added to HTB94 cells for 24 h, after which conditioned media were harvested and transferred to naïve HTB94 cells for a further 0-24 h.
Radioactivity in the cell-associated fraction (▲), as well as in the TCA-insoluble (◼) and TCA-soluble (●) fractions of the conditioned media were quantified by counting radioactivity (this result is representative of three separate experiments). C, [35S]TIMP-3 (1 nM) was added to PEA-13 cells for 24 h, after which conditioned media were harvested and transferred to fresh MEF-1 cells. Radioactivity in the cell-associated fraction (▲), as well as in the TCA-insoluble (◼) and TCA-soluble (●) fractions of the conditioned media were quantified by scintillation counting (representative of two separate experiments). D, [35S]TIMP-3 (1 nM) was added to MEF-1 cells for 24 h, after which conditioned media were harvested and transferred to naïve MEF-1 cells. Radioactivity in the cell-associated fraction (▲), as well as in the TCA-insoluble (◼) and TCA-soluble (●) fractions of the conditioned media were quantified by scintillation counting (this result is representative of two separate experiments). E, Conditioned medium harvested from HTB94 cells at different times was analyzed by SDS-PAGE and immunoblotting using 8G1 anti-LRP-1 antibody. F, Conditioned medium collected from HTB94 cells was incubated with or without TIMP-3-FLAG (1 nM, overnight, RT) applied to an M2 anti-FLAG resin. The resin was extensively washed with TBS and then with 1 M NaCl in TBS to remove unspecific binding. Subsequently the resin was sequentially eluted with 200 µg/ml FLAG peptide in TBS (2 x 0.5 ml, lanes 1 and 2). Eluted samples were analyzed by SDS-PAGE and immunoblotting using M2 anti-FLAG and 8G1 anti-LRP-1 antibodies. G, Different concentrations of TIMP-3 (0 to 25 nM) were incubated with HTB94 cells (24 h, 37 °C). Conditioned media were harvested and analyzed by SDS-PAGE and immunoblotting using 8G1 anti-LRP-1 antibody. H, TIMP-3-FLAG was incubated with HTB94 cells (1 nM, 24 h, 37 °C). The conditioned medium containing TIMP-3-FLAG remnants was harvested and applied to an M2 anti-FLAG resin. The resin was extensively washed with TBS and then with 1 M NaCl in TBS to remove unspecific binding. Subsequently the resin was sequentially eluted with 200 µg/ml FLAG peptide in TBS (2 x 0.5 ml, lanes 1 and 2). Eluted samples were analyzed by SDS-PAGE and immunoblotting using M2 anti-FLAG and 8G1 anti-LRP-1 antibodies.
Figure 1
A: MEF-1

B: PEA-13

C

D

E

Figure 2
Figure 3
Figure 4
Differential Regulation of Extracellular Tissue Inhibitor of Metalloproteinases-3 Levels by Cell Membrane-bound And Shed Low Density Lipoprotein Receptor-related Protein 1
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