Low Tissue Inhibitor of Metalloproteinases 3 and High Matrix Metalloproteinase 14 Levels Defines a Subpopulation of Highly Invasive Foam-Cell Macrophages

Jason L. Johnson, Graciela B. Sala-Newby, Yasmin Ismail, Concepción M. Aguilera, Andrew C. Newby

Objective—An excess of metalloproteinases (MMPs) over tissue inhibitors of metalloproteinases (TIMPs) may favor atherosclerotic plaque rupture. We compared TIMP levels in nonfoamy and foam-cell macrophages (FCM) generated in vivo.

Methods and Results—In vivo generated rabbit FCM exhibited 84% reduced TIMP-3 protein compared to nonfoamy macrophages, and immunocytochemistry revealed a TIMP-3 negative subset (28%). Strikingly, only TIMP-3 negative FCM invaded a synthetic basement membrane, and invasion was inhibited by exogenous TIMP-3. TIMP-3 negative FCM also had increased proliferation and apoptosis rates compared to TIMP-3 positive cells, which were retarded by exogenous TIMP-3; this also reduced gelatinolytic activity. TIMP-3 negative FCM were found at the base of advanced rabbit plaques and in the rupture-prone shoulders of human plaques. To explain the actions of low TIMP-3 we observed a 26-fold increase in MT1-MMP (MMP-14) protein in FCM. Adding an MT1-MMP neutralizing antibody reduced foam-cell invasion, apoptosis, and gelatinolytic activity. Furthermore, MT1-MMP overexpressing and TIMP-3 negative FCM were found at the same locations in atherosclerotic plaques.

Conclusions—These results demonstrate that TIMP-3 is downregulated in a distinct subpopulation of FCM which have increased MMP-14. These cells are highly invasive and have increased proliferation and apoptosis, all properties expected to destabilise atherosclerotic plaques. (Arterioscler Thromb Vasc Biol. 2008;28:1647-1653)

Key Words: atherosclerosis ■ tissue inhibitors of matrix metalloproteinases ■ macrophages ■ foam cells ■ plaque vulnerability

Macrophages have been proposed to be involved in both atherosclerotic plaque fibrous cap formation and disruption.1-4 Matrix metalloproteinases (MMPs) are one group of proteases produced by macrophages that also appear to have a dual role in plaque cap building and destruction. Consistent with this, MMPs-2 and -9 in particular are implicated in intima formation after vascular injury. On the other hand, MMPs-1, -2, -3, -7, -9, -11, -12, -13, -14, and -16 are upregulated in human plaques in macrophage-rich areas that show a high propensity for plaque rupture.5 Numerous MMPs are also upregulated in the plaques of cholesterol-fed rabbits6,7 and ApoE null mice.8 Studies with ApoE/MMP compound knockout mice and other transgenic models show clear effects of individual MMPs on both fibrous cap formation and rupture.5 What seems to determine the outcome is the level and spectrum of MMPs produced and activated. There is therefore significant interest in the factors regulating MMP activity in macrophages. Conversion of macrophages to foam-cells is one important factor. For example, in vivo generated rabbit foam-cells have increased levels of MMPs-1 to 3 and -12.6,9,10

Tissue inhibitors of MMPs (TIMPs) are a family of specific protein inhibitors of MMPs, 4 of which have been demonstrated in vascular cells. Smooth muscle cells (SMCs) in human atherosclerotic plaques harbor abundant TIMP-1 and -2 protein expression.11 TIMP-1 and -2 expression is increased at the base of atherosclerotic plaques in cholesterol-fed rabbit aortas, and this correlates with areas of low MMP activity measured by in situ zymography.12 TIMP-3 has been detected more selectively in plaque macrophages at the shoulder regions and between the fibrous cap and necrotic core.13 Subsequently it was suggested that TIMP-3 may serve as a protective mechanism against plaque rupture by dampening local proteolysis.13 Thus, upregulation of TIMPs could have an important plaque stabilizing effect. Consistent with this, gene transfer of TIMP-1 or -2 reduces atherosclerosis and stabilizes plaques in the apoE knockout mouse.14,15 The effect of foam-cell formation on TIMP production has not...
been systematically investigated. We therefore undertook a study to compare the expression of TIMPs during macrophage foam cell formation.

Methods
Please see expanded details in the Online Data Supplement (available online at http://atvb.ahajournals.org).

Generation and Isolation of Macrophages and Foam-Cell Macrophages
New Zealand White rabbits (Harlan, UK) fed a normal chow diet or 1% cholesterol-supplemented diet had sterile sponges placed under the dorsal skin to generate nonfoamy macrophages (NFM) or foam-cell macrophages (FCM), respectively, as described previously. Endarterectomy specimens were made available from the AtheroExpress biobank.

Analysis of TIMP mRNA Expression by Quantitative-Polymerase Chain Reaction
Total RNA was extracted from rabbit macrophage and foam-cell macrophages. Procedures for RT-PCR have been described previously. Results are expressed in arbitrary units and are adjusted for 18S mRNA levels.

Western Blotting
Cell lysates and concentrated supernatants (10×) from both human and rabbit NFM and FCM were subjected to Western blotting and TIMP-3 or MT1-MMP levels determined.

In Vitro Studies on Macrophages and Macrophage-Derived Foam Cells
The effect of recombinant TIMP-3 (10 nmol/L) on migration, proliferation, and apoptosis were determined in rabbit NFM and FCM as previously described. Additionally parallel studies using a MT1-MMP neutralizing antibody (10 nmol/L) were performed. To induce apoptosis, rabbit NFM and FCM were either cultured in the presence of lipopolysaccharide (LPS) or subjected to serum withdrawal for 72 hours, with or without the addition of either recombinant TIMP-3 (10 nmol/L) or a MT1-MMP neutralizing antibody (10 nmol/L).

Immunocytochemistry
Immunocytochemistry was conducted on rabbit NFM and FCM using goat polyclonal cleaved caspase-3, monoclonal antirabbit RAM-11, monoclonal antihuman/rabbit TIMP-3, monoclonal antihuman MT1-MMP, or Alexa Fluor 488-conjugated phalloidin to detect filamentous actin.

In Situ Zymography
Rabbit NFM and FCM were cultured on glass coverslips and incubated in the presence of fluorescein quenched DQ gelatin substrate (40 μg/mL). Parallel experiments with either recombinant TIMP-3 (10 nmol/L) or a MT1-MMP neutralizing antibody (10 nmol/L) were performed. Gelatinolytic activity was visualized under fluorescence microscopy.

Immunohistochemistry
Immunohistochemistry was conducted for macrophages, TIMP-3, MT1-MMP, and cleaved caspase-3 (apoptosis). Dual immunostaining was performed using the same antibodies and visualized by confocal microscopy.

Statistical Analysis
Data were analyzed for normality and compared using the unpaired Student t test or the Mann-Whitney test, as appropriate. Statistical differences between apoptotic foam-cell macrophages were analyzed by Student paired t test. Normally distributed data are presented as mean±SEM.

Results
TIMP-3 mRNA and Protein Expression of Nonfoamy and Foam-Cell Macrophages
Foam-cell macrophages (FCM) were isolated from subcutaneous sponges placed in vivo in cholesterol-fed rabbits for 4 to 6 weeks and compared to nonfoamy macrophages (NFM) isolated from sponges in chow-fed rabbits. Western blotting revealed similar TIMP-1 and TIMP-2 protein levels in rabbit FCM and NFM (supplemental Figure IA and IB). By contrast, TIMP-3 protein levels were significantly reduced (84%; P<0.0001; supplemental Figure IA and IB) in FCM compared to NFM. Real-time PCR demonstrated no significant difference in TIMP-3 mRNA levels between FCM and NFM (supplemental Figure IC), which suggests a posttranscriptional mechanism.

TIMP-3 Downregulation in Rabbit Foam-Cell Macrophages Promotes Their Invasion Through Basement Membranes and Increases Their Gelatinolytic Activity
RAM-11 immunocytochemistry stained 98% of all rabbit FCM and NFM, as previously demonstrated. TIMP-3 immunocytochemistry revealed that all rabbit NFM expressed TIMP-3 protein at similar levels (Figure 1A). By contrast FCM showed a wide spectrum of TIMP-3 protein; indeed, a subset (28±5%) was TIMP-3 negative (arrows in Figure 1B). When the invasion of rabbit FCM through a synthetic basement membrane was tested in vitro, remarkably 100% of the macrophages that penetrated were TIMP-3 negative (Figure 1D and 1E) and these cells spread out to adopt an elongated morphology. By contrast, 100% of the cells that did not penetrate after 48 hours were TIMP-3 positive, failed to spread, and kept a rounded morphology (Figure 1D and 1E). These results were reproduced in 5 separate experiments. Immunocytochemistry for RAM11 confirmed that all the cells that had invaded were macrophages (Figure 1F). Immunocytochemistry for TIMP-1 and TIMP-2 showed no difference between migrated and nonmigrated FCM (results not shown). Addition of recombinant TIMP-3 to the Matrigel significantly reduced the invasion of FCM (65%, P<0.05; supplemental Table I) and the nonmigrated cells now all failed to spread and were a mixture of TIMP-3 positive and negative cells (Figure 1C). TIMP-3 addition had no effect on the invasion of NFM (supplemental Table I).

Using in situ zymography, proteolytic activity around FCM was reduced by adding exogenous TIMP-3 (75%; P<0.01; Figure 3A). Adding the MMP inhibitor BB94 abolished the proteolytic activity (results not shown).

Effect of TIMP-3 on Nonfoamy and Foam-Cell Macrophage Proliferation and Apoptosis
Recombinant TIMP-3 did not affect NFM proliferation but significantly reduced FCM proliferation by 73% (P<0.05; supplemental Table I). The rate of apoptosis induced by either LPS or serum deprivation in NFM was unchanged by treatment with recombinant human TIMP-3 (supplemental...
However, the rate of apoptosis in FCM (20% of cells) was significantly inhibited by exogenous TIMP-3 (50%, P < 0.05; supplemental Table I and Figure 2A). Subsequently, we used dual immunocytochemistry for cleaved caspase-3 to colocalize apoptosis with TIMP-3 protein in rabbit FCM. A significantly higher proportion of TIMP-3 negative FCM exhibited signs of apoptosis (76 ± 7%; P < 0.05; Figure 2B) compared to TIMP-3 positive cells (25 ± 7%; Figure 2B).

Localization of TIMP-3 Protein in Rabbit and Human Atherosclerotic Plaques

We sought histological evidence in plaques to corroborate our in vitro findings of variable TIMP-3 expression in FCM and its relation with invasion. The early fatty streak lesions formed after feeding rabbits cholesterol for 4 weeks demonstrated areas rich in macrophages and FCM (Figure 4A), which colocalized with cell-specific TIMP-3 protein expression (Figure 4B). Whereas most NFM were strongly TIMP-3 positive (arrows, Figure 4C), a more divergent expression pattern was observed within FCM, throughout the lesion (Figure 4C). Additionally, extracellular TIMP-3 protein was detected at the lesion/media interface (Figure 4C). Based on the use of nonimmune mouse IgG as a primary antibody, TIMP-3 staining was highly specific (Figure 4D). In advanced plaques formed after 8 weeks of cholesterol feeding, FCM within the upper aspect of the lesions (Figure 4E) demonstrated TIMP-3 immunoreactivity (arrows, Figure 4F). However, the FCM found in the deeper layers of the plaque (Figure 4E) showed little or no TIMP-3 protein expression (Figure 4G). Interestingly, dual immunohistochemistry revealed that the NFM in the media below the lesion (presumably resident macrophages) are TIMP-3 positive (arrowheads; Figure 4H). Also, adventitial macrophages underlying the lesion are clearly nonfoamy and express abundant TIMP-3 (arrows; Figure 4H). TIMP-1 and TIMP-2 expression was observed in NFM and FCM throughout the lesion (data not shown).

A mixture of FCM with intense TIMP-3 protein expression (Figure 5C and 5D, black arrows) and with little or no TIMP-3 expression (white arrows) were also observed intermingled at the shoulder regions toward the lipid core of these advanced human atherosclerotic plaques. From dual stained images (Figure 5E through 5H), the TIMP-3 negative FCM appeared to form discrete islands or nodules within surrounding TIMP-3 positive cells. Additionally, the frequency of TIMP-3 positive FCM undergoing apoptosis was extremely low (4 ± 1%, Figure 5I), mirroring the in vitro findings (Figure 2B).

MT1-MMP mRNA and Protein Expression of Nonfoamy and Foam-Cell Macrophages

Considering the effects observed with TIMP-3 and that we have previously observed similar in vitro effects on migration, proliferation, and apoptosis with TIMP-2 but not TIMP-1, we investigated the expression patterns of MT1-MMP, which is inhibited by TIMP-2 and TIMP-3 but not TIMP-1.17 Both MT1-MMP mRNA (2.6-fold; P = 0.0138; supplemental Figure IC) and protein (26-fold; P < 0.0001; supplemental Figure IA and IB) were significantly elevated in FCM compared to NFM control macrophages.
MT1-MMP Upregulation in Rabbit Foam-Cell Macrophages Promotes Their Invasion, Gelatinolytic Activity, and Susceptibility to Apoptosis

Only 23% of the rabbit FCM that failed to penetrate the synthetic basement membrane were MT1-MMP positive (Figure 3B), whereas 100% of the cells were TIMP-3 positive (Figure 1C). Conversely 100% of the FCM that invaded the membrane were MT1-MMP positive (Figure 3C), whereas none were TIMP-3 positive (Figure 1E). This implies that lack of TIMP-3 combined with upregulated MT1-MMP (and possibly other MT-MMPs) in these FCM permits them to invade. Consistent with this, proteolytic activity was reduced in FCM by adding an MT1-MMP neutralizing antibody (60%; P<0.01; Figure 3A). Addition of MT1-MMP neutralizing antibody to the Matrigel also tended to reduce the invasion of FCM (71%, P=0.09). Furthermore, the rate of apoptosis in foam-cell macrophages was significantly inhibited by a MT1-MMP neutralizing antibody (6±2 versus 19±9%, P<0.05; Figure 2A).

We went on to use immunocytochemistry to relate our findings with isolated cells to human and rabbit plaques. MT1-MMP positive FCM were found at the shoulder regions of human atherosclerotic plaques. Moreover, the frequency of such cells undergoing apoptosis was significantly higher than observed for TIMP-3 positive FCM (27±9% v 4±1%, P<0.05, Figure 5J). MT1-MMP demonstrated a converse pattern of localization in advanced rabbit atherosclerotic plaques compared to TIMP-3 (Figure 6). The majority of macrophages (Figure 6A and 6D) within the upper aspect of the lesion were MT1-MMP negative, whereas FCM within the deepest region of the plaque were MT1-MMP positive (Figure 6B and 6E). MT1-MMP positive foam-cells were TIMP-3 negative (Figure 6C and 6F).

Discussion

It was previously shown that the phenotypic transformation of monocytes to macrophages in culture coincides with upregulation of TIMP-3 mRNA and protein.13 Consistent with this, TIMP-3 protein expression was demonstrated in macrophages in human atherosclerotic plaques and was suggested to act as a protective factor against plaque rupture.13 Our studies illustrate, for the first time, that transformation of differentiated macrophages to FCM causes a subsequent decrease in TIMP-3 protein expression, and that the protective effect of TIMP-3 is completely lost in a subpopulation of FCM that also overexpresses MMP-14. We observed no difference in TIMP-3 mRNA expression between NFM and FCM, consistent with previous observations that posttranscriptional mechanisms can regulate TIMP expression during macrophage development.18

Our most interesting and unexpected finding was that only the subset of FCM that became TIMP-3 negative was capable of invading through a synthetic extracellular matrix. Furthermore, TIMP-3 negative FCM had an increased propensity to undergo proliferation and apoptosis. It was important to determine whether the results obtained in FCM and NFM derived from granulomas are relevant to plaque macrophages. To do this we showed that FCM with high and low TIMP-3 expression are found in early fatty streak lesions in rabbit aortas, whereas in the deep layers of advanced atherosclerotic plaques, most FCM had little or no TIMP-3 protein expression. The fact that TIMP-3 negative macrophages occurred in the deeper layers of the plaque is consistent with their greater invasive ability. Interestingly, we recently demonstrated up-regulation of MMP-12 and downregulation of arginase-1 occurs in the same location as TIMP-3 down regulation in advanced rabbit plaques.10

It is possible that TIMP-3 downregulation marks a distinct phenotype of macrophages. Functionally divergent macrophage subtypes have been ascribed to other inflammation-related pathologies19,20 and recently demonstrated to populate murine21,22 and human atherosclerotic lesions.23,24 Further studies beyond the scope of the present experiments would be needed to establish the relationship between the TIMP-3 negative subpopulation described here and these previously described phenotypes.

We also identified wide variation in TIMP-3 levels in FCM in human plaques. Looking specifically at the shoulder-regions of advanced plaques, islands/nodules of FCM with either low or no TIMP-3 expression were apparent, surrounded by TIMP-3 positive FCM. These areas were predom-
inantly around the periphery of the lipid/necrotic core, below the thinnest regions of the fibrous cap. Although it was not commented on, the immunocytochemical data previously reported by Fabunmi and colleagues also shows a subpopulation of macrophages that appears TIMP-3 negative at similar regions.13

The most obvious explanation for the effect of TIMP-3 deficiency in FCM is that it reveals the actions of metalloproteinases that facilitate invasion and proliferation and induce susceptibility to apoptosis. Consistent with this explanation, exogenous TIMP-3 blocked reduced invasion, proliferation, and apoptosis of FCM. Despite reported findings that TIMP-3 is proapoptotic in some cells,25,26 prosurvival effects have also been described in other cell types.27–29 We recently demonstrated that TIMP-2 similarly reduces FCM migration, proliferation, and apoptosis, although exogenous TIMP-1 has no effect.15 These findings suggest the involvement of a membrane type MMP, MT1-MMP (MMP-14), for example, is inhibited by TIMP-2 and -3 but very poorly by TIMP-1.17 Furthermore, MMP-14 is upregulated during foam cell formation in vitro.30 We demonstrated here that mRNA and protein are elevated in in vivo generated FCM compared to NFM. Additionally, it has recently been observed that MMP-14 regulates intercellular adhesion molecule-1 (ICAM-1) mediated monocyte/macrophage migration through an endothelial monolayer in vitro; TIMPs-2 and -3 blocked migration, whereas TIMP-1 was ineffective.31 We now illustrate that blocking MMP-14 activity retards FCM invasion, diminishes their proteolytic potential, and reduces their susceptibility to undergo apoptosis, similar to that observed with exogenous TIMP-3. Supportive findings have shown that MMP-14 can induce apoptosis in several cell types.32,33 Thus we propose that transformation of macrophages to FCM causes a significant decrease in TIMP-3 protein while increasing MMP-14 levels. This inverse correlation implies that the protective effect of TIMP-3 is reduced.
and the disruptive nature of MMP-14 is heightened. Our studies cannot rule out that other MT-MMPs also contribute to these effects, because several are also inhibited by TIMP-2 and -3 but not TIMP-1.

Consistent with our data, a previous study also found prominent MMP-14 expression in FCM at the base of advanced rabbit lesions. It is interesting that the subpopulation of FCM that have diminished TIMP-3 expression reside in a similar area to where increased MMP-14 (Figure 6) is detected in both the rabbit and human advanced plaques. MMP-14 expression has been postulated to accelerate the progression of atherosclerotic plaques and promote plaque instability. Moreover, MMP-14 has been shown to be fundamental for macrophage-mediated proteolysis and invasion. Thus the reduction in TIMP-3 and increased MMP-14 expression suggests that a more invasive and destructive subpopulation of FCM resides at the base of rabbit and the shoulder region of human plaques.

In summary our results show that TIMP-3 is downregulated in a subset of FCM, which results in increased invasion through ECM, proliferation, and apoptosis. Additionally, the loss of TIMP-3 appears associated with an increase in MMP-14 expression and activity. The ability of FCM to degrade ECM has been previously associated with morphological features of plaque instability. Increased foam-cell proliferation and apoptosis, resulting in increased extracellular lipid content, are also associated with plaque instability. Hence all these properties promoted by reducing the TIMP-3/MMP-14 balance would be expected to increase the potential of FCM to destabilise atherosclerotic plaques. The identification of FCM with differing proteolytic potential may aid future development of therapies to promote atherosclerotic plaque stability.

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**Disclosures**

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*Generation and isolation of macrophages and foam-cell macrophages*

Foam-cell macrophages were isolated from subcutaneous granulomas in New Zealand White rabbits fed an enriched cholesterol diet as previously described \(^1,^2\). Briefly 2 weeks after starting cholesterol feeding 2-6 polyurethane sponges (Baxter Scientific) were implanted under the dorsal skin for 6-8 weeks. The recovered sponges were gently squeezed over sterile test tubes and the exudates were layered onto a discontinuous metrizamide gradient (bottom cushion 10 mL of 10 % metrizamide w/v, top 3 ml cell suspension) and centrifuged 1200 x g for 15 minutes at 10°C. Foam cells were recovered from the floating layer, washed 3 times and aliquots taken for oil red O staining to confirm lipid content and immunocytochemistry using the rabbit macrophage specific marker RAM 11. One rabbit yielded approximately 2 x 10^7 foam-cells. The similar procedure performed in rabbits fed a chow-diet yielded non-foamy macrophages, as previously described \(^1\).

*Quantitative analysis of TIMP-3 and MT1-MMP mRNA expression by RT-PCR*

Total RNA was extracted using RNaseasy Mini kit (Qiagen, UK) according to the manufacturer’s instructions. Quantity and purity were assessed by absorbance at 260 and 280 nm. cDNA was generated using 40U of RT enzyme (Promega, UK) per 1 µg of RNA. The cDNA was amplified by Q-PCR utilising a Roche Light Cycler using 55 cycles for TIMP-3, MT1-MMP and 18S, of 94°C for 15sec, 58°C for 20sec and 72°C for 20sec. The forward and reverse primers (Sigma, UK) were 5’-TCTGCAACTCCGACATCGTG-3’ and 5’-CGGATGCAGGCGTAGTGTT-3’ for TIMP-3, 5’-TCTGGAGGGTGAGGAACAAC-3’ and 5’-GCTCCTCGTTGAATCGGTAG-3’ for MT1-MMP, and 5’-
CTTCAACCTCGACTTTCG-3’ and 5’- CTCGATGCTTTAGCTGAGT-3’ for 18S. Data was analysed using LightCycler Data Analysis software.

**Western Blotting**

Total cell lysates and concentrated supernatants (10x) from rabbit macrophages and foam-cell macrophages were prepared in SDS-lysis buffer (50 mmol/L Tris-HCl, pH 6.8, 10% glycerol, 1% SDS) and analysed for protein content (Micro BCA assay kit, Pierce, UK). Samples were run on Bis-Tris gels and transferred to nitrocellulose. After blocking in 5% skimmed milk powder, blots were incubated overnight at 4°C with either, 0.75 µg/mL anti-TIMP-3 antibody (TCS CellWorks, UK) or 5 µg/mL anti-MT1-MMP (Calbiochem, UK), in Starting Block™ blocking buffer (Pierce, UK). Bound antibodies were detected with goat anti-mouse IgG horseradish peroxidase-conjugated antibody (Dako, UK) and enhanced chemiluminescence (Amersham International, UK). Detected bands were quantified using a Bio-Rad GS-690 scanning densitometer (Bio-Rad, UK) and expressed as a percentage of the control value.

**In vitro studies on rabbit macrophages and foam-cell macrophages**

Migration of macrophages and foam-cell macrophages isolated from rabbits was measured by the invasion of cells through Matrigel-coated Corning™ transwell inserts (Appleton Woods, Birmingham, UK). Transwell inserts containing membranes with 5 µm pores were coated with Matrigel (40 µL/well; BD Biosciences, UK). Either purified human recombinant TIMP-3 (10 nmol/L; Chemicon International, Harrow, UK) or a MT1-MMP neutralising antibody (10 nmol/L; Chemicon International, Harrow, UK) was added to the Matrigel of some inserts. Cell suspension (100 µL; 1 x 10^5 cells) in RPMI 1640 medium (supplemented with 100 IU/mL penicillin, 100
µg/mL streptomycin and 2 mmol/L L-glutamine) was added to the upper chambers. The same medium (600 µL) supplemented with 8.5 ng/mL of human recombinant monocyte chemoattractant protein-1 (MCP-1) (R&D Systems, Abingdon, UK) was placed in the lower wells. Cultures were incubated for 48 hours and then cells on the under surface of the transwell membrane were fixed with cold methanol, stained with haematoxylin, counted, and expressed as a percentage of the control value.

Additionally, immunocytochemistry for TIMP-3 or MT1-MMP was performed on both the upper and under surface of transwell membranes, and freshly isolated cells subjected to cytopinning. Briefly, after fixing with 3% (w/v) paraformaldehyde and blocking with 20% (v/v) goat serum, cells were incubated overnight with either 0.75 µg/ml of anti-TIMP-3 antibody (TCS Cellworks, UK) or 5 µg/mL anti-MT1-MMP (Calbiochem, UK). Bound antibody was detected with anti-mouse biotinylated IgG (Dako, UK) followed by Extravidin-peroxidase conjugate (Sigma, UK) and developed with 0.06% diaminobenzidine in the presence of 0.03% H₂O₂ and counterstained with haemotoxylin. Controls with corresponding nonimmune IgG were conducted in parallel. The number of positive cells was counted and expressed as a percentage of the number per field (10 field per sample). For each treatment, data was expressed as a percentage of the control value.

Proliferation of macrophages and foam-cell macrophages was measured by BrdU incorporation as previously described.

Fluorescent immunocytochemistry for cleaved caspase-3

Cleaved caspase-3 was detected in rabbit macrophage and foam-cell macrophages cultured with serum-free RPMI 1640 media alone or with 10nmol/L recombinant human TIMP-3 and with 200 ng/ml lipopolysaccharide (LPS) to induce apoptosis.
Cells were trypsinised and combined with any floating cells and subjected to cytopinning. After fixing with 3% (w/v) paraformaldehyde and blocking with 20% (v/v) goat serum, cells were incubated overnight with 1µg/ml of anti-cleaved caspase-3 antibody (R&D Systems, Abingdon, UK) and 0.75 µg/ml of anti-TIMP-3 antibody (TCS Cellworks, UK). Bound antibody was detected with anti-goat IgG labelled with Alexa Fluor488 and anti-mouse IgG labelled with Alexa Fluor594 (Molecular Probes, Paisley, UK), respectively. After mounting with Vectashield containing DAPI, cells were visualized with a fluorescent microscope. The number of positive cells was counted and expressed as a percentage of the number per field (10 fields per sample). For each treatment, data was expressed as a percentage of the control value.

Immunohistochemistry

Female New Zealand White Rabbits (from 6 weeks of age) were fed a diet containing 1% cholesterol, for 4 weeks to produce fatty streaks that are rich in macrophages or 8 weeks plus a further 8 weeks on chow diet to yield advanced atherosclerotic plaques in the aortic arch that are rich in foam-cell macrophages. Age matched control rabbits were fed a standard laboratory chow. The aortic arch was removed and fixed in 4% phosphate buffered formaldehyde and then wax-embedded. Slides from human endarterectomy specimens were made available from the Athero-Express biobank. Briefly, serial 3 µm paraffin sections were dewaxed and rehydrated. Endogenous peroxidase activity was inhibited by incubation with 3% (v/v) hydrogen peroxide. After blocking sections with 20% (v/v) goat serum in PBS, sections were incubated overnight at 4°C with either purified mouse monoclonal antibody against human/rabbit TIMP-3 (TCS Cellworks, UK), or mouse monoclonal antibody against human/rabbit MT1-MMP (Calbiochem, UK), mouse monoclonal antibody against α-
smooth muscle actin (Sigma, UK), mouse monoclonal antibody against rabbit macrophage (RAM11) (Dako, UK), and mouse monoclonal antibody against human macrophage (CD68) (Dako, UK) in 1% (w/v) bovine serum albumin (BSA) in PBS. After mounting with Vectashield containing DAPI, cells were visualized with a fluorescent microscope. A negative control, where the primary antibody was replaced with mouse IgG at the same dilution, was always included.

**Dual immunohistochemistry**

For dual immunostaining, slides were prepared and incubated with purified mouse monoclonal antibody against human/rabbit TIMP-3 (TCS Cellworks, UK). Bound antibody was detected with anti-mouse IgG labelled with Alexa Fluor488 (Molecular Probes, Paisley, UK). They were then incubated with Image-iT FX™ signal enhancer (Molecular Probes, Paisley, UK) and subsequently incubated overnight with the second primary antibodies, RAM-11 for rabbit sections or CD68 for human sections. Sections were then incubated with the appropriate fluorescently-conjugated secondary antibodies (Molecular Probes, UK). After mounting with Vectashield containing DAPI, cells were visualized using a Leica confocal imaging spectrophotometer system (TCS-SP2). TIMP-3 immunoreactivity was observed as green, macrophages as red and nuclei as blue. Consequently, macrophages with co-localised TIMP-3 expression appear yellow whilst macrophages with low or no TIMP-3 expression appear red.

For dual immunostaining of apoptosis, slides were incubated with mouse monoclonal antibody against either human/rabbit TIMP-3 (TCS Cellworks, UK) or human MT1-MMP (Calbiochem, UK). Bound antibody was detected with anti-mouse IgG labelled with Alexa Fluor594 (Molecular Probes, Paisley, UK). They were then
incubated with Image-iT FX™ signal enhancer (Molecular Probes, Paisley, UK) and subsequently incubated overnight with goat polyclonal antibody against cleaved caspase-3 (R&D Systems, UK). Bound antibody was detected with anti-goat IgG labelled with Alexa Fluor488 (Molecular Probes, Paisley, UK). After mounting with Vectashield containing DAPI, cells were visualized using a Leica confocal imaging spectrophotometer system (TCS-SP2). TIMP-3 and MT1-MMP immunoreactivity was observed as red, apoptotic cells as green and nuclei as blue. Consequently, apoptotic cells with co-localised TIMP-3 or MT1-MMP expression appear yellow.
References


**Expanded Figure Legends**

**Figure 1: Effect of foam-cell macrophage formation and their subsequent migration on TIMP-3 protein expression**

(A and B) Cytospin preparations of in vivo generated macrophage and foam-cell macrophages were subjected to immunocytochemistry for TIMP-3, (A) macrophages and (B) foam-cell macrophages, white arrowheads indicate cells with low TIMP-3 expression. (C – E) TIMP-3 expression of non-and migrated foam-cell macrophages from Matrigel-coated transwell inserts; (C) foam cells on top of insert after TIMP-3 addition to the Matrigel, arrows indicate TIMP-3 negative cells. (D) Non-migrated foam-cells on top of insert, and (E) migrated foam-cells on bottom of the same insert. Black arrows in panels D and E indicate TIMP-3 positive non-migrated cells and white arrowheads highlight TIMP-3 negative migrated cells. Brown colour represents TIMP-3 immunopositivity. (F) RAM11 expression for macrophages on migrated foam-cell macrophages. Black arrows indicate RAM11 cells (brown) that have migrated to the underside of the invasion chamber. (G) Non-immune IgG on migrated foam-cell macrophages.

**Figure 2: Effects of TIMP-3 expression and addition on foam-cell macrophage proliferation, migration, and apoptosis**

(A) Effect of exogenous TIMP-3 or a MT1-MMP neutralising antibody on serum-starvation-induced foam cell macrophage apoptosis, as assessed by cleaved caspase-3 immuno-positivity (* denotes p<0.05, n=3).

(B) Dual immunocytochemical labelling of rabbit in vivo generated foam-cell macrophages for TIMP-3 and a marker of apoptosis (cleaved caspase-3) visualised by confocal microscopy. Nuclei were stained with DAPI (blue). White arrows indicate...
TIMP-3 positive foam cell macrophages (red) and white arrowheads highlight TIMP-3 negative foam-cell macrophages undergoing apoptosis (green). Quantification of the proportion of apoptotic cells in TIMP-3 positive and negative cells is summarised in the adjoining graph (* denotes p<0.05, n=4).

**Figure 3: Effect of exogenous TIMP-3 and MT1-MMP inhibition on foam-cell macrophage gelatinolytic activity**

(A) Rabbit in vivo generated foam-cell macrophages were cultured in the presence of a fluorescently-quenched gelatin substrate with and without the addition of exogenous TIMP-3 (10nmol/L) or a MT1-MMP neutralising antibody (10nmol/L) for 24 hours. Green fluorescence represents gelatinolytic activity, as indicated by white arrows. Nuclei were stained with DAPI (blue). Quantification of the proportion of foam-cell macrophages exhibiting gelatinolytic activity is summarised in the adjoining graph (* denotes p<0.05, n=3).

(B and C) MT1-MMP expression of non-and migrated foam-cell macrophages from Matrigel-coated transwell inserts; (B) foam-cells on top of insert after MT1-MMP addition to the Matrigel, and. (C) migrated foam-cells on bottom of the same insert. Black arrows in pane B highlight MT1-MMP negative non-migrated cells and arrowheads in panels B and C indicate MT1-MMP positive non-migrated cells and white. Brown colour represents MT1-MMP immunopositivity.

**Figure 4: TIMP-3 localisation in rabbit atherosclerotic plaques**

Immunohistochemical labelling of early (A - C) and advanced (D - H) rabbit atherosclerotic plaques, for macrophages with RAM11 (A and E), for TIMP-3 (B – D, F and G) or both (H). Box in panel (B) represents higher magnification in panel (C).
Large box in panel (E) represents higher magnification in panel (F). Box in panel (F) represents higher magnification in panel (G). (D) Advanced rabbit atherosclerotic plaque incubated with mouse IgG as a negative control. (H) Dual immunohistochemistry for macrophages (blue/green) and TIMP-3 (brown) in an advanced rabbit atherosclerotic plaque, demonstrating TIMP-3 positive macrophages (arrows) within the adventitia. Scale bar in panel (A) represents 200µm in panels (A, B, D and F), 500µm in panel (E) and 100µm in panels (C, G, and H).

**Figure 5: TIMP-3 localisation in human advanced atherosclerotic plaques**

(A and B) Immunohistochemical labelling for macrophages with CD68 in advanced human carotid atherosclerotic plaques. (C and D) Immunohistochemical labelling for TIMP-3 in serial sections from (A) and (B) respectively, at higher magnification of boxed areas in panels (A and B). Black arrows indicate TIMP-3 positive foam-cell macrophages and white arrows indicate TIMP-3 negative foam-cell macrophages.

(E and F) Immunohistochemical labelling for macrophages (red, F) and TIMP-3 (green, G) in an advanced human carotid atherosclerotic plaque, visualised by confocal microscopy. Nuclei were stained with DAPI (E). In the merged image (H), yellow arrowheads indicate TIMP-3 positive foam-cell macrophages (yellow) encircling invading TIMP-3 negative foam-cell macrophages (red) indicated by white arrows.

(I and J) Dual immunohistochemical labelling for cells undergoing apoptosis (green) and either TIMP-3 (red, I) or MT1-MMP (red, J) in advanced human carotid atherosclerotic plaques, visualised by confocal microscopy. Nuclei were stained with DAPI. White arrows in panel (I) indicate TIMP-3 negative cells undergoing apoptosis (green). White arrows in panel (J) indicate MT1-MMP positive cells undergoing
apoptosis (yellow). Scale bar in panel A represents 500µm in panel (A and B) and 100µm in panels (C) through to (J).

**Figure 6: MT1-MMP localisation in rabbit atherosclerotic plaques**

Immunohistochemical labelling of advanced rabbit atherosclerotic plaques, for macrophages with RAM11 (A and D), for MT1-MMP (B and E), or TIMP-3 (C and F). Box in panels (A, B and C) represents respective higher magnification in panels (D, E and F). Arrows indicate immunopositive cells (brown). Dotted lines represent the atherosclerotic plaque medial boundary. Scale bar in panel A represents 100µm.
Online Table I: Effect of exogenous TIMP-3 on non-foamy macrophage (NFM) and foam-cell macrophage (FCM) behaviour

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>TIMP-3</th>
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<tbody>
<tr>
<td><strong>Migration</strong></td>
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</tr>
<tr>
<td>NFM (n=6)</td>
<td>69±19</td>
<td>57±14</td>
</tr>
<tr>
<td>FCM (n=6)</td>
<td>45±17</td>
<td>16±2*</td>
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<tr>
<td><strong>Proliferation</strong></td>
<td></td>
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</tr>
<tr>
<td>NFM (n=4)</td>
<td>22±4</td>
<td>18±4</td>
</tr>
<tr>
<td>FCM (n=4)</td>
<td>15±5</td>
<td>4±1*</td>
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<tr>
<td><strong>Apoptosis</strong></td>
<td></td>
<td></td>
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<tr>
<td>(LPS-induced)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NFM (n=6)</td>
<td>19±4</td>
<td>18±4</td>
</tr>
<tr>
<td>FCM (n=6)</td>
<td>20±3</td>
<td>10±3*</td>
</tr>
<tr>
<td><strong>Apoptosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(serum withdrawal)</td>
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</tr>
<tr>
<td>FCM (n=3)</td>
<td>19±9</td>
<td>6±1*</td>
</tr>
</tbody>
</table>

Values are mean±SEM

*p<0.05, TIMP-3 versus control
Online Figure I: TIMP-1, -2, -3 and MT1-MMP mRNA and protein expression in foam-cell macrophages

(A) Cell lysates were generated from \textit{in vivo} generated rabbit non-foamy macrophages (black bars, n=6) and foam-cell macrophages (white bars, n=6) and subjected to Western blot analysis for TIMP-1, -2, -3 and MT1-MMP.

(B) Representative Western blots for TIMP-1, -2, -3 and MT1-MMP. NFM represent non-foamy macrophages and FCM represents foam-cell macrophage.

Data are representative of 6 independent experiments. * denotes p<0.05 versus non-foamy macrophages.

(C) Total RNA was extracted from \textit{in vivo} generated rabbit non-foamy macrophages (black bars, n=6) and foam-cell macrophages (white bars, n=6) and subjected to Q-PCR for TIMP-3 and MT1-MMP.