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ORIGINAL PAPER

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Tissue inhibitor of metalloproteinases 4 (TIMP4) is involved in inflammatory processes of human cardiovascular pathology

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Abstract Tissue inhibitors of matrix metalloproteinases (TIMPs) comprise a family of four members, of which TIMP4 is characterized by being primarily restricted to cardiovascular structures. We demonstrate with immunohistochemical analysis of healthy human tissue that TIMP4 is present in medial smooth muscle cells and adventitial capillaries of arteries as well as in cardiomyocytes. Animal studies have suggested a role for TIMP4 in several inflammatory diseases and cardiovascular pathologies. We therefore examined whether TIMP4 is involved in human inflammatory cardiovascular disorders, specifically atherosclerosis, giant cell arteritis and chronic rejection of heart allografts. TIMP4 was most clearly visible in cardiovascular tissue areas populated by abundant inflammatory cells, mainly macrophages and CD3+ T cells. Using western blotting and immunocytochemistry, human blood derived lymphocytes, monocytes/macrophages and mast cells were shown to produce TIMP4. In advanced atherosclerotic lesions, TIMP4 was detected around necrotic lipid cores, whereas TIMP3 and caspase 3 resided within and around the core regions, indicating different roles for TIMP3 and TIMP4 in inflammation-induced apoptosis and in matrix turnover. In conclusion, the data demonstrate upregulation of TIMP4 in human cardiovascular disorders exhibiting inflammation, suggesting its future use as a novel systemic marker for vascular inflammation.

Keywords TIMP4 · Inflammation · Atherosclerosis · Giant cell arteritis · Heart allograft rejection

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Introduction

Endogenous inhibitors of matrix metalloproteinases (MMPs) called tissue inhibitors of matrix metalloproteinases (TIMPs) form a family of four members, TIMP1–4. Although possessing many similarities, TIMPs show several structural and biochemical differences (Baker et al. 2002). Furthermore, their expression patterns differ. Unlike the other three TIMPs, TIMP4 appears to have a highly restricted tissue expression (Baker et al. 2002; Rahkonen et al. 2002). TIMP4 is considered to be the most characteristic of cardiovascular structures (Spinale 2002), but its function in cardiovascular biology and pathology is still poorly understood. Based on animal models, several lines of evidence suggest that TIMP4 has a role in cardiovascular disorders exhibiting an inflammatory component. First, overexpression of TIMP4 has been observed in the neointima, media and adventitia of large vessels after balloon injury in rats (Dollery et al. 1999). Second, an imbalance between TIMP4 and MMP2 has been detected after myocardial reperfusion injury in rats (Schulze et al. 2003). Third, TIMP4 expression is upregulated in rat cardiac myocytes in response to proinflammatory cytokines, such as tumour necrosis factor (TNF) and interleukin 1, beta (IL1B) (Li et al.

1999). TIMP4 expression is restricted not only to inflammatory processes in cardiovascular structures, but also a link between TIMP4 and inflammation has been seen, e.g. in adjuvant-induced arthritis in a rat model (Celiker et al. 2002). Furthermore, inflammatory cells, especially peripheral blood mononuclear cells (PBMC) from cattle have been shown to upregulate their TIMP4 expression when infected by *Mycobacterium paratuberculosis* (Coussens et al. 2002).

It is now widely accepted that atherosclerosis is an inflammatory disease and that inflammatory cells, i.e. macrophages, T lymphocytes and mast cells are all involved in the pathogenesis and progression of atherosclerosis (Libby 2002; Ross 1999). In the present study, we examined whether TIMP4 is involved in inflammatory human cardiovascular diseases, namely atherosclerosis of coronary arteries, giant cell arteritis (GCA) of temporal arteries and rejection of heart allografts. We also examined whether human blood derived inflammatory cells, specifically monocytes/macropages, T lymphocytes and mast cells, are capable of producing TIMP4. Furthermore, because TIMPs, including TIMP4, have been shown to contribute not only to MMP inhibition, but also to the regulation of apoptosis (Guo et al. 2004; Tummalapalli et al. 2001), we examined the spatial locations of TIMP4, TIMP3 and caspase 3 in human atherosclerotic coronary arteries using immunohistochemistry. This is the first published data on TIMP4 expression in either normal human coronary vessels or in various human inflammatory cardiovascular diseases, including atherosclerosis.

Materials and methods

Human tissue samples

Fresh human atherosclerotic coronary arteries were collected from recipient hearts ($n = 10$) at the time of cardiac transplantation at Helsinki University Central Hospital (HUCH), Helsinki, Finland. Non-atherosclerotic coronary arteries were collected from healthy hearts ($n = 6$) of organ donors who had no history of cardiovascular disease but who had been excluded from transplantation due to size or tissue type mismatch with potential recipients. Human heart endomyocardial biopsy (EMB) samples were taken from right ventricles of cardiac allograft recipients ($n = 8$) at the time of routine rejection surveillance examinations. Institutional ethical review board of the HUCH approved the study protocol, and informed consent was obtained from all patients.

Temporal artery biopsies were derived from patients under clinical examination due to high blood sedimentation rate. Samples ($n = 12$) fulfilling the criteria of GCA (Hunder et al. 1990) served as arteritis specimens and samples ($n = 6$) showing no signs of inflammation were used as controls.

Human inflammatory cells

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from the blood of healthy human donors. The buffy coats were provided by the Finnish Red Cross Blood Bank (Turku/Helsinki, Finland). Immunomagnetic cell separation (MACS, Miltenyi Biotech, Bergisch Gladbach, Germany) of PBMC was performed to obtain purified monocytes and lymphocytes.

Macrophages were generated from isolated monocytes by incubating the monocytes on culture plates for 7–10 days in a medium containing 10 ng/ml colony stimulating factor 1 (CSF1, R&D Systems, Abingdon, UK) or 100 μg/ml oxidized low-density lipoprotein (oxLDL, a gift from Dr Markku Ahotupa, University of Turku, Turku, Finland) (Nagy et al. 1998).

Human mast cells (HuMC) were derived from cord blood as described previously in detail (Saito et al. 1995).

Immunohistochemistry

Tissue samples used for immunohistochemical stainings were fixed in 10% neutral-buffered formalin or methyl Carnoy's fixative, embedded in paraffin and cut into 5 μm thick serial sections. For the detection of TIMP4 and TIMP3 immunoreactivity, rabbit polyclonal antibodies (NeoMarkers, Fremont, CA, USA) in dilutions 1:2,000 and 1:400, respectively, were used. Cleaved caspase 3 was detected using a rabbit polyclonal antibody (Cell Signaling Technology, Inc., Beverly, MA, USA) in dilution 1:2,000. For the detection of macrophages, T lymphocytes and smooth muscle cells, antibodies against CD68 (DAKO, Glostrup, Denmark), CD3 (DAKO) and αSMA (alpha smooth muscle actin; Sigma, St. Louis, MO, USA) were used, respectively, as described earlier (Nelimarkka et al. 2001). For the detection of leucocyte infiltration in EMB samples, a mouse monoclonal antibody against leucocyte common antigen (CD45; DAKO) in dilution 1:10 was used. The reactivity of the antibodies was visualized using the avidin–biotin method as recommended by the supplier (Histostain-Plus Kit, Zymed, South San Francisco, CA, USA). Brown colour was developed with 3,3′-diamino-benzidine (DAB-Plus Kit, Zymed), and the sections were counterstained with haematoxylin and mounted (Aquamount improved Gurr, BDH Laboratory Supplies, Poole, UK). The specificity of the immunoreactions was controlled by omitting the primary antibody or for TIMP4 by incubating the antibody overnight at 4°C with an excess of specific blocking peptide (NeoMarkers). For the visualization of elastin, Verhoef-van Gieson staining was applied.

Immunodetection of TIMP4 in cultured cells

Cultured human macrophages and HuMC were fixed and stained with the above TIMP4 antibody (NeoMar-

kers; dilution 1:2,000) using the avidin–biotin method (Histostain-Plus Kit, Zymed).

Western blot analysis

Western blot analyses were performed as described earlier (Oksjoki et al. 2004). Briefly, the coronary artery and heart samples from organ transplantations were collected, snap-frozen in liquid nitrogen and stored at −80°C until used. Frozen specimens were homogenized and mixed with 300 µl of ice-cold extraction buffer (1:3 w/v) containing cacodylic acid (10 mmol/l), NaCl (0.15 mol/l), $ZnCl_2$ (1.0 mmol/l), $CaCl_2$ (20 mmol/l), NaN_3 (3.0 mmol/l), and 0.01% Triton X-100 (pH 5.0). The homogenate was centrifuged at 800×*g* for 10 min at 4°C, the supernatant decanted and saved on ice. Protein concentration of the myocardial extracts was determined with a standardized colorimetric assay (Bio-Rad DC Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA). Equal amount (20 µg) of protein extracts was resolved by SDS-PAGE on a 10% gel and transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membranes were blocked with SuperBlock blocking buffer (Pierce Biotechnology, Rockford, IL, USA) in Tris-buffered saline (TBS) at room temperature for 1 h and probed with a polyclonal antibody to human TIMP4 (NeoMarkers; dilution 1:2,000). Next, the membranes were subjected to three 5 min washes with Tween in TBS (T-TBS, 0.1% [v/v]) and probed with a secondary antibody [horseradish-peroxidase-conjugated anti-rabbit secondary antibody (Bio-Rad Laboratories; dilution 1:30,000)]. The bound antibodies were detected by an ECL kit as recommended by the supplier (SuperSignal West Pico Chemiluminescent Substrate, Pierce Biotechnology). Western blot analyses on protein extracts from cultured human monocytes/macrophages, lymphocytes and mast cells were performed similarly. Only the amounts of proteins loaded on SDS-PAGE gels were different.

Results

Localization of TIMP4 in human non-atherosclerotic and atherosclerotic coronary arteries

In human non-atherosclerotic coronary artery wall exhibiting no substantial inflammation as assessed by the lack of immunoreaction for macrophages (Fig. 1c) and CD3+ T cells (Fig. 1d), TIMP4 was detected in the medial smooth muscle cell layer and in capillaries in the outermost layer of the vascular wall, the adventitia (Fig. 1a, b). In intermediate (Fig. 1e–h) and advanced (Fig. 1i, j) types of atherosclerotic lesions of human coronary arteries (Stary et al. 1995), strong immunoreaction for TIMP4 was also detected in the intima (Fig. 1e, i), in the same areas as macrophages and CD3+ T cells (Fig. 1g, h, j).

TIMP4 is co-localized with macrophages in human inflamed temporal artery wall

The above results show that TIMP4 is involved in the inflammatory process within human coronary arterial wall. To confirm that this finding represents a broader phenomenon, temporal artery biopsy specimens obtained from patients with GCA and control patients were compared. Arterial wall tissue from control patients (Fig. 2a–c) showed no immunoreaction for TIMP4 (Fig. 2a), while temporal artery wall from GCA patients (Fig. 2d–f) stained positive for TIMP4, particularly in the intimal areas where macrophages were dominating (Fig. 2d, e). This result verified our notion that inflammatory process causes a local accumulation of TIMP4 within human arterial wall, and that this is a general phenomenon in human arteries.

TIMP4 in human heart allografts exhibiting rejection

Next, EMB specimens derived from human heart allografts exhibiting rejection were used to examine whether TIMP4 accumulation is coupled with inflammation not only in arterial wall, but also in other parts of the vascular system. Strong TIMP4 staining was seen in infiltrating leucocytes (Fig. 3a, c). Cardiomyocytes were also positive for TIMP4 in areas with and without leucocyte infiltration (Fig. 3a, c).

Confirmation of the specificity of the TIMP4 antibody

The specificity of TIMP4 immunostainings was confirmed by blocking the primary antibody with a specific peptide, by omitting the primary antibody, and with western blot analyses. Preincubation of the TIMP4 antibody with a blocking peptide completely abolished immunostaining (Fig. 4a, b). Western blot analyses of protein extracts derived from either human heart or from coronary arteries showed one intense band typical in size for the TIMP4 protein (Fig. 4c).

TIMP4 is synthesized by human inflammatory cells in vitro

Besides demonstrating that TIMP4 is abundant at sites of arterial wall inflammation, the above results also indicate that human inflammatory cells, especially macrophages and CD3+ T lymphocytes are capable of producing TIMP4. To examine TIMP4 protein production by inflammatory cells in more detail, mononuclear cells were isolated from human peripheral blood and cultured on plastic for various time periods with or

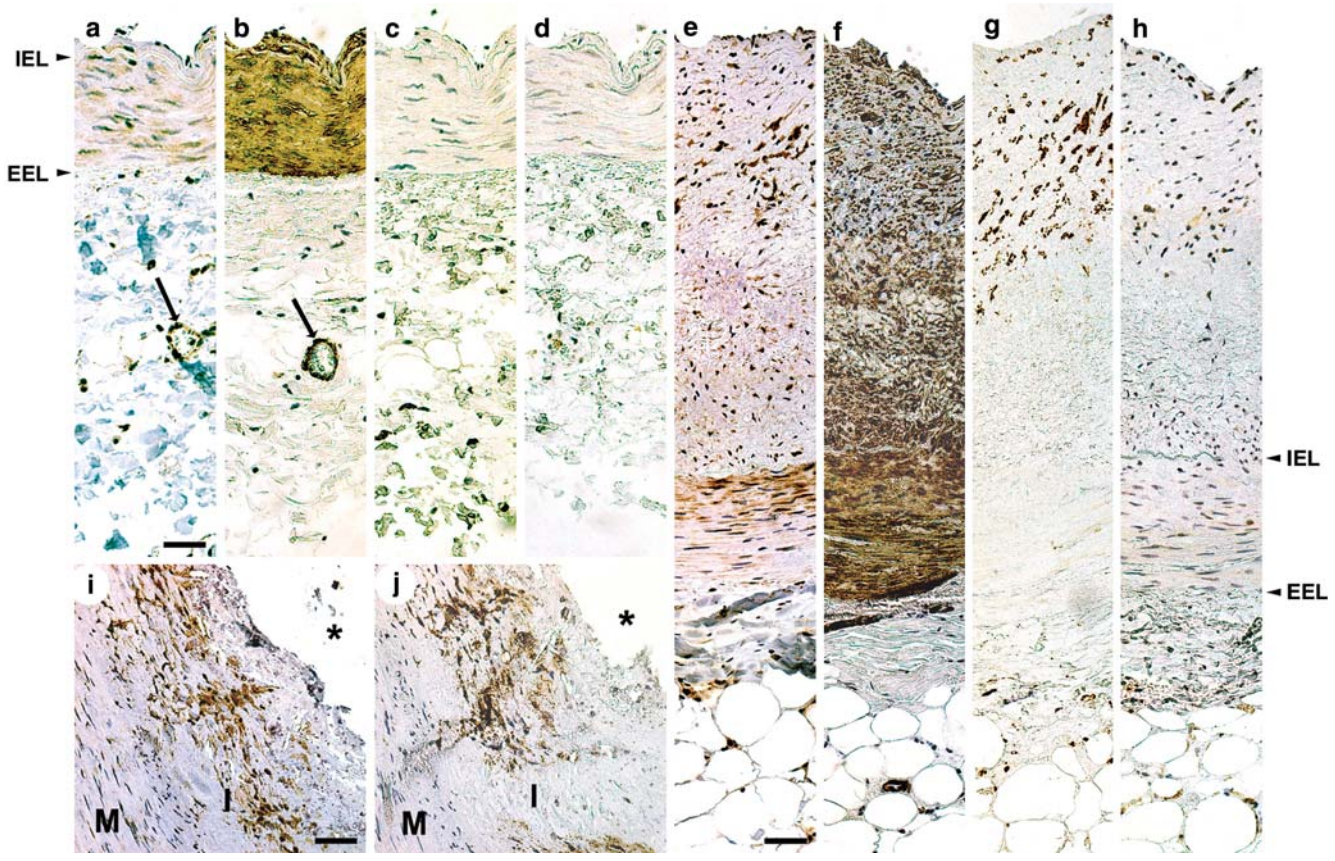

Fig. 1 Immunolocalization of TIMP4 in human coronary artery wall. Immunodetection of TIMP4 (**a**, **e**, **i**) in human non-atherosclerotic (**a**–**d**) coronary artery wall and in intermediate (type III, **e**–**h**) and advanced (type Vb, **i** and **j**) types of human coronary artery atherosclerotic lesions. Smooth muscle cells (**b**, **f**), macrophages (**c**, **g**, **j**) and lymphocytes (**d**, **h**) identified with antibodies against αSMA, CD68 and CD3, respectively. Immunocytochemistry reactions are *brown* and counterstain for nuclei by haematoxylin is *blue*. *Arrowheads* indicate internal (*IEL*) and external (*EEL*) elastic laminae. Small blood vessels in the adventitia (**a**, **b**) are indicated by *arrows*. *Asterisk*, *M* and *I* indicate calcified region, medial and intimal layers, respectively (**i**, **j**). Scale bars: 25 μm (**a**–**d**); 50 μm (**e**–**j**)

without additional stimuli. Furthermore, lymphocytes derived from human peripheral blood were included in the analyses. By western blotting it was shown that protein extracts derived from cultured human macrophages (monocytes stimulated with CSF1 or oxLDL) contain TIMP4 (Fig. 5a). TIMP4 protein was also detected in the media of the above cultures (data not shown). The above results were confirmed by positive immunoreaction for TIMP4 in cultured human monocytes (data not shown) and macrophages (Fig. 5b, c). Furthermore, lymphocytes were demonstrated to produce TIMP4 (data not shown). In addition, similarly to monocytes/macrophages and lymphocytes, HuMC were shown by western blotting to synthesize TIMP4 protein (Fig. 5d) and a portion of cultured HuMC was positive for TIMP4 immunostaining (Fig. 5e, f).

Differential location of TIMP4 and TIMP3 in advanced atherosclerotic coronary artery lesions

To examine the possible function of TIMP4 in apoptosis, serial sections of human atherosclerotic coronary arteries were analysed by immunohistochemistry for TIMP4, TIMP3 and caspase 3. The results demonstrated that TIMP4 and TIMP3 are differentially located. While TIMP3 (Fig. 6b) and caspase 3 (Fig. 6c) are present in and around the necrotic lipid core, TIMP4 resides only in areas surrounding the necrotic lipid core (Fig. 6a).

Discussion

Inflammation plays a key role in various forms of cardiovascular diseases including not only true vasculitic syndromes such as GCA (Weyand and Goronzy 2003) but also atherosclerosis (Libby 2002; Ross 1999). Therefore, molecules that contribute to the inflammatory process within the cardiovascular system have been the focus of numerous studies. In this work we provide evidence that TIMP4 is involved in several inflammatory processes in human cardiovascular tissue. This study is the first to show that TIMP4 is present in significant amounts in human atherosclerotic coronary artery lesions and in human temporal artery specimens exhibiting GCA. TIMP4 was immunohistochemically localized

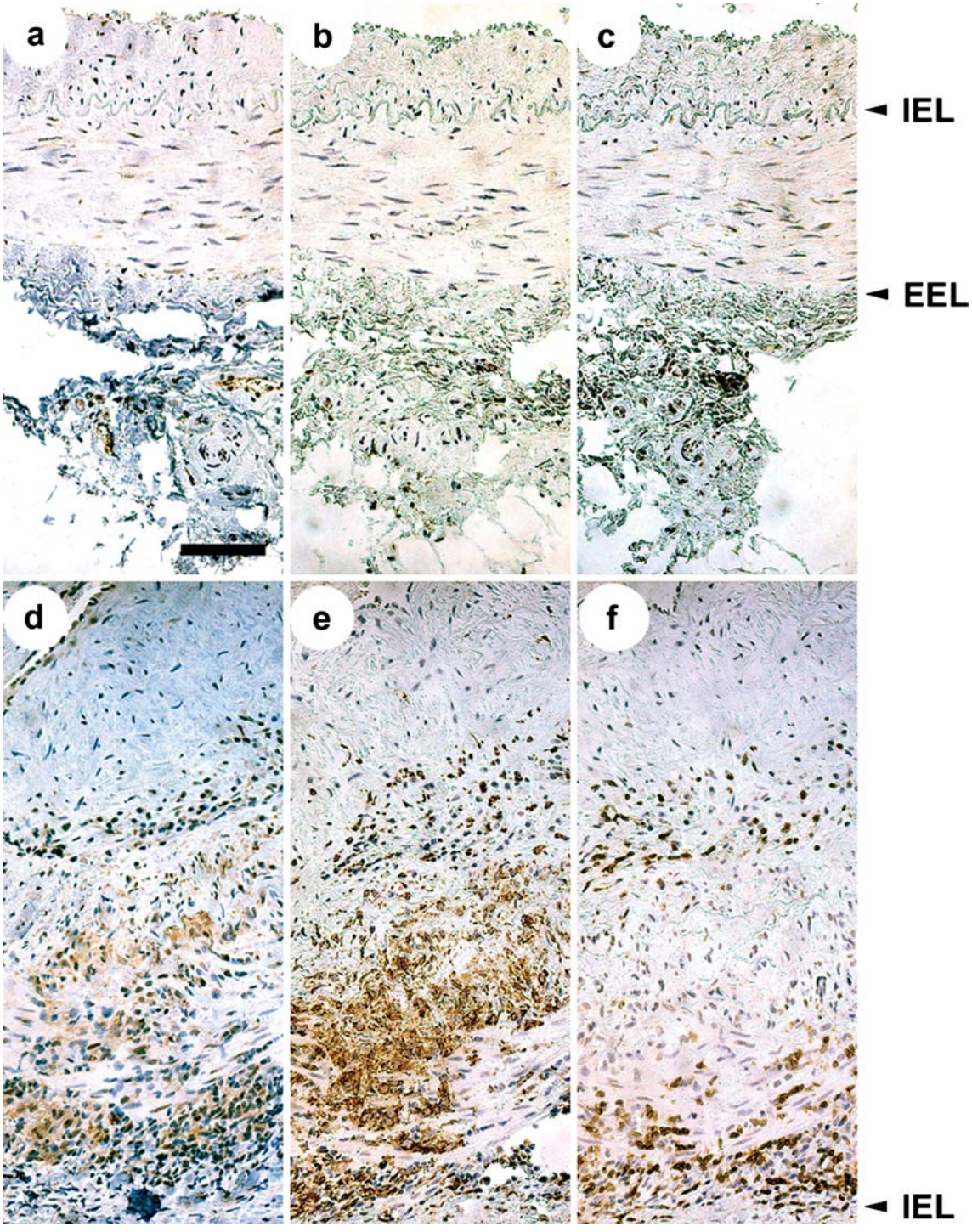

Fig. 2 Immunodetection of TIMP4 in human inflamed and uninvolved temporal artery wall. Immunoperoxidase staining for TIMP4 (**a**, **d**) in temporal artery specimens from an uninvolved control patient (**a**–**c**) and a patient with GCA (**d**–**f**). Immunohistochemical localization of macrophages (**b**, **e**) and T lymphocytes (**c**, **f**). *Arrowheads* indicate internal (*IEL*) and external (*EEL*) elastic laminae. Scale bar: 50 μm (**a**–**f**)

to inflammatory cells, mainly macrophages and CD3+ T lymphocytes. Furthermore, TIMP4 not only appeared in cardiomyocytes, as expected (Rahkonen et al. 2002), but was also observed in leucocyte infiltrations in myocardial specimens from human heart allografts exhibiting chronic rejection. Interestingly, upregulation of TIMP4 in response to inflammation is not restricted to vascular tissue. In fact, similar findings have been described in inflamed joints (Celiker et al. 2002) and in cerebrospinal fluid from patients with tropical spastic paraparesis or myelopathy associated with human T cell lymphotrophic virus (Kettlun et al. 2003). Furthermore,

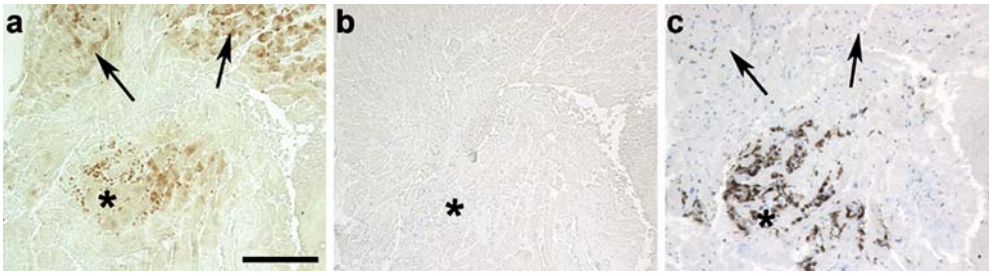

Fig. 3 Immunodetection of TIMP4 in a representative EMB sample derived from a human heart allograft exhibiting rejection. Immunoperoxidase staining for TIMP4 (**a**). Immunostaining for leucocyte common antigen (CD45) to reveal leucocyte infiltration (**c**). **b** is a control slide where no primary antibody for TIMP4 was applied. The *asterisk* in **a**–**c** indicates the corresponding area in separate sections. The *arrows* in **a** indicate TIMP4-positive cardiomyocytes in areas with no substantial inflammation as assessed by the lack of leucocyte infiltration (see the *arrows* in corresponding areas in **c**). Scale bar: 300 μm (**a**–**c**)

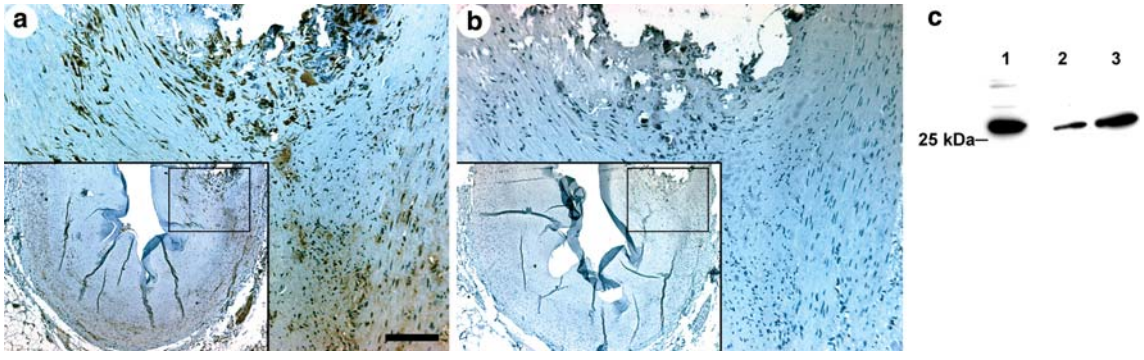

Fig. 4 Confirmation of the specificity of TIMP4 antibody. Immunoperoxidase stainings of an advanced (type Vb) atherosclerotic lesion of human coronary artery using TIMP4 antibody without (**a**) or with (**b**) a preincubation of the antibody with a blocking peptide. In **a** and **b**, *insets* display immunohistochemical stainings at a lower magnification and *squares* highlight the areas shown at a higher magnification. Scale bar: 100 μm (**a**, **b**). Western blotting (**c**) of protein extracts derived from human left ventricular myocardium (*lane 1*) and from human non-atherosclerotic (*lane 2*) and atherosclerotic (*lane 3*) coronary arteries revealed a single band corresponding to the typical size of TIMP4 protein. Equal amounts of protein extracts were loaded in each lane

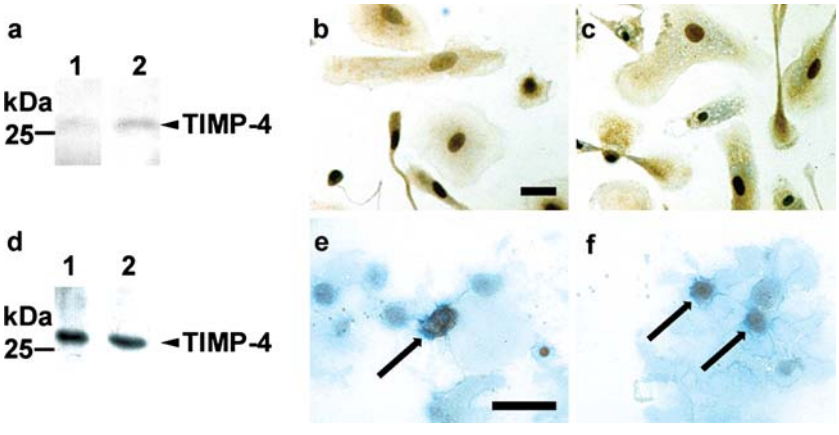

Fig. 5 TIMP4 is synthesized by human monocytes and mast cells (*HuMC*) in culture. Western blotting (**a**) and immunocytochemistry for TIMP4 (**b** and **c**) were performed for human monocytes cultured on plastic for 7–10 days and stimulated with CSF1 (**a**, *lane 1*; **b**) or oxLDL (**a**, *lane 2*; **c**). Western blotting (**d**) and immunocytochemistry for TIMP4 (**e** and **f**) were performed for unstimulated (**d**, *lane 1*; **e**) and IgE-stimulated (**d**, *lane 2*; **f**) HuMC. *Arrows* indicate mast cells that are positive for TIMP4 immunostaining. Scale bar: 25 μm (**b**, **c**, **e**, **f**)

while Greene et al. (1996) have shown that TIMP4 is not expressed by latent inflammatory cells, Coussens et al. (2002) have recently demonstrated that mononuclear cells infected with *M. paratuberculosis* upregulate their TIMP4 expression. Together with our present findings the data collectively suggest that TIMP4 contributes to pathological inflammation where extracellular matrix remodelling is usually included.

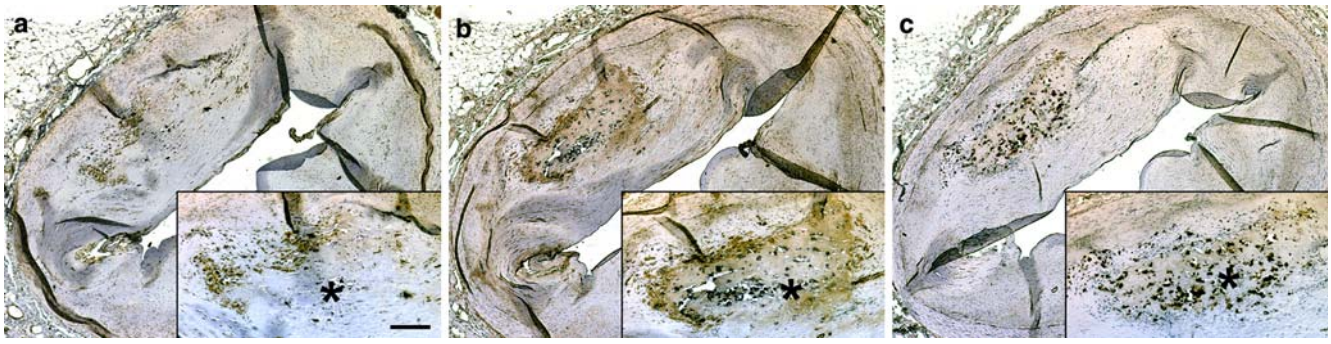

Fig. 6 Immunodetection of TIMP4, TIMP3 and caspase 3 in advanced atherosclerotic lesion of human coronary artery wall. Immunoperoxidase staining for TIMP4 (**a**) and TIMP3 (**b**). Apoptotic cells identified with an antibody against cleaved caspase 3 (**c**). In **a**–**c**, *insets* represent magnified illustrations of the lesion areas. The *asterisk* indicates the corresponding area in separate sections (**a**–**c**). Scale bar: 100 μm (**a**–**c**)

In addition to demonstrating that TIMP4 and inflammation are coupled within human cardiovascular structures, we have shown that macrophages (Libby et al. 1996), lymphocytes (Hansson et al. 1989) and mast cells (Kaartinen et al. 1994), which are known to accumulate in diseased vascular wall, produce TIMP4. Previously, TIMP4 has been shown to be synthesized by monocytes (Bar-Or et al. 2003) as well as by interstitial macrophages in lungs affected by idiopathic pulmonary fibrosis (Selman et al. 2000). Furthermore, in endometrium, strong immunostaining for TIMP4 has been observed in areas of inflammatory cells (Chegini et al. 2003). In contrast, no data on the production of TIMP4 by human lymphocytes or mast cells have been reported earlier.

We have also demonstrated in this study that TIMP4 is expressed in the medial smooth muscle cell layer and in adventitial capillaries of non-atherosclerotic coronary arteries. Interestingly, while TIMP4 is expressed in healthy coronary vessels, we observed no TIMP4 staining in normal temporal arteries. Expression differences could be due to environmental differences between the two types of vessels. Studies of cultured vascular smooth muscle cells have shown that MMP activity increases in response to mechanical strain (Asanuma et al. 2003). Furthermore, high intraluminal pressures in isolated arteries increase activities of MMP2 and MMP9 (Lehoux et al. 2004). Higher blood pressure in coronary arteries probably stimulates MMP expression, and thereby increased TIMP4 expression may be required to inhibit inappropriate remodelling of the arterial wall. In temporal arteries where pressure is lower, TIMP4 is apparently not necessary under normal physiological situations.

There are many known functions attributed to TIMP4. TIMP4 has been shown to inhibit all MMPs studied so far. TIMP4 has been demonstrated to variably influence the growth and metastasis of tumours depending on the tumour type (Celiker et al. 2001; Jiang et al. 2001; Wang et al. 1997). TIMP4 has also been shown to regulate platelet aggregation and recruitment (Radomski et al. 2002). Furthermore, TIMP4 has been found to play a role in hormonal regulation and endometrial tissue remodelling (Chegini et al. 2003). There is also evidence that TIMP4 may play a role in apoptosis. Accordingly, Tummalapalli et al. (2001) have shown that addition of TIMP4 to cultured human cardiac fibroblasts transformed by polyoma virus middle T-antigen leads to cell death, and this effect of TIMP4 could be reversed by anti-TIMP4 antibody. Furthermore, in recent studies it has been demonstrated that the loss of TIMP4 from cardiac myocytes in an ischaemia injury model results in an increase in the net myocardial MMP activity (Schulze et al. 2003; Wilson et al. 2003), which in turn contributes to the acute myocardial injury. Recently, adenovirus-mediated TIMP4 overexpression has been shown to inhibit migration and induce apoptosis of rat aortic SMC in vitro (Guo et al. 2004). In rat carotic balloon injury, TIMP4 overexpression reduced neointimal hyperplasia and increased SMC apoptosis (Guo et al. 2004). In the present study, TIMP3 was co-localized with caspase 3 positive, i.e. apoptotic, cells in and around necrotic lipid cores of atherosclerotic plaques. TIMP4 co-localized with TIMP3 and caspase 3 only in areas surrounding the necrotic lipid cores, whereas the necrotic lipid cores were completely negative for TIMP4. The differential location of TIMP4 and TIMP3 strongly supports the idea that TIMP4 and TIMP3 display distinct roles in inflammation-induced apoptosis during coronary atherogenesis.

In conclusion, in the present study we have provided strong supportive evidence for a role of TIMP4 in inflammatory processes of human cardiovascular pathology. We have also demonstrated that inflammatory cells, specifically macrophages, T lymphocytes and mast cells residing in human cardiovascular structures are capable of producing TIMP4. Finally, evidence is presented that the functional role of TIMP4, at least in inflammation-induced apoptosis, is different from that of TIMP3. It remains to be examined whether the serum/plasma concentration of TIMP4 could be used as a new marker for vascular inflammation. The definite function and the potential therapeutic value of TIMP4 during inflammatory processes within human cardiovascular structures also remain to be explored.

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