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ORIGINAL ARTICLE

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Metallothionein in human atherosclerotic lesions: a scavenger mechanism for reactive oxygen species in the plaque?

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Abstract Oxidative stress is important in the genesis of atherosclerotic lesions. The extracellular effects of reactive oxygen species (ROS), such as oxidative modification of lipoproteins and upregulation of matrix degrading enzymes, are considered crucial in this context. The effects of ROS are counteracted by antioxidant scavenging systems; metallothioneins (MTs) may serve as such. This study was designed to see whether MTs occur in human atherosclerotic plaques and which cell types are involved. The immunohistochemical study focuses on smooth muscle cells (SMCs), macrophages, and T cells. MT immunoreactivity was seen only within SMCs, which occurred usually in small clusters and were found mostly near lipid cores and occasionally in the media. Double immunostaining showed MT-positive SMCs and matrix metalloproteinase (MMP)-9 in the same area but not within the same cell. Electron microscopy was done to evaluate the subtype of MT-positive cells and revealed that the majority consisted of synthetic SMCs. Thus, atherosclerotic plaques in humans contain MT known to act as a scavenger for ROS. The observation that MT was expressed only in SMCs, particularly those of synthetic phenotype, suggests that MT plays a role in protecting these active matrix-producing cells.

Keywords Oxidative stress · Metallothionein · Smooth muscle cells · Matrix metalloproteinase-9 · Immunohistochemistry

Introduction

Oxidative stress is widely considered to play a role in the pathogenesis of atherosclerosis. The generation of reactive oxygen species (ROS), such as superoxide anion (O_2^-) or hydroxyl radical (OH), may directly or indirectly modify or damage proteins and DNA [2, 4, 40, 44]. Lipid peroxidation, a crucial phenomenon in atherosclerosis, is known to occur either via enzymatic mechanisms, as found in macrophage derived foam cells, or via non-enzymatic, direct actions of ROS on lipids present in the plaque [8, 11]. Furthermore, in vitro experiments have shown that ROS can upregulate matrix digesting enzymes [6, 26], thus contributing to plaque destabilization and subsequent plaque rupture. In this context, it is of clinical relevance that a recent experimental study in hypercholesterolemic rabbits revealed that treatment with a ROS scavenger, *N*-acetyl-cysteine, decreased the gelatinolytic activity and the expression of matrix metalloproteinase (MMP)-9 by macrophage-derived foam cells isolated from plaque tissue [13].

Under normal conditions, cells are equipped with antioxidant systems that prevent the reaction of ROS with other structures, such as proteins and lipids [9, 19]. In case of oxidative stress, however, the balance between ROS production and scavenging is impaired either by an increase in the amount of ROS or by a decrease in scavenger capabilities. One of the potential protective mechanisms may involve metallothionein (MT), a small protein of 6–10 kDa characterized by a high number of cysteine residues. MT is capable of binding metals, such as zinc and copper ions. Therefore, its main function has long been seen as a storage and transport vehicle for these metal ions within the cell [17]. Recently, a study showed that MT also has a high capacity to scavenge OH and, albeit to a somewhat lesser degree, O_2^- in vitro [36]. In addition, the cysteine residues are important for this capacity [29] In fact, MT was found to be able to protect cells against ROS [25, 41].

This study was designed to evaluate the presence of MT in human atherosclerotic lesions. We investigated which cell types are associated with the presence of MT and the topographic relationship between MT, lipids, and MMP-9 (gelatinase B) in plaques. For this purpose, we employed immunohistochemical techniques at both light- and electron microscopical levels.

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Table 1 Monoclonal antibodies used in the study. *SMC* smooth muscle cell; *MMP* matrix metalloproteinase; *Ig* immunoglobulin

[a] Dako (Glostrup Denmark)
[b] Calbiochem Novabiochem Corp. (San Diego, Calif.)

Antibody	Clone	Ig-subclass	Working dilution	Source
Anti-metallothionein	E9	IgG1	1:200	Dako[a]
Anti-SMC-α-actin	1A4	IgG2a	1:100	Dako[a]
Anti-macrophages	PGM1	IgG3	1:50	Dako[a]
Anti-CD45RO(T-cells)	UCHL-1	IgG2a	1:100	Dako[a]
Anti-MMP-9	7–11C	IgG1	1:50	Calbiochem Novabiochem[b]

Materials and methods

Tissue specimens

Arterial wall segments containing atherosclerotic plaques were obtained from patients who underwent surgery for atherosclerotic disease [carotid arteries (n=5); abdominal aorta (n=6); femoral artery (n=3); aortocoronary vein graft (n=2)]. Coronary arteries (n=31) were obtained upon autopsy (postmortem interval less than 24 h). The tissue samples were fixed in phosphate-buffered formalin and embedded in paraffin.

The plaques were grouped to be either fibrous, fibrolipid, or lipid-rich [39]. This classification was chosen because of the relevance of the size of the lipid core for the clinical stability of the plaque. Lipid-rich lesions have a greater propensity to develop plaque complications, such as plaque rupture, than fibrous lesions [10]. Fibrous plaques consisted predominately of fibrocellular tissue with a lipid core of less than 25% per whole plaque area, and lipid-rich plaques showed more than 75% extracellular lipid core. Plaques with 25–75% extracellular lipid covered by a fibrous cap were classified as fibrolipid.

Immunohistochemistry

Paraffin sections 5-mm thick were mounted on organosilan coated slides, dried, and stored until use. By application of a three-step streptavidin–biotin complex-method according to standard protocols, single immunostainings were performed. Briefly, sections were deparaffinized in xylene and alcohol. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol for 20 min at room temperature. Unspecific background was blocked with normal goat serum (Dako, Glostrup, Denmark). MTs were detected using the monoclonal antibody E9 (Dako). This antibody reacts against a shared N-terminal epitope of MT I and MT II isoforms. After washing in Tris/HCl-buffered saline, goat anti-mouse antibody conjugated with biotin was applied. This was followed by the application of streptavidin–biotin horseradish peroxidase complex (SABC/HRP, Dako). Peroxidase activity was visualized with 3.3 diaminobenzidine (Sigma Chemical Co., St. Louis, Mo.). After counter-staining with hematoxylin, sections were dehydrated in a graded series of alcohol and xylene and then, a coverslip was placed on top. The primary antibodies used in the study are listed in Table 1.

Double immunostaining procedures using anti-MT/anti-CD68 and anti-MT/anti-αactin were performed according to the indirect/indirect procedure based on the immunoglobulin (Ig) subclass difference of the primary antibodies [37, 38]. Briefly, the primary antibodies were applied according to the single staining procedure, whenever possible as a cocktail, and incubated for 60 min at room temperature or at 4°C overnight in humidified chambers. The secondary antibodies were applied as a cocktail using an alkaline phosphatase (AP) and a biotin label for 30 min at room temperature (goat anti-mouse-IgG1/biotin, goat anti-mouse-IgG2a/AP, goat anti-mouse-IgG3/AP (Southern Biotechnology Associates, Birmingham, Ala.). The biotinylated secondary antibody was further detected with SABC/HRP. The enzymatic activities of HRP and AP were visualized with amino-ethylcarbazole, yielding a red reaction product and fast blue BB/Naphthol-ASMX-phosphate with levamisole, yielding a blue reaction product, respectively.

Table 2 Lesion types of the investigated specimens and immunoreactivity (IR) distribution of metallothionein (MT) and matrix metalloproteinase (MMP)-9

Lesion type	No. of segments	MT-positive IR per segment	MMP-9-positive IR per segment
Fibrous	17	6/17 (35.3%)	6/17 (35.3%)
Fibrolipid	23	15/23 (65.2%)	11/23 (47.8%)
Lipid-rich	7	6/7 (85.7%)	5/7 (71.4%)
Total	47	29/47 (61.7%)	22/47 (46.8%)

Colocalization of two antigens in a single cell results in a distinct purple staining. In case of double immunostaining, nuclear counterstaining with hematoxylin was not applied in order to avoid confusion in the interpretation of the immunostaining.

The immunodouble staining procedure for MT/MMP-9 was performed in a sequential approach because of the same IgG-subclass of the primary antibodies. Reagents from the Dako EnVision Doublestain System (Dako) were used. Briefly, MT-staining was done according to the single staining protocol using diaminobenzidine (DAB) plus as the final chromogen. Microwave antigen retrieval in citrate buffer (pH 6) was applied to visualize MMP-9-immunoreactivity. This step enhances the MMP-9 immunoreactivity and also removes the first set of antibody reagents, just leaving the precipitate of DAB plus at the specific site [5, 18], thus preventing unwanted cross-reactions of the second staining sequence with the first sequence. As a secondary reagent in the second staining sequence, Dako EnVision linked with AP was applied, and the AP-enzymatic activity was visualized with fast red. Here, we counterstained the sections with hematoxylin and then placed a coverslip on top with an aqueous mounting medium.

Tissue obtained from human kidney and colonic carcinoma was used as positive controls for MT immunoreactivity. Controls showed a distinct staining of tubular epithelium (kidney) and tumor epithelium of carcinomas as previously described [31]. For negative controls, the primary antibody was replaced by a non-immune isotype specific reagent (anti-*aspergillus niger* glucose oxidase, Dako) with a protein concentration matched to the concentration of the original primary antibody. Negative controls were invariably negative.

Immunoelectron microscopy

Immunoelectron microscopy was used according to standard protocols described elsewhere [20]. Briefly, small pieces of plaque tissue were fixed for 1 h in freshly prepared 4% paraformaldehyde (pH 7.4) at 4°C. After treatment with 50 mM glycine for 10 min, the material was dehydrated with an ethanol gradient using the progressive lowering of temperature procedure and embedded in LR-Gold. Ultra-thin sections were collected on formvar-coated 50 mesh copper grids or, in the case of two-face double staining procedures, on uncoated 400 or 700 mesh hexagonal copper grids.

Free aldehyde groups were reduced by treating the sections for 10 min with 50 mM glycin in phosphate-buffered saline (PBS), followed by rinsing (two 5-min washes) with 0.2% acetylated bovine serum albumin (BSA-C) (Aurion, Wageningen, The Nether-

lands) in PBS. The sections were incubated with primary antibody [either anti-MT or anti-smooth muscle cell (SMC) actin], diluted in 0.2% BSA-C/PBS (Table 1) for 1 h at 30°C, and rinsed with 0.2% BSA-C/PBS (five 1-min washes). After incubating with the bridging antibody (rabbit anti-mouse Ig), the immunoreactivity was visualized with protein A/15 nm gold.

In case of double staining according to the two face method [3, 20], two different sized gold particles were used: anti-MT was labeled with 15 nm gold and anti-SMC actin was labeled with 10 nm gold. Finally, the sections were contrasted with uranyl and lead.

Light microscopical assessment of the MT staining

For evaluation of the amount of MT-positive immunoreactivity, a semiquantitative approach with a grading from 0 to 3 per cluster area was chosen. No MT positivity in the plaque was graded 0, less than 1% of MT-positive cells per all plaque cells was graded 1, between 1% and 10% of the MT-positive cells per all plaque cells was graded 2, and more than 10% of the MT-positive cells per all plaque cells was graded 3.

Results

Immunohistochemical localization of MT

According to the classification of plaques with respect to their histologic composition, 17 lesions were classified as fibrous, 23 were fibrolipid lesions, and 7 were lipid-rich lesions (Table 2). No major differences were seen in the distribution of these morphologic variants when different arterial sites (i.e., aorta, coronary, etc.) were compared.

Cytoplasmic staining of MT was observed in 29 of 47 plaque segments (61.7%; Table 2). MT positivity occurred in distinct clusters of cells. In almost all cases, the amount of MT-positive cells accounted for 1–10% of all plaque cells. In two cases, the plaques consisted of a large extracellular lipid core with a thin fibrous cap in which >50% of cells stained with anti-MT. Within the clusters, the amount of positive cells varied from 10 % to 60% of all the cells seen in this particular area. Extracellular MT immunoreactivity was never seen. Occasionally, nuclear staining was present.

The MT-positive foci were found in the media or in the intimal plaque. In 20 of 29 MT-positive plaques (69%), MT-positive cells were located near lipid-rich cores (Fig. 1). With respect to the lesion type, we found MT-positive clusters in 6 of 17 fibrous plaques, in 15 of 23 fibrolipid, and in 6 of 7 lipid-rich lesions (Table 2). Rarely, singular positive cells were located in the media or near to necrotic cores. Nerves present in the adventitia stained with anti-MT, which is in accordance with previous studies of brain tissue [30]. In arteries without atherosclerotic plaques (internal mammary artery and temporal artery), MT was detected also in nerves outside the arterial wall, but not in cells of the intima or media.

MT is expressed by SMCs

Most cells that expressed MT in atherosclerotic plaques are spindle-shaped or with the morphology of a lacunar cell as described for SMCs [14, 28]. A few cells showed cytoplasmic lipid droplets. With double staining methods, we demonstrated that the spindle-shaped MT-positive cells were all negative for CD68 (macrophages) (Fig. 2) and CD45RO (T cells), whereas a number of the spindle-shaped MT-positive cells showed staining with anti-α-actin (Fig. 3). Immunodouble-electron microscopy for MT and α-actin showed that particularly SMCs of the synthetic phenotype, with only small amounts of α-actin, were MT positive. Some of these cells clearly showed matrix production, thus accounting for the lacunar cells as previously described [14]. MT-positive granules (15 nm) were located near membranes and within the cytoplasm but without any connections with cellular organelles. The α-actin-positive granules (10 nm) were located along the actin fibers, as anticipated (Fig. 4). In all instances, the cells were located near lipid-rich areas. Neither T cells nor macrophages could be detected as MT positive. No MT-immunoreactivity was found in endothelial cells. Scarce MT-positive cells in the media were also of SMC origin.

MT in Relation to MMP-9

Single stainings revealed MMP-9-immunoreactivity in 22 of 47 segments (47%) (Table 2). MMP-9-immunoreactivity was mostly related to ($CD68^+$) macrophages around and in lipid cores. In 19 of the 29 segments (65.5%) containing MT-positive cells, MMP-9 immunoreactivity around lipid cores was also found in close proximity with MT immunoreactivity. This was confirmed in double staining experiments in selected cases (Fig. 5). Because of the presence of extracellular staining for MMP-9, reliable quantification of cellular distribution for both MT and MMP-9 was no longer feasible.

Fig. 1 Single immunostaining for metallothionein (MT) in the fibrous cap of a right coronary artery plaque. The *brown color* indicates MT-positivity of smooth muscle cells (SMCs) in fibrous tissue of the plaque. *Bar* 100 µm ▶

Fig. 2 Metallothionein immunostaining of an aortic atherosclerotic plaque. Double immunostaining for metallothionein (MT; *red*) and macrophages (*blue*) of an atherosclerotic plaque, showing absence of MT immunoreactivity in macrophages. *Bar* 100 µm

Fig. 3 Double staining for metallothionein (MT; *red*) and smooth muscle cells (SMCs; *blue*). Several cells are double immunostained, indicating presence of MT in SMCs (*arrows*). In addition, SMCs that do not stain for MT are present. *Bar* 100 µm

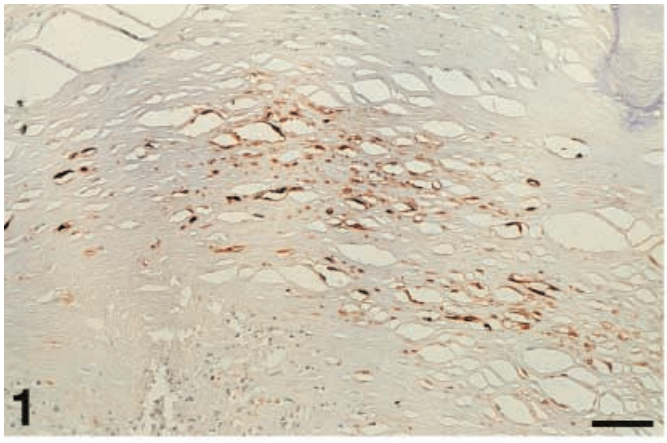
Fig. 4 Double immunoelectron microscopy of an ultra-thin atherosclerotic plaque section showing co-localization of anti α-actin and anti-metallothionein (MT) in a smooth muscle cell (SMC). **B** Enlargement of boxed area in (**A**). Small gold particles (10-nm gold, *arrow*) were used for α-actin labeling, large gold particles (15-nm gold, *arrowhead*) were used for MT labeling, *Bars* 0.1 µm

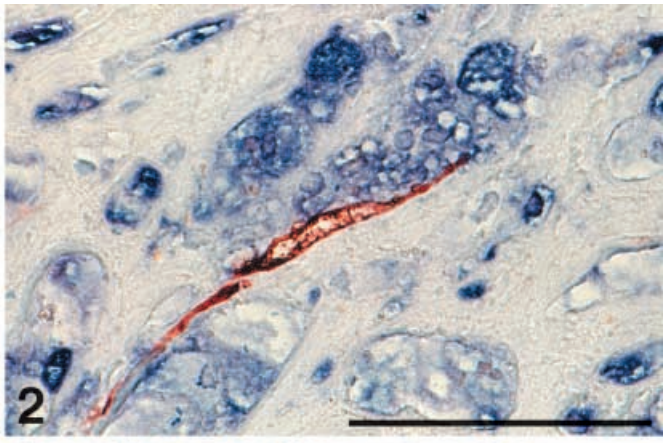
Fig. 5 Double immunostaining for metallothionein (MT; *brown*) and matrix metalloproteinase (MMP)-9 (*red*). Both proteins are in the same plaque area (near the atheroma), but there is no double staining of cells. Nuclear counterstain with hematoxylin in *blue*. *Bar* 100 µm

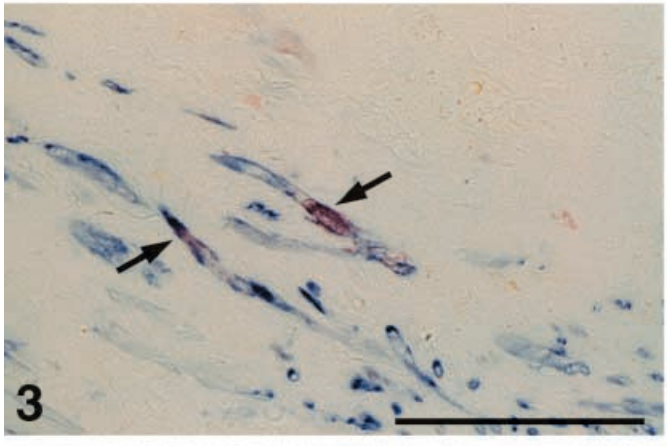
Discussion

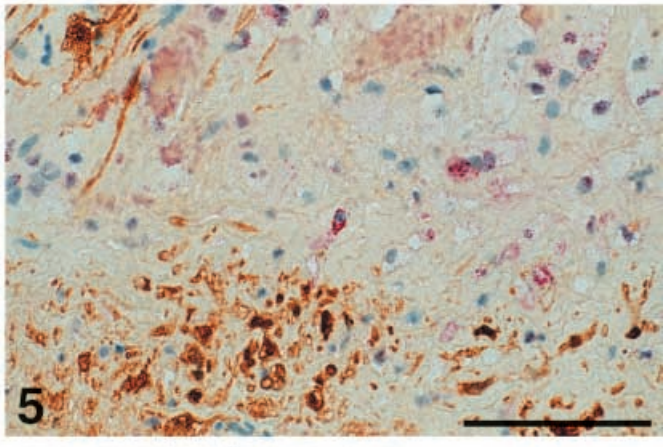
Oxidative stress is receiving more attention in atherosclerosis research. The radicals involved have a broad spectrum of action leading to cell dysfunction or even cell death [7, 42], and MT was shown to have a protective role for cells exposed to ROS [1, 23, 25, 36]. Patterns of MT immunoreactivity have been well documented in various normal tissues and also in pathological conditions, such as tumors [16, 21, 24, 31]. Our findings show for the first time that MT is present in human atherosclerotic lesions.

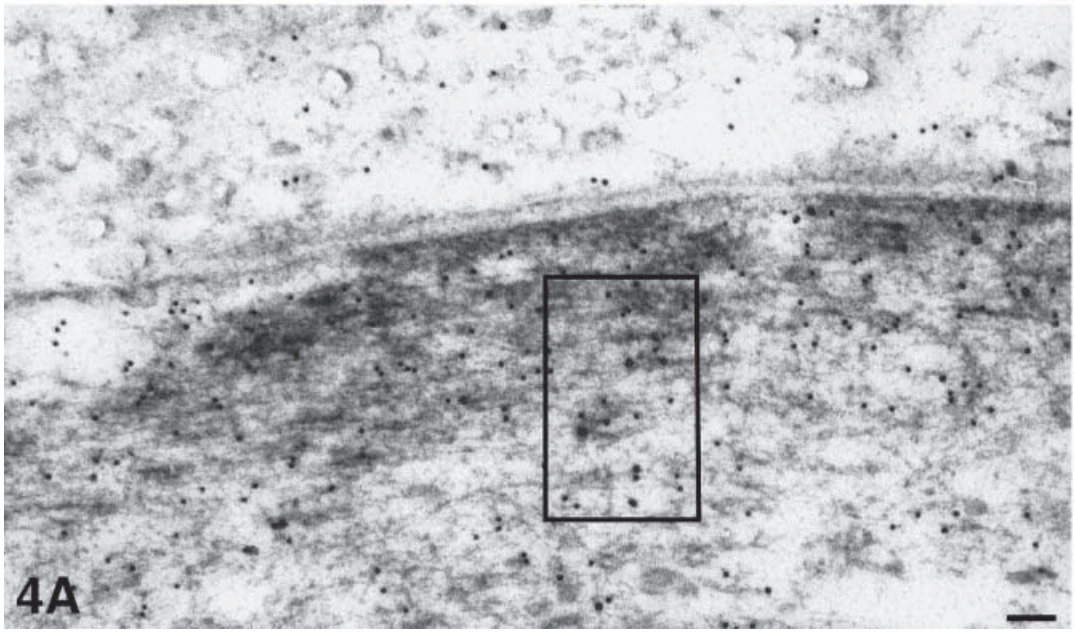
The MT-positive cells express α-actin and, hence, appear to be of SMC origin. The overall low amounts of immunostainable α-actin in these MT and SMCs fit with

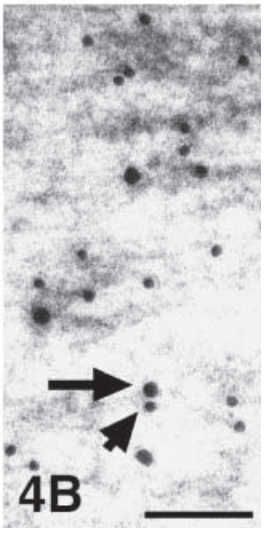












a synthetic phenotype. There may be several explanations for the presence of MT-positive cells with no α-actin staining. First, these cells are completely of a synthetic phenotype with no contractile elements to be stained. Second, they may be of fibroblast origin, a spindle cell type also not staining with anti-SMC α-actin antibodies, or with anti-macrophage antibodies.

It is intriguing that the cells expressing MT are SMCs and not macrophages since macrophages produce huge quantities of Zn-dependent molecules, such as metalloproteinases, and MT has long been considered as the main transport vehicle involved with transmembrane movement of such metal ions. Our findings suggest that MT, – at least in atherosclerotic plaques – may play a protective role for SMCs against ROS.

Indeed, MT immunoreactivity was more prominent in plaques containing large amounts of lipids (Table 2). The localization of the MT-positive cells near lipid cores could indicate a relationship with oxidation of lipids in plaques. ROS, produced by endothelial or SMCs, may oxidize low density lipoprotein (LDL) particles already present in the vessel wall in the early stages of atherosclerosis [43]. In advanced lesions, activated macrophages near lipid cores produce an oxidative burst leading to enhanced lipid peroxidation within cells and in the extracellular matrix [45]. Moreover, oxidized lipids are chemoattractants for monocyte macrophages [12] and are also able to induce a specific immune response [15, 32, 46]. Taken together, the antioxidant character of MT and the localization of MT near lipid-rich areas indicate a possible role in the protection of SMCs against lipid peroxidation by ROS.

ROS are thought to be involved as secondary messengers in gene activation [27, 35]. In the atherosclerotic lesion, the presence of MMP-9 immunoreactivity in the same region as MT immunoreactivity could be interpreted as an indirect sign for such oxidative reactions, since it is known that ROS are able to induce MMP production, at least in vitro [6, 26], and ROS are capable also of directly degrading collagens [22]. A recent experimental study in hypercholesterolemic rabbits, moreover, has shown that treatment with a ROS scavenger decreases the production of MMP-9 by lipid-laden macrophage-derived foam cells from plaques [13]. Because atherosclerotic plaque rupture is the main event causing myocardial infarction, most therapeutic strategies aim at preventing extracellular matrix breakdown and, hence, keeping the lesion stable. Antioxidant therapies with vitamins, such as α-tocopherol (vitamin E) or vitamin C, for chronic inflammatory diseases have been known for many years. However, the applicability in patients with atherosclerotic disease is of a more recent date [33, 34]. The concept underlying this therapeutic approach is that diminution of active oxidative radicals could prevent further tissue damage. Since MMPs are major enzymes involved in matrix degradation, a reduction of their activity and/or presence should lead to a more stabile plaque. In this context, MT may act as a protective agent, since MT occurred predominantly in SMCs of the synthetic phenotype, and these are the cells involved in the production of the extracellular matrix.

This observation, together with the fact that these MT-positive SMCs are located closely to the lipid core, suggests that MT serves as a protective mechanism for SMCs. The localization of MMP-9 in close proximity to MT suggests that ROS may be responsible for the upregulation of both proteins.

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