INTRODUCTION

Extracellular matrix (ECM) degradation is tightly regulated within the normal vessel wall through a balance between proteinases and their endogenous inhibitors. However, within the atherosclerotic plaque the balance may become shifted towards matrix degradation since accumulating macrophages and phenotypically altered smooth muscle cells secrete a plethora of proteinases, including matrix metalloproteinases (MMPs) (1). The MMPs are inhibited by specific endogenous tissue inhibitors of metalloproteinases (TIMPs), which comprise a family of four protease inhibitors: TIMP-1, TIMP-2, TIMP-3 and TIMP-4. Although undetectable in normal arteries, MMP-1 expression has been localized to the fibrous cap and the shoulder regions of carotid atherosclerotic lesions (2). In the latter tissue, the cellular sources of MMP-1 are mainly represented by macrophages, smooth muscle cells SMCs, and endothelial cells. Morphological analysis of the plaques have, in addition, revealed higher MMP-1 transcript levels in carotid lesions with a large lipid core and thin fibrous cap as compared with fibrous lesions with thick fibrous caps (3). The latter findings suggest an increased MMP-1 expression associated with plaque vulnerability, which has also been supported by the MMP-1 messenger RNA (mRNA) levels detected in carotid lesions derived from patients with recent ischemic manifestations. Finally, a study of carotid lesions derived from patients undergoing repeated vascular intervention have shown that an increased MMP-1 expression correlated with the more foam cell-dominated late lesions compared with early restenotic lesions, which were characterized by increased SMC content (4). Taken together, these studies support a role of MMP-1 derived from inflammatory cells in ECM degradation associated with plaque rupture.

In line with the aforementioned mouse studies of the collagenases, important information about the role of gelatinases in atherogenesis and/or atherothrombotic events has also been obtained from knocking out MMPs genes in mouse models of atherosclerosis. A significant reduction in atherosclerotic plaque has been observed in MMP-2-/- x ApoE-/- mice compared to MMP-2+/+ x ApoE-/- mice. These mice presented also concomitant reduction in macrophages and collagen in the aortic sinus (5). Exogenous synthetic inhibitors generally contain a chelating group which binds the catalytic zinc atom in the enzyme active site. Doxycycline, already at subantimicrobial doses, inhibits MMPs activity, and has been used in various experimental setups for this purpose (6). It is used clinically for the treatment of periodontal disease and is the only MMP inhibitor which is widely available clinically (7). Therefore, the aim of the current study was to examine whether doxycycline treatment results in a development of less advanced atherosclerotic lesions in apolipoprotein E (apoE)-knockout mice and to confirm whether this effect is associated with decreased metalloproteinase activity.

MATERIALS AND METHODS

Animals and treatment

Female apoE-knockout mice on the C57BL/6J background were obtained from Taconic (Ejby, Denmark). Mice were maintained on 12-h dark/12-h light cycles in air-conditioned rooms (22.5±0.5°C, 50±5% humidity) and access to diet and water ad libitum. At the age of 8 weeks mice were put on chow diet made by Ssniff (Soest, Germany) for 4 months.
Experimental group received the same diet, mixed with doxycycline (Sigma Aldrich, St. Louis, MO, USA) at a dose 1.5 mg per kg of body weight per day.

This study complied with domestic and international guidelines of animal welfare and was approved by the Jagiellonian University Ethical Committee on Animal Experiments.

Animal procedures

At the age of 6 months mice were sacrificed under anesthesia and 1000 UI of fraxiparine (Sanofi-Synthelabo, France) was injected into the peritoneal cavity. The blood was collected from the right ventricle. Plasma was separated by centrifugation at 1000g at 4°C for 10 min and stored in -80°C. Then, right atrium was incised and the heart was perfused by PBS through the apex of the left ventricle of the heart at a constant pressure of 100 mm Hg. Next, the heart and the whole aorta were dissected.

Plasma lipids

Total cholesterol and triglycerides were assayed using commercially available kits (Roche Molecular Biochemical, USA).

Quantitation of atherosclerosis

The heart and ascending aorta were embedded in OCT compound (CellPath, UK) and snap-frozen. Ten micrometer-thick cryosections were cut from the aortic root using a standardized protocol (9-12).

Serial sections were cut from the proximal 1 mm of the aortic root. Eight adjacent sections were collected at 100-µm intervals starting at a 100-µm distance from the appearance of the aortic valves. Sections were thaw-mounted on poly-L-lysine coated slides and air dried. After fixation in 4% buffered formaldehyde (pH=7), sections were stained with Meyer’s hematoxylin and oil red-O (Sigma-Aldrich, USA) or left unfixed for proteolytic activity.

The alterations of the ECM induced by MMPs are dependent on several factors (14, 15). First, in addition to MMP production by the structural components of the vascular wall, for example, endothelial and smooth muscle cells (SMCs), the infiltration of inflammatory cells into the atherosclerotic lesion results in a constitutive expression of MMPs for proteolytic activity.

Based on the above, MMPs have been considered as putative therapeutic targets in the prevention of atherogenesis. Synthetic inhibitors generally contain a chelating group which binds the catalytic zinc atom at the MMP active site tightly. Common chelating groups include hydroxamates, carboxylates, thiols, and phosphinyls. Hydroxamates are

intensity/green channel: range 0-255) were measured in eight sections from each sample applying analySIS FIVE software.

Results are expressed as mean±S.E.M. The nonparametric Mann-Whitney U test was used for analysis of the data. P value less than 0.05 was considered as statistically significant.

RESULTS

Doxycycline did not change the level of cholesterol and triglycerides in blood, as compared to the control group (Table 1).

Table 1. Cholesterol (TCH) and triglycerides (TG) levels in control and doxycycline-treated groups, presented as mean ±S.E.M. NS: non-significant difference between groups.

<table>
<thead>
<tr>
<th>group</th>
<th>TCH (mmol/l)</th>
<th>TG (mmol/l)</th>
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<tbody>
<tr>
<td>control (n=10)</td>
<td>15.3±1.1</td>
<td>1.94±0.1</td>
</tr>
<tr>
<td>doxycycline-treated</td>
<td>15.2±0.9(NS)</td>
<td>1.96±0.1(NS)</td>
</tr>
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Measured by the "en face" method, percentage of area occupied by atherosclerotic lesions in aortas in the control group was 15.7±2.0%, whereas in doxycycline-treated group was 10.25±1.7% (p<0.05).

Lesion area measured by "cross-section" of aortic roots was 90,687±8,521 µm² in the control group (Fig. 1A) vs. 66,254±7,468 µm² in doxycycline-treated group (p<0.05) (Fig. 1C). In situ zymography showed decrease of total area of gelatinase activity in doxycycline-treated mice (32,786±3,334 µm²) (Fig. 1D) in comparison to control group (65,996±11,480 µm², p<0.001) (Fig. 1B). Also intensity of fluorescence was lower in experimental group (77±15) than in untreated animals (132±32, p<0.05).

DISCUSSION

The composition of the extracellular matrix (ECM) may affect plaque progression. Matrix metalloproteinases (MMPs) are a group of endopeptidases with capacity to cleave several components of the ECM, such as collagen, elastin, gelatins, casein and others.

The alterations of the ECM induced by MMPs are dependent on several factors (14, 15). First, in addition to MMP production by the structural components of the vascular wall, for example, endothelial and smooth muscle cells (SMCs), the infiltration of inflammatory cells into the atherosclerotic lesion results in a major increase of MMP activity. Second, MMPs are subdivided into different groups, according to what components of the ECM they degrade, and the profile of MMPs expressed within an atherosclerotic lesion hence has consequences for its ECM composition. Third, although some of the MMPs are constitutively expressed, others are highly dependent on transcriptional regulation for their expression. Finally, most MMPs are secreted in a latent proform, which require activation for proteolytic activity.

Based on the above, MMPs have been considered as putative therapeutic targets in the prevention of atherogenesis. Synthetic inhibitors generally contain a chelating group which binds the catalytic zinc atom at the MMP active site tightly. Common chelating groups include hydroxamates, carboxylates, thiols, and phosphinyls. Hydroxamates are
particularly potent inhibitors of MMPs and other zinc-dependent enzymes, due to their bidentate chelation of the zinc atom. Other substituents of these inhibitors are usually designed to interact with various binding pockets on the MMP of interest, making the inhibitor more or less specific for given MMPs.

Doxycycline at subantimicrobial doses inhibits MMP activity in a non-specific way, and has been used in various experimental systems for this purpose. Moreover, it is the only MMP inhibitor which is widely available clinically (16, 17). So far, we have no data indicating other mechanisms, involved in antiatherogenic effect of doxycycline.

The results published so far on this topic are contradictory. Manning et al. showed no effect of doxycycline in angiotensin II - infused LDL receptor +/- mice (18). However, the conditions of his experiments were far from similar to ours. On the other hand, doxycycline proved efficacy in rat model of aneurysm (19, 20). Castro et al. showed that MMPs inhibition by doxycycline in hypertensive rats ameliorates hypertension and prevents vascular dysfunction (21). Finally, in experiments made by Madan et al. there was a positive effect of doxycycline on atherogenesis in a special apoE heterozygote murine model, infected with Porphyromonas gingivalis (22). Therefore, our report, provided on apoE-knockout mice, seems to broaden the current knowledge about the positive effect of doxycycline in subantimicrobial doses on animal experimental models of atherogenesis.

To sum up, the concept of matrix metalloproteinases as valid clinical targets seems to be promising, however, there is still a long way to establish proper drugs and conditions in which this kind of treatment could have the best clinical effect (23-28).

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REFERENCES


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