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Short communication

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THE EFFECT OF DOXYCYCLINE ON ATHEROGENESIS IN APOE-KNOCKOUT MICE

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Doxycycline at subantimicrobial doses inhibits matrix metalloproteinases (MMPs) activity, and is the only MMP inhibitor which is widely available in clinical practice. The aim of the study was to reveal whether non-specific MMPs inhibition by tetracycline could ameliorate development of atherosclerosis in apolipoprotein E (apoE)-knockout mice. Doxycycline (1.5 mg/ kg b.w./day) administered orally attenuated atherogenesis, measured both by "en face" method (10.25±1.7% *vs*. 15.7±2.0%, $p<0.05$) and "cross-section" method (66,254±7,468 μm^2 *vs*. 90,687±8,521 μm^2, $p<0.05$). *In-situ* zymography showed decrease of the extent of non-specific gelatinase activity in doxycycline-treated mice This is the first report to date describing the effect of doxycycline on atherogenesis in apoE-targeted mice.

Key words: *atherosclerosis, apoE-knockout mice, doxycycline*

INTRODUCTION

Extracellular matrix 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 inhibitor 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, 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-/-. 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 set-ups 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 (8) 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 1000×g 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 and stored in -20?C (*in situ* zymography).

Oil red O-stained sections were examined under Olympus BX50 (Olympus, Tokyo, Japan) microscope and used for quantitative evaluation. Images of the aorta were recorded using Olympus Camedia 5050 digital camera and stored as TIFF files of resolution 1024×768 pixels. Total area of the lesion was measured semiautomatically in each slide using LSM Image Browser 3 software (Zeiss, Jena, Germany). For each animal a mean lesion area was calculated from eight sections, reflecting the cross-section area covered by atherosclerosis.

The aorta from arch to bifurcation was fixed in 4% formaldehyde, opened longitudinally, pinned onto black wax plates and stained with Sudan IV (Sigma-Aldrich, St. Louis, MO, USA). Aortic lesion area and total aortic area were calculated using LSM Image Browser software.

In situ zymography was performed to demonstrate non-specific activity of gelatinases (mostly MMP-2 and MMP-9) in tissue sections (13). Unfixed sections were thawed and incubated for 2 hours at 37°C in a dark humid chambers with reaction buffer containing 50 mg/ml of FITC-labelled DQ-gelatin (Invitrogen, Eugene, OR). Sections were rinsed in PBS and fixed in 4% formaldehyde for 5 min, then mounted in glycerin/PBS.

The fluorescence of gelatinase activity was observed under BX50 microscope equipped with mercuric burner and appropriate filter set (U-MNIBA), and recorded with DP71 camera. The area displaying fluorescence resulting from enzymatic break-down of FITC-gelatin is representative for local tissue gelatinase activity. Areas of MMP activity as well as intensities of fluorescence (reflecting enzyme activity; pixel intensity/green channel: range 0-255) were measured in eight sections from each sample applying analySIS FIVE software.

Statistical analysis

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.

group	TCH (mmol/l)	TG (mmol/l)
control (n=10)	15.8±1.1	1.94±0.1
doxycycline-treated (n=10)	15.2±0.9(NS)	1.96±0.1(NS)

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 µm^2 in the control group (*Fig. 1A*) *vs.* 66,254±7,468 µm^2 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 µm^2) (*Fig. 1D*) in comparison to control group (65,996±11,480 µm^2, $p<0.001$) (*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 are degraded, and the profile of MMPs expressed within an atherosclerostic 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. Hydroxymates are

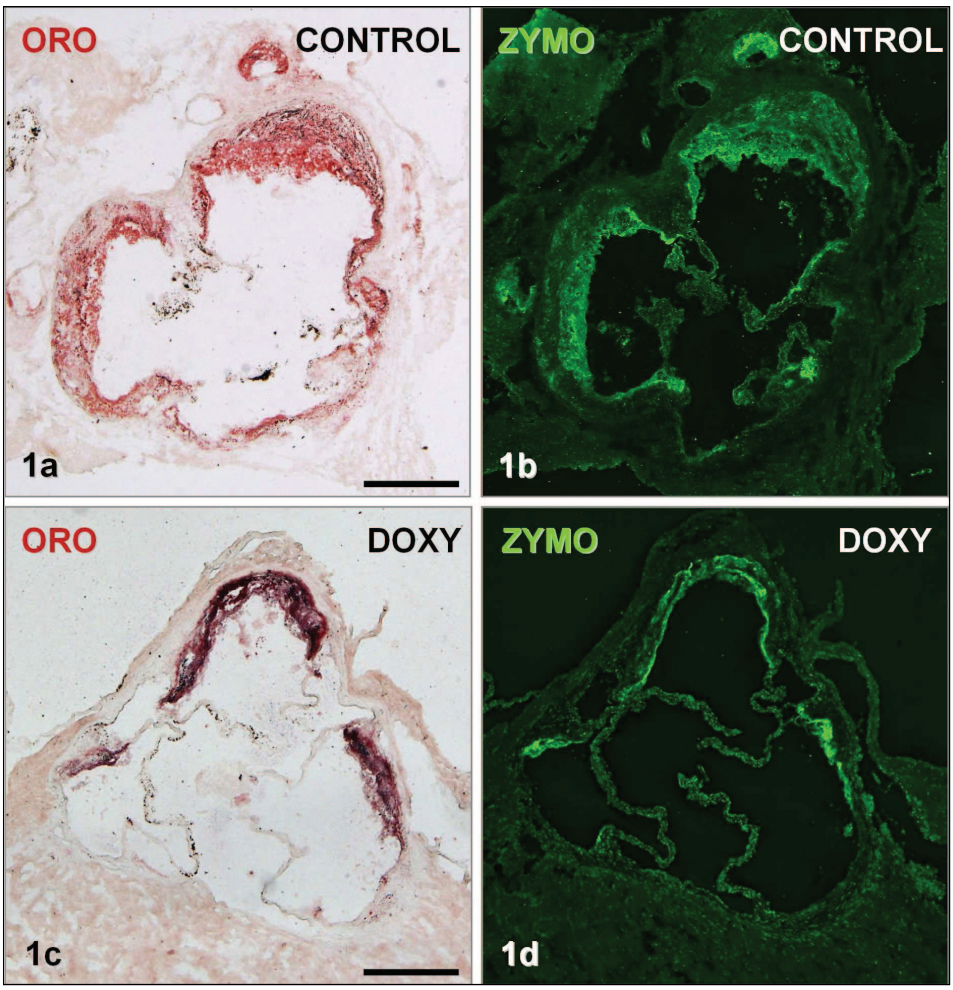

Fig. 1. Aortic roots from control (*A, B*) and doxycycline-treated mice (*C, D*) stained with oil red-O (*A, C*). Consecutive sections (*B, D*) show zymographic reaction for non-specific gelatinase activity. Scale bar=500 μm.

particularly potent inhibitors of MMPs and other zinc-dependent enzymes, due to their bidentate chelation of the zinc atom. Other substitutents 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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