It is widely appreciated that inflammation and oxidant stress contribute to atherogenesis. Curcumin, a polyphenolic natural compound has been reported to possess anti-inflammatory and anti-oxidant actions. We hypothesized that curcumin could inhibit the development of atherosclerosis in the apoE/LDLR - double knockout mice fed with Western diet (21% fat, 0.15% cholesterol w/w, without cholic acid). Curcumin (purity ≥ 98%), premixed with diet, was given for 4 months at a dose of 0.3 mg/per day/per mouse. In this model curcumin inhibited atherogenesis, measured both by "en face" method (25.15±2.9% vs. 19.2±0.6%, p<0.05) and "cross-section" method (565867±39764 µm² vs. 299201±20373 µm², p<0.05). Importantly, curcumin influenced neither the concentrations of cholesterol and triglycerides in blood nor animal body weight. To our knowledge, this is the first report that shows the anti-atherogenic effect of low dose of curcumin in fine model of atherosclerosis: gene-targeted apoE/LDLR - double knockout mice.

Key words: atherosclerosis, apoE/LDLR - knockout mice, curcumin

INTRODUCTION

Atherosclerosis is currently considered as a chronic and progressive disease arising from the inflammatory processes and oxidative stress within vessel wall (1,2,3). Over the last 10 years, a significant body of evidence has emerged that chemically diverse classes of plant-derived constituents possess potent anti-inflammatory and antioxidant action (4-6).
Curcumin (Fig. 1) is a naturally occurring yellow pigment isolated from ground rhizomes of the plant *Curcuma longa* L. (*Zingiberaceae*). As a powder, called turmeric, it has been in continuous use as a coloring and flavoring spice in foods as well as in folk medicine in the management of various inflammatory disorders and wound healing (7). Although molecular mechanisms of action of curcumin are not fully understood, in several animal models it has been demonstrated to exert potent antioxidant, anti-inflammatory and anti-tumor properties (7-9). The question arises, whether curcumin could suppress inflammatory component of atherogenesis. Indeed, recently curcumin derivatives have been demonstrated to reduce aortic fatty streak formation in cholesterol-fed rabbits (10). However, this animal model has many limitations and there are no data pertaining to antiatherogenic potential of curcumin, as far as more relevant models of atherosclerosis are considered (11).

In 1992 the new, excellent animal models became available for experimental atherosclerosis research. At that time the first line of gene targeted animal models, namely apolipoprotein E (apoE) - knockout mice was developed (12). More recently, apoE and LDLR - double knockout (apoE/LDLR - DKO) mice have been created, representing a model that develop more severe hyperlipidaemia and atherosclerosis than mice deficient for apoE alone (13). In both strains of gene-targeted mice, lesion formation is greatly accelerated and lesion size is increased by feeding with atherogenic Western diet. Thus, apoE/LDLR - DKO mouse is nowadays considered as a one of the most relevant models to study the anti-atherogenic potential of drugs.

The present study was designed to examine whether curcumin could prevent atherosclerosis in apoE/LDLR - DKO mice fed with Western diet.

**MATERIAL AND METHODS**

**Animals and treatment**

All animal procedures were approved by the Jagiellonian University Ethical Committee on Animal Experiments. Female apoE/LDLR - DKO mice on the mixed C57BL/6J x 129/SvJ background were obtained from Taconic (Bomholt, 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 Western diet (consisting of 21% fat by
weight, 0.15% cholesterol by weight and no cholic acid, made by ssniff, Germany) for 4 months. Experimental group received the same diet, mixed with curcumin (Cayman Chemical Co., USA) without heating at a dose of 0.3 mg/per day/per mouse. Compliance with curcumin supplementation was confirmed by measuring the consumption of curcumin-supplemented chow every day.

**Procedures**

At the age of 6 months mice were sacrificed under anesthesia and 1000 UI of fraxiparine (Sanofi-Synthelabo, France) was injected into the peritoneum. The blood was collected from the right ventricle. Plasma was separated by centrifugation at 1000 x 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 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 (PZ Cormay, Poland).

**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. Serial sections were cut from the proximal 1 mm of the aortic root. Eight 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% paraformaldehyde (pH=7), sections were stained with Meyer’s hematoxylin and oil red-O (Sigma-Aldrich, USA) (12). 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 1024x768 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.

**Statistical analysis**

Results are expressed as mean±SEM. The nonparametric Mann-Whitney U test was used for analysis of the data. P<0.05 was considered as statistically significant.

**RESULTS**

The apoE/LDLR - DKO mice fed for 4 months with Western diet developed massive atherosclerotic changes as evidenced both by "en face" and cross-section methods (**Fig. 2A, Fig. 4A**). Importantly, the aortas differed between control and curcumin-treated animals (**Fig. 2**). Measured by "en face" method, percentage of total aortic area occupied by Sudan IV-stained changes was significantly lower in
curcumin-treated group as compared to control animals (19.2±0.6% vs. 25.15±2.9%, both groups n=7, p<0.05) (Fig. 3).

Cross-sectioning of aortic roots revealed also the difference in lesions area (Fig. 4). Counting by 8 consecutive sections the mean area±SEM, occupied by
Fig. 5. Lesion size in the aortic root ($\mu$m$^2$) stained by ORO in 6-month-old control and curcumin-treated apoE/LDLR-double knockout mice (n=10 per group). Results presented as mean±SEM. *p<0.05.
ORO-stained lesions was $565867\pm39764\,\mu m^2$ in control group versus $291695\pm30384\,\mu m^2$ in curcumin-treated group ($p<0.05$) (Fig. 5).

Importantly, treatment with curcumin did not influence the concentrations of cholesterol and triglycerides in blood (Table 1). There were no differences in body-weight between the control and curcumin-treated groups during the study period.

**DISCUSSION**

In the present study we demonstrated that curcumin, given orally at a relatively low dose, was able to decrease formation of atherosclerotic changes in apoE/LDLR - DKO mice fed with Western diet. Importantly, in our hands the effect of curcumin was not due to changes in lipid metabolism.

One could argue that the anti-atherosclerotic action of curcumin in our model was relatively weak, as it decreased the lesion formation by c.a. 20 % only, as evidenced by "en face" method. However, the presented data pertain to whole vessel and the effect of curcumin was more pronounced in distal portions of aorta. Also, more accurate cross-section analysis of aortic root showed almost two-fold decrease of ORO-staining.

It should be noted that action of curcumin was present in apoE/LDLR - DKO mice even despite feeding them by Western diet, what greatly accelerates lesion formation, increases lesion size and promotes development of advanced lesions at a significantly earlier age (14). Taking into granted poor curcumin bioavailability due to its rapid metabolism in the liver and intestinal wall (15) as well as relatively low dose used in our study (0,3 mg/mouse/day) the anti-atherogenic action of curcumin seems to be quite strong.

In present work, we are not able to demonstrate mechanisms responsible for anti-atherosclerotic action of curcumin, which theoretically may interfere with atherosogenesis in several critical points.

Many authors addressed NF κB as a important therapeutic target in atherosclerosis (16). Recently, we have reported that PDTC, an NF κB inhibitor significantly decreased atherosclerosis in apoE/LDLR - DKO mice fed with Western diet (17). It was demonstrated that oral supplementation of caffeic acid phenethyl ester (CAPE), compound similar to curcumin, attenuates the atherosclerotic process in apoE-knockout mice due to inhibition of transcription.

**Table 1.** Cholesterol and triglycerides levels in control and curcumin-treated groups. NS: non-significant difference between groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>TCH (mmol/l)</th>
<th>TG (mmol/l)</th>
</tr>
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<tbody>
<tr>
<td>CONTROL (n=10)</td>
<td>26.8±1.3</td>
<td>2.01±0.1</td>
</tr>
<tr>
<td>CURCUMIN-TREATED (n=10)</td>
<td>25.9±1.1 (NS)</td>
<td>1.8±0.1 (NS)</td>
</tr>
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</table>
factor NFκB (18). Interestingly, curcumin has been demonstrated to inhibit activity of NFκB in stimulated endothelial cells (19). However, whether inhibition of NFκB could be responsible for anti-atherogenic action of curcumin remains to be elucidated.

Another plausible mechanism responsible for anti-atherogenic action of curcumin may depend on the induction of heme oxygenase-1 (HO-1), a potent antioxidant and vasculo-protective enzyme (20,21). Induction of HO-1 has been claimed to inhibit development of atherosclerosis in ApoE deficient mice (22,23). Interestingly, it was shown that curcumin may induce HO-1 in endothelial cells in vitro (24). Thus, the possible involvement of HO-1 induction in anti-atherogenic action of curcumin in our model requires further investigation.

Natural, polyphenolic compounds present wide spectrum of biological activities (25-27) and may represent also promisive group of antiatherosclerotic compounds (28,29). For example, flavonoids protect LDL from oxidation (30), enhance endothelium-derived nitric oxide bioactivity (31-33), inhibit endothelial activation (34-36) and platelet oxide bioactivity (37). It may well be that all above mechanisms could be partly involved in anti-atherosclerotic action of curcumin in our model.

In summary, we demonstrated that oral treatment with curcumin decreases development of atherosclerosis in apoE/LDLR- DKO mice. To our knowledge, this is the first report that shows the effect of curcumin on atherogenesis in gene-targeted apoE/LDLR - double knockout mice.

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