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Rapid regression of atherosclerosis with MTP inhibitor treatment

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Abstract

Objective—Regression of atherosclerosis is a vital treatment goal of atherosclerotic vascular disease. Inhibitors of the microsomal triglyceride transfer protein (MTP) have been shown to reduce apolipoprotein B (apoB)-containing lipoproteins in animals and humans effectively. Therefore, the major aim of our study is to evaluate the effect of MTP inhibition on atherosclerotic plaque regression.

Methods—LDL-receptor-deficient (*LDLr*^{−/−}) mice were fed a Western diet for 16 weeks and then harvested for baseline (n=8), switched to chow diet (n=8) or chow diet containing MTP inhibitor (BMS 212122; n=8) for 2 weeks before harvesting.

Results—Treatment with MTP inhibitor led to rapid reduction in plasma lipid levels, which were accompanied by a significant decrease in lipid content and monocyte-derived (CD68+) cells in atherosclerotic plaques compared to baseline and chow diet control groups. MTP inhibitor-treated mice had increased collagen content, a marker associated with increased stability in human plaques. Furthermore, plaques of these mice showed a significant decrease in tissue factor and pro-inflammatory M1 macrophage marker monocyte chemoattractant protein-1 (MCP-1) and an increase in anti-inflammatory M2 macrophage markers arginase-I and mannose receptor 1.

Conclusion—Reversal of hyperlipidemia in atherosclerotic mice by inhibition of MTP leads to rapid and beneficial changes in the composition and inflammatory state of the plaque.

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Disclosures
None.

Keywords

Atherosclerosis; Regression; Hyperlipidemia; Microsomal triglyceride transfer protein

Introduction

Lowering of non-HDL-cholesterol (VLDL-C and LDL-C) is the most common approach to decrease coronary artery disease (CAD) risk. For example, various clinical trials have shown that lowering of LDL-C by statins reduces event rates in all age groups [1]. In the ASTEROID trial, aggressive LDL-C lowering with rosuvastatin therapy resulted in regression of coronary atherosclerosis assessed by intravascular ultrasound (IVUS) [2]. Statins increase the clearance of LDL-C. Another approach of lowering LDL-C is to reduce the production of its precursor, VLDL. Microsomal triglyceride transfer protein (MTP) is expressed in the liver, intestine and the heart and is required for the proper assembly of VLDL and chylomicrons [3]. In the Reversa mouse (*Ldlr*^{-/-}*Apob*^{100/100}*Mtp*^{fl/fl}*Mx1Cre*^{+/+}), reversal of hyperlipidemia by conditional inactivation of MTP led to regression of atherosclerosis accompanied by favorable changes in the composition and the inflammatory state of the atherosclerotic plaque [4,5]. MTP inhibitors have been shown to reduce apolipoprotein B (apoB)-containing lipoproteins in animals and humans effectively, and are undergoing clinical testing in select populations; but chronic treatment has been associated with hepatic fat accumulation, though this may lessen over time [3,6].

Long term treatment of *apoE*^{-/-} mice on a high cholesterol diet with MTP inhibitor, Implitapide, resulted in decreased progression of atherosclerosis [7]. In the present study, we used the MTP inhibitor, BMS 212122, on a short-term basis (2 weeks) to test its ability to induce regression of atherosclerosis in *LDLr*^{-/-} mice. As will be presented, mice treated with MTP inhibitor had a significant decrease in plaque macrophage and lipid content and an improved inflammatory status of the plaque compared to control mice.

Methods

Animals

All procedures were approved by the NYU Institutional Animal Use and Care Committee. *LDL*^{-/-} mice (B6.129S7-*LDL*^{tm1Her}/J; The Jackson Laboratories, Bar Harbor, USA) were weaned at 4 weeks and placed onto a Western diet (Dyets Inc., Bethlehem, USA, Dyet #101977) for 16 weeks. Mice were then harvested for baseline analysis (baseline group), switched to a chow diet (chow group; this group served as a control for the diet change that was also performed in the MTP inhibitor group) or to a chow diet containing MTP inhibitor (BMS 212122; concentration: 25 mg per kg chow diet) (MTPi group) for 14 days before harvesting (n=8 in each group). To achieve a maximal and rapid reduction in plasma lipids the MTP inhibitor treatment was combined with a diet switch to a normal chow diet in the present study, since in a previous study, feeding hyperlipidemic *apoE*^{-/-} mice with a western-type diet along with MTP inhibitor treatment maintained high plasma levels of cholesterol (~500 mg/dL) and failed to result in regression of atherosclerosis. In addition, we wished to simulate the clinical scenario, in which lipid lowering drugs are considered to be

an adjunct to life style changes, a major one being the adoption of a healthier diet. The mean body weight of the mice was 26.8 ± 1.3 g after 16 weeks of Western diet and did not differ significantly between all three groups.

Lipid and Lipoprotein Analysis

Plasma and hepatic total cholesterol, triglyceride and plasma high-density lipoprotein cholesterol (HDL-C) levels were determined by enzymatic assays (Wako Chemicals, Richmond, USA). Hepatic lipids were normalized to the hepatic protein content, which was measured with the DC Protein Assay Kit (Bio-Rad, Hercules, USA) according to the manufacturer's instructions.

Hepatic lipid content

Hepatic triglyceride and cholesterol content were determined as previously described [8]. Briefly, frozen livers were homogenized, and lipids were extracted. After evaporation of the final chloroform phase, the lipids were dissolved in isopropanol. Triglyceride and cholesterol concentrations were determined as described above.

Liver transaminases

Levels of liver transaminases (AST and ALT) were measured by ANTECH Diagnostics (Farmingdale, New York, USA).

Histology and Morphometrics

At harvesting, aortic roots were removed, perfused with 10% sucrose/saline and embedded in OCT. Serial sections were cut and stained for CD68 (AbD Serotec, Oxford, UK) as previously described [9]. Additional sections were stained with Oil Red O (Sigma), and for monocyte chemoattractant protein-1 (MCP-I antibody; BioLegend, San Diego, USA), arginase-I (arginase-I antibody; Santa Cruz Biotechnology, Santa Cruz, USA) mannose receptor 1 (CD206 antibody; AbD Serotec, Oxford, UK) and tissue factor. The tissue factor antibody (TF632) was generously provided by Dr. Mark B. Taubman (University of Rochester, USA) [10]. For arginase-I and MCP-I stainings, immunofluorescence microscopy was used. Collagen content of lesions was assessed with Sirius Red-stained sections under polarizing light [4]. Negative controls were performed for all stainings. Intimal lesions and stained areas were quantified by computer-aided morphometric analysis of digitized images (ImagePro Plus 3.0 software, Silver Spring, USA).

Grading of steatohepatitis was performed according to Brunt et al. [11] on paraffin embedded liver sections after hematoxylin and eosin (HE) staining.

Statistical Analysis

Data are expressed as mean $\pm$ SEM. Data were analyzed by one-way ANOVA with post hoc multiple comparison Tukey tests (Prism 5.0, GraphPad Software Inc., San Diego, USA). Additional comparisons between just the chow and MTPi groups were performed by t-test (Supplemental Fig. 1F), which did not change the general conclusions. For comparison of plasma total cholesterol and triglyceride levels between the chow and MTPi groups at each time-point the unpaired t-test was used; $p < 0.05$ was considered significant.

Results

The MTP inhibitor BMS 212122 was well tolerated by the mice; none of them developed signs of a severe steatohepatitis (see Supplemental Fig. 1).

The MTP inhibitor rapidly lowered total cholesterol levels by 83% (201.1 ± 53.8 mg/dl) at 1 week and by 94% (70.0 ± 11.8 mg/dl) at 2 weeks of treatment compared to the baseline group (1160.0 ± 87.0 mg/dl). The chow group, although exhibiting a significant decrease in total cholesterol compared to the baseline group, still had levels >250 mg/dl at 2 weeks, while the MTPi group achieved levels around 70 mg/dl (~ wild-type mouse levels) in the same period (Fig. 1A). TC/HDL ratio decreased in both diet-switched groups compared to baseline (26.3 ± 3.4), while the MTPi group had a ~50% decrease in the TC/HDL ratio compared to the chow group (4.3 ± 0.2 vs. 2.1 ± 0.2 , respectively) (Fig. 1B). Plasma triglyceride levels were significantly lower in the MTPi group compared to the baseline and chow group (Supplemental Fig. 1A). There was no significant change in absolute plaque size between all groups (Fig. 1C), but lipid content (i.e., Oil Red O staining) was significantly lower in the MTPi group ($27.8 \pm 1.9\%$ of plaque area) compared to the chow group ($35.9 \pm 1.9\%$ of plaque area, $P<0.05$) and the baseline group ($39.5 \pm 2.0\%$ of plaque area, $p<0.001$) (Fig. 1 D–E). The preservation of plaque size in the MTPi group could be explained by an $80 \pm 18\%$ increase in plaque collagen content compared to baseline ($p<0.01$); there was a $30 \pm 5\%$ ($p>0.05$) increase of plaque collagen in the chow diet group (Fig. 2A). The plaque macrophage (CD68+) content did not differ significantly between the baseline and the chow group ($39.4 \pm 1.5\%$ versus $34.7 \pm 2.0\%$ of plaque area; $p>0.05$). In contrast, MTPi-treated mice showed a statistically significant $32 \pm 5\%$ decrease in plaque macrophages compared to the baseline group ($p<0.001$) and $23 \pm 5\%$ decrease compared to the chow group ($p<0.05$) (Fig. 2B).

In addition to the changes in macrophage content, we were interested in evaluating the inflammatory state of the plaque. We evaluated the expression of tissue factor, which is induced by inflammatory factors [12], in the plaques. Expression of tissue factor was significantly lower in plaques of MTPi-treated mice ($21.4 \pm 2.1\%$ of plaque area) compared to the baseline group ($33.3 \pm 3.2\%$ of plaque area; $p<0.05$), while there was no significant difference between the chow ($30.4 \pm 2.3\%$ of plaque area) and baseline group ($p>0.05$) (Fig. 2C).

A simple classification system of tissue macrophages is M1 (pro-inflammatory) and M2 (anti-inflammatory) [13]. We stained for MCP-I (M1 marker), arginase-I (M2 marker) and mannose receptor 1 (M2 marker). We found a significant decrease in MCP-I protein in plaques of the MTPi group ($18.4 \pm 2.5\%$ of plaque area) compared to the baseline ($34.9 \pm 2.6\%$ of plaque area; $p<0.001$) and the chow ($28.0 \pm 2.3\%$ of plaque area; $p<0.05$) groups, but the chow data were not statistically different from baseline ($p>0.05$) (Fig. 2D). Arginase-I was significantly increased in the plaques of the MTPi group ($17.2 \pm 2.5\%$ of plaque area) compared to the baseline group ($8.1 \pm 1.4\%$ of plaque area; $p<0.05$). As with the MCP-I data, the increase in arginase-I in the chow group ($12.6 \pm 2.1\%$ of plaque area) did not reach statistical significance ($p>0.05$) (Fig. 2E). The expression of mannose receptor 1 was significantly higher in the MTPi group ($20.4 \pm 1.0\%$ of plaque area) compared to the

baseline ($14.6 \pm 0.5\%$ of plaque area; $p < 0.01$) and chow group ($13.6 \pm 1.7\%$ of plaque area; $p < 0.01$) (Fig. 2F).

Discussion

Inhibition of MTP has previously been shown to effectively lower the level of apoB-containing lipids in the plasma of animals and humans [3]. The MTP inhibitor (BMS 212122) used in the present study was previously evaluated in Golden Syrian hamsters and cynomolgus monkeys, where it led to a dose-dependent reduction of non-HDL-C plasma levels by over 80% although atherosclerosis was not evaluated [14]. To date MTP inhibition has only been shown to decrease progression of atherosclerosis with long-term treatment in *apoE*^{-/-} mice [7].

In the present study we show for the first time that reversal of hyperlipidemia by treatment with a MTP inhibitor leads to the regression of atherosclerosis as judged by lipid and macrophage contents of the plaques. This was undoubtedly related to the severe reduction in non-HDL-C plasma levels (~ 38 mg/dl; wild-type mouse level); In contrast, the non-HDL-C levels in the chow-fed group were ~ 211 mg/dl, explaining the more mild changes we observed in these mice. Two other notable findings of the regression process induced by MTP inhibitor treatment were changes in plaque composition (more collagen, which in human plaques is considered to be stabilizing) and in the inflammatory state, with evidence that the phenotype of the remaining macrophages resembled that of the M2 state. These results are in accordance with our previous studies in which the plasma lipoprotein profile was modified in a similar way in Reversa mice (i.e., non-HDL-C reduction) [4] and in *LDLr*^{-/-} mice treated with the LDL receptor by adenovirus [15], or in a different way (selective raising of HDL-C or lowering of non-HDL-C and raising of HDL-C) [16], arguing that the M2 state may be a general characteristic of regressing plaques.

The reduction in plaque size did not follow the decreases in lipid and macrophage contents, but there was a significant increase in collagen [4,17]. We hypothesize that the increase of extracellular matrix counterbalances the decrease in plaque lipids and macrophages. This would be consistent with previous studies, including those in Reversa mice, that have shown an increase in plaque collagen after correction of hyperlipidemia [4,15]. The net content of collagen depends on the balance of its synthesis and degradation. We have previously shown in Reversa mice that after reversal of hyperlipidemia, macrophages become capable of producing collagen, a known property of the M2 phenotypic state [17], thought to be the result of the increase in arginase-I activity, which provides metabolic substrates for collagen synthesis [18]. Similarly, we observed in the present study that in MTP inhibitor treated mice, there was enrichment in plaque macrophage arginase-I. In addition, we have previously reported that the expression of matrix metalloproteinases-2 and -9 mRNA is reduced in laser captured plaque CD68⁺ cells (macrophages) of Reversa mice after reversal of hyperlipidemia, indicating a decreased degradation of collagen [4,17]. Thus, the increase in collagen content we observed may represent either increased synthesis, decreased degradation, or a combination of both after plasma lipid levels were lowered by inhibiting MTP. It is unlikely to reflect the direct effects of inhibiting MTP in the cells in the plaque,

given that we have previously shown that the MTP expressing status of plaque cells did not affect the regression process after lipid lowering [4].

Lowering of non-HDL-C plays a central role in the prevention of cardiovascular disease. Several studies using intravascular ultrasound have demonstrated a strong linear relationship between the degree of LDL-C lowering and the change in atheroma volume [19]. Therefore, the current consensus is that aggressive LDL-C lowering will promote a limited amount of clinical regression if the decrease is chronically maintained below 60 mg/dl [2]. This rationale is supported by our results that demonstrated that an aggressive non-HDL-C lowering is followed by tremendously beneficial changes in the plaque composition. What is particularly attractive about the model we have presented is the ease of simulating aggressive statin trials in man - except for starting with a readily available mouse model: the *LDLr*^{-/-} mouse - no other genetic or technically challenging manipulations are needed to achieve significant quantitative and qualitative changes in plaques over a relatively short time.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

A) Total plasma cholesterol levels, B) plasma TC/HDL ratios, and C-E) aortic root plaque sizes and Oil Red O staining (ORO) of plaques from *LDLr*^{-/-} mice before (Baseline) and after switch to either a chow diet (Chow) or a chow diet containing MTP inhibitor (MTPi) for 2 weeks (magnification: 10×). Values are mean ± SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n = 8$ in each group.

Fig. 2.

Plaques of aortic root sections stained for A) collagen (Sirius Red), B) macrophages (CD68+), C) tissue factor (TF), D) monocyte chemoattractant protein-1 (MCP-I), E) arginase-I and F) mannose receptor 1 (MR) from *LDLr*^{-/-} mice before (Baseline) and after switch to either a chow diet (Chow) or a chow diet containing MTP inhibitor (MTPi) for 2 weeks (magnification: 10×; arginase-I: 20×). Values are mean ± SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n = 7-8$ mice in each group.