T-0901317, a synthetic liver X receptor ligand, inhibits development of atherosclerosis in LDL receptor-deficient mice

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Abstract Liver X receptors (LXRα and LXRβ) are nuclear receptors, which are important regulators of cholesterol and lipid metabolism. LXRs control genes involved in cholesterol efflux in macrophages, bile acid synthesis in liver and intestinal cholesterol absorption. LXRs also regulate genes participating in lipogenesis. To determine whether the activation of LXR promotes or inhibits development of atherosclerosis, T-0901317, a synthetic LXR ligand, was administered to low density lipoprotein receptor (LDLR)$^{-/-}$ mice. T-0901317 significantly reduced the atherosclerotic lesions in LDLR$^{-/-}$ mice without affecting plasma total cholesterol levels. This anti-atherogenic effect correlated with the plasma concentration of T-0901317, but not with high density lipoprotein cholesterol, which was increased by T-0901317. In addition, we observed that T-0901317 increased expression of ATP binding cassette A1 in the lesions in LDLR$^{-/-}$ mice as well as in mouse peritoneal macrophages. T-0901317 also significantly induced cholesterol efflux activity in peritoneal macrophages. These results suggest that LXR ligands may be useful therapeutic agents for the treatment of atherosclerosis.

Key words: Liver X receptor; Nuclear receptor; ATP binding cassette A1; Cholesterol efflux; Macrophage; Atherosclerosis

1. Introduction

Liver X receptors (LXRs), LXRα and LXRβ, are members of the nuclear receptor superfamily and are involved in regulation of cholesterol and lipid metabolism. LXRs bind to DNA as obligate heterodimers with retinoid X receptors and are activated by oxysterols [1,2].

In the liver, LXRs contribute to regulation of the gene encoding cholesterol 7α-hydroxylase, the rate-limiting enzyme in conversion of cholesterol to bile acids [3]. In intestine, LXRs increase expression of ATP binding cassette (ABC) A1 and ABCG1, which are involved in intestinal cholesterol absorption [4–6]. ABCA1 also plays an important role in apolipoprotein (apo) A-I-dependent cholesterol efflux in macrophages [7]. The importance of ABCA1 and reverse cholesterol transport is highlighted by the linking of Tangier disease, a genetic disorder of high density lipoprotein (HDL) deficiency, with a mutation in the gene encoding ABCA1 [8–10]. Patients with Tangier disease exhibit reduction in plasma HDL levels and massive accumulation of cholesterol in the peripheral tissues, and are at increased risk for atherosclerosis [11–14].

Recent studies have also revealed that LXRs are involved in the regulation of triglyceride metabolism [15,16]. It has been reported that a synthetic LXR ligand increased expression of genes participating in lipogenesis such as sterol response element binding protein 1c (SREBP-1c), fatty acid synthetase (FAS), stearoyl CoA desaturase 1 (SCD-1) and lipoprotein lipase and induced hypertriglyceridemia in mice [17,18].

These findings raised the question of whether the activation of LXRs promotes or inhibits atherosclerosis. In the present study, we administered T-0901317, a synthetic LXR ligand, to atherogenic diet fed low density lipoprotein receptor (LDLR)$^{-/-}$ mice, an animal model of progressive atherosclerosis. The effect of T-0901317 on lipid profiles, extent of atherosclerosis, and gene expression was investigated.

2. Materials and methods

2.1. Animals and diet

Male LDLR$^{-/-}$ and C57BL/6 mice were obtained from Crea Japan, Inc. The LDLR$^{-/-}$ mice were fed an atherogenic diet (1.25% cholesterol, 7.5% cocoa butter and 0.5% sodium cholate). The LXR ligand T-0901317 at doses of 3 and 10 mg/kg was orally administered daily in propylene glycol/Tween 80 (4/1) formulation to LDLR$^{-/-}$ mice for 8 weeks. Blood was drawn and total cholesterol (TC) and triglyceride (TG) levels were determined. At week 8, the mice were sacrificed, blood was obtained and tissues were collected for further analysis.

2.2. Isolation of mouse peritoneal macrophages

Thioglycolate-elicited peritoneal macrophages were isolated from the C57BL/6 mice 4 days after peritoneal injection of thioglycolate broth medium [19].

2.3. Lipoproteins and their modification

Human LDL (d = 1.019–1.063) and HDL_3 (d = 1.125–1.21) were

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Abbreviations: LXR, liver X receptor; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; ABC, ATP binding cassette; apoA-I, apolipoprotein A-I; TC, total cholesterol; TG, triglycerides; HPLC, high performance liquid chromatography; SREBP-1c, sterol response element binding protein 1c; FAS, fatty acid synthetase; SCD-1, stearoyl CoA desaturase 1

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isolated by sequential ultracentrifugation from the plasma obtained after overnight fasting of normolipidemic subjects who had given their consent. Acetyl-LDL was prepared by chemical modification of LDL with acetic anhydride [20].

2.4. RNA isolation and analysis of gene expression by real-time quantitative PCR

Total RNA from the mouse peritoneal macrophages was isolated using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. To generate cDNA, 1 μg of total RNA was used with the oligo dT primer following the protocol for First Strand cDNA Synthesis kit (Amersham Bioscience). Real-time quantitative PCR analysis was performed using the ABI Prism 7700 system (Applied Biosystems). The level of the ABCA1 mRNA expression was normalized to cyclophilin mRNA expression level. The sequences of forward primers, reverse primers and TaqMan probes, respectively, were as follows: ABCA1: GCTCTCAGGTGGGATGCAG, GGCTCGTCCAGAATGACAAC, FAM-CTTGGCCTTCGTGGGTGGATCC-TAMRA; cyclophilin: CGATGACGAGCCCTTGG, TCTGCTGTCTTTGGAACTTTGTC, FAM-CGCGTCTCCTTTGAGCTGTTTGCA-TAMRA.

2.5. Cholesterol efflux

Mouse peritoneal macrophages were labeled with 0.2 μCi/ml [^{14}C]cholesterol and 50 μg/ml acetyl-LDL for 48 h. Labeled cells were washed, and efflux was initiated in medium with or without 200 μg/ml HDL in the absence or presence of T-0901317. Radioactivity of [^{14}C]cholesterol in medium was monitored for 48 h.

2.6. Lipid and lipoprotein analyses

Plasma TC and TG levels were determined using commercial kits (Wako Pure Chemical Industries, Ltd.). Lipoprotein profiles were analyzed with high performance liquid chromatography (HPLC) [21].

2.7. Preparation of histological sections and analysis of atherosclerosis

Preparation of histological sections from the aortic root was essentially according to a technique as described by Paigen et al. [22] and modified by Calleja et al. [23]. The heart was fixed in periodate lysine paraformaldehyde fixative at 4°C for 24 h and paraffin-embedded. The atherosclerotic lesion area of Elastica Masson-stained sections was determined with image analysis software (Qwin, Leica AG). The results are represented as the average of four sections each separated by 100 μm.

Macrophages were detected with monoclonal rat anti-mouse antibody MOMA-2 (Biosource International). Tissue sections were incubated in Tris-buffered saline containing 0.1% trypsin and 0.1% $CaCl_2$ for 30 min at room temperature to retrieve the antigenicity. Endogenous peroxidase activity was quenched by incubation with 3% H_2O_2. The sections were incubated with MOMA-2 diluted 1:10 at 4°C overnight. Then, the sections were incubated with biotinylated rabbit anti-rat IgG (1:500, Dako Japan Co., Ltd.) for 30 min at room temperature. After horseradish peroxidase-conjugated streptavidin (Nichirei Corporation) was applied to the sections, antibody binding was visualized with diaminobenzidine (Dako Japan Co., Ltd.). The sections were counter-stained with methyl green.

Expression of ABCA1 was determined by polyclonal anti-ABCA1 antibody (Novus Biologicals, Inc.). Tissue sections were incubated with anti-ABCA1 antibody diluted 1:500 at 4°C overnight. Then the sections were incubated with biotinylated donkey anti-rabbit IgG (1:500, Amersham Bioscience) for 30 min at room temperature. Immunoactivities were detected as well as MOMA-2.

2.8. Determination of plasma concentration of T-0901317

Plasma concentrations of T-0901317 were determined using the HPLC system (LC10 System, Shimazu Corporation) with a Luna 5μ C18 column (Phenomenex).

2.9. Statistical analysis

Values are represented as mean ± S.E.M. Statistical significance from the control was assessed using Dunnett's multiple comparison test and analysis of variance (ANOVA). A difference was considered to be statistically significant when the *P*-value was less than 0.05.

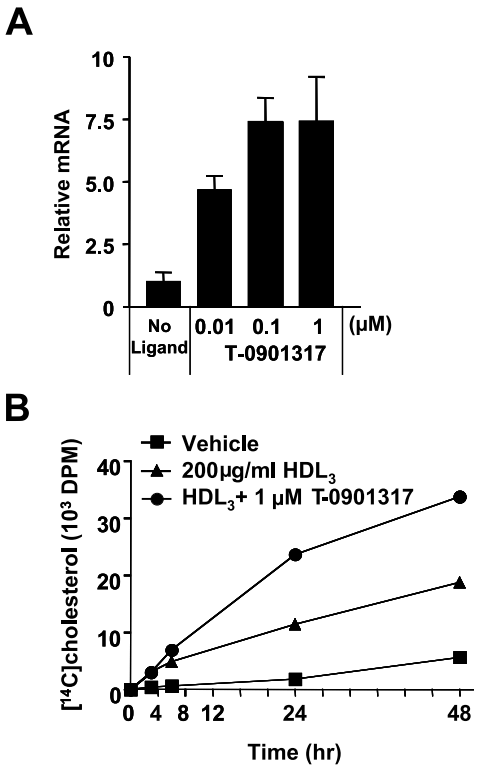

Fig. 1. Induction of ABCA1 mRNA expression and cholesterol efflux in mouse peritoneal macrophages by T-0901317. A: ABCA1 mRNA assay. Mouse peritoneal macrophages were treated for 24 h in the absence or presence of 0.01, 0.1, or 1 μM of the LXR ligand T-0901317, and total RNA was isolated. ABCA1 mRNA expression levels were determined by real-time quantitative PCR and normalized to cyclophilin. Data are presented as mRNA expression relative to the vehicle control. B: Cholesterol efflux assay. Mouse peritoneal macrophages were incubated with acetyl-LDL for 48 h. Labeled cells were washed, and efflux was initiated in medium with or without 200 μg/ml HDL in the absence or presence of T-0901317. Radioactivity of [^{14}C]cholesterol in the medium was monitored for 48 h.

3. Results

3.1. Expression of ABCA1 mRNA and cholesterol efflux

Treatment of mouse peritoneal macrophages with T-0901317 resulted in a dramatic increase in ABCA1 mRNA expression (Fig. 1). T-0901317 was significantly more effective than HDL_3 in inducing cholesterol efflux. ApoA-I-dependent cholesterol efflux was also induced by T-0901317 (data not shown).

3.2. Plasma lipid and lipoprotein profiles

$LDLR^{-/-}$ mice were orally administered T-0901317 at doses of 3 and 10 mg/kg for 8 weeks. The atherogenic diet elevated plasma TC levels from approximately 300 to 2400 mg/dl and decreased HDL-C levels from approximately 100 to 30 mg/dl in the control group at week 1 (Table 1).

Treatment with T-0901317 had no influence on plasma TC levels. T-0901317 significantly increased chylomicron-cholesterol levels at weeks 1 and 5, but these completely recovered to the same levels as the control group by week 8. However, treatment with T-0901317 at 10 mg/kg significantly decreased very low density lipoprotein (VLDL)+LDL-C (exposure: 20 943.1–17 524.7 mg/week/dl). On the other hand, HDL-C

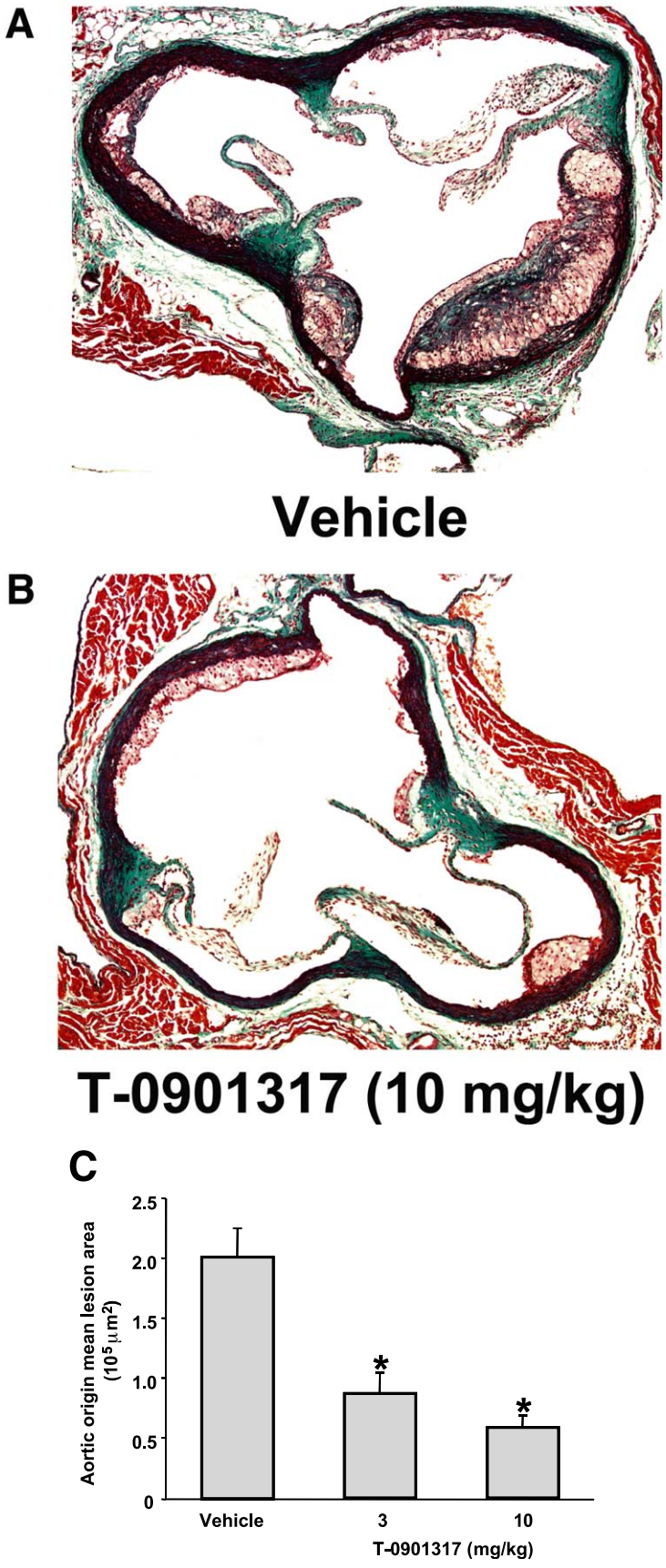

Fig. 2. Inhibitory effect of T-0901317 on the development of atherosclerotic lesions in LDLR$^{-/-}$ mice. A,B: Representative Elastica Masson staining of aortic root lesions from atherogenic diet-fed LDLR$^{-/-}$ mice after administration of the LXR ligand T-0901317 for 8 weeks. C: Quantitative analysis of atherosclerosis lesion areas. Mean ± S.E.M. were: control, 201615 ± 23346 μm^2 ($n = 9$); T-0901317 3 mg/kg, 86899 ± 17271 μm^2 ($n = 8$); T-0901317 10 mg/kg, 58667 ± 9835 μm^2 ($n = 8$). *$P < 0.05$, as compared with the control group using Dunnett's multiple comparison test.

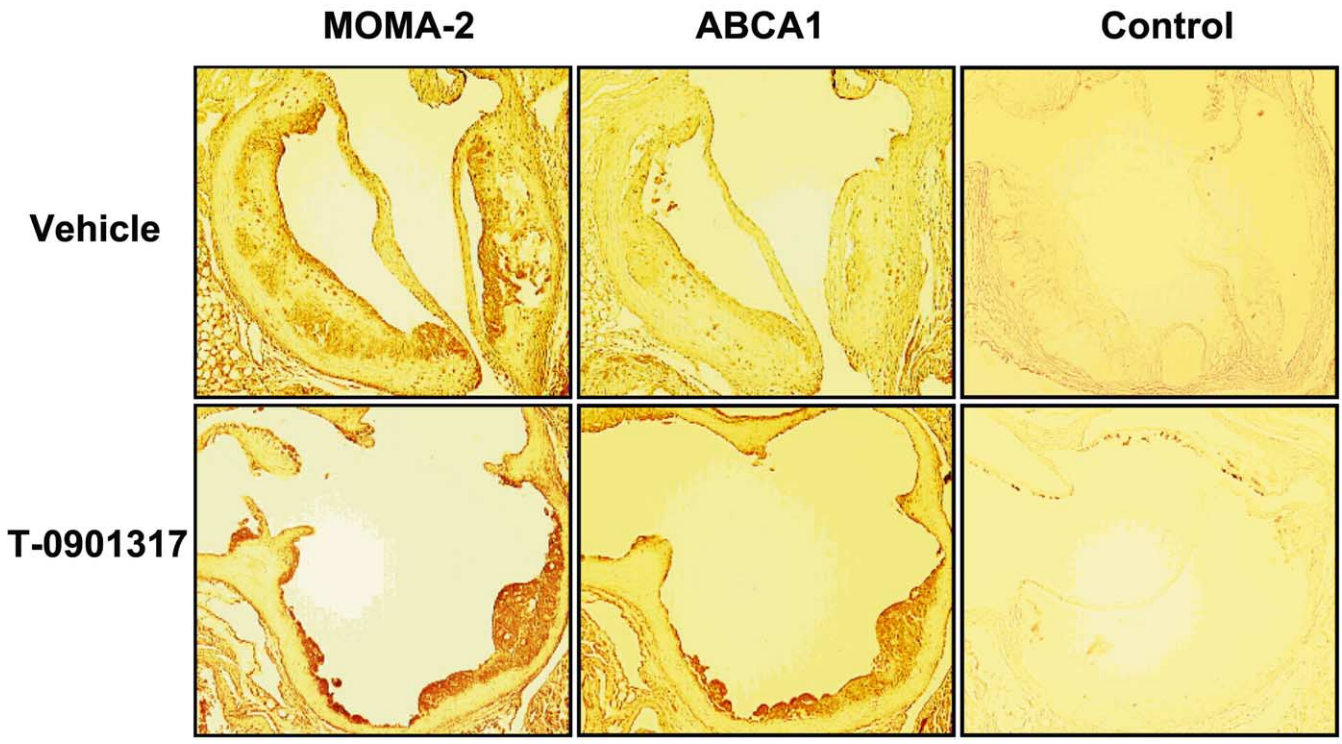

Fig. 3. Expression of ABCA1 protein within macrophages in atherosclerotic lesions from LDLR$^{-/-}$ mice. A,B: Representative immunostaining of aortic root lesions from atherogenic diet-fed LDLR$^{-/-}$ mice with anti-ABCA1 and anti-MOMA-2 (macrophage) after administration of the LXR ligand T-0901317 for 8 weeks.

levels were increased by T-0901317 in a dose-dependent manner (exposure: 301.3, 543.0 and 667.9 mg/week/dl for control, 3 and 10 mg/kg, respectively). TG elevation by the treatment with T-0901317 was observed at week 1, but gradually declined during the administration period and completely recovered to the same level as the control group by week 8.

3.3. Analysis of atherosclerosis lesions

The atherosclerotic lesions were dominantly composed of foamed macrophages. Treatment with T-0901317 at 3 and 10 mg/kg dramatically inhibited the development of atherosclerosis compared with the control group (57 and 71% reduction, respectively; Fig. 2). We also analyzed expression of ABCA1 protein in the lesions using anti-ABCA1 antibody (Fig. 3). The immunostaining of aortic root sections treated with T-0901317 revealed that significant expression of ABCA1 protein was co-localized with the macrophages. Plasma concentrations of T-0901317 were measured by use of HPLC. There was a negative correlation between mean lesion area and plasma concentration of T-0901317, but no correlation between mean lesion area and HDL-C level (Fig. 4).

Table 1
Effect of T-0901317 on plasma lipid and lipoprotein levels in LDL receptor-deficient mice

	TC (mg/dl)	Chylomicron-C (mg/dl)	VLDL+LDL-C (mg/dl)	HDL-C (mg/dl)	Triglycerides (mg/dl)
Vehicle ($n=9$)					
Week 0	276.5 ± 7.2	0.2 ± 0.2	170.1 ± 6.4	106.2 ± 1.8	195.0 ± 11.5
Week 1	2 419.9 ± 143.4	88.2 ± 19.1	2 300.1 ± 134.8	31.6 ± 3.0	300.2 ± 35.7
Week 5	3 741.0 ± 241.8	225.3 ± 49.6	3 477.1 ± 214.0	38.7 ± 6.9	404.1 ± 80.2
Week 8	1 981.2 ± 60.3	0.0 ± 0.0	1 958.6 ± 60.1	22.6 ± 1.8	95.8 ± 4.4
Exposure	22 253.4 ± 887.0	1 009.2 ± 213.1	20 943.1 ± 784.7	301.3 ± 30.3	2 405.9 ± 334.9
T-0901317 3 mg/kg ($n=8$)					
Week 0	273.6 ± 7.5	5.3 ± 5.3	172.0 ± 10.4	96.3 ± 3.5	208.0 ± 7.0
Week 1	2 911.4 ± 115.9	541.1 ± 69.7*	2 290.8 ± 92.5	79.5 ± 4.9*	2 811.4 ± 460.6*
Week 5	3 793.3 ± 194.1	463.7 ± 66.9*	3 263.0 ± 137.6	66.6 ± 5.6*	749.3 ± 135.5
Week 8	1 882.5 ± 94.7	0.9 ± 0.9	1 840.4 ± 92.7	42.1 ± 3.3*	140.3 ± 27.6
Exposure	23 515.5 ± 855.1	2 979.7 ± 294.4*	19 993.9 ± 642.8	543.0 ± 27.4*	9 965.3 ± 1 330.8*
T-0901317 10 mg/kg ($n=8$)					
Week 0	277.8 ± 8.0	0.0 ± 0.0	177.5 ± 6.6	100.2 ± 3.3	199.7 ± 12.4
Week 1	2 934.9 ± 363.6	754.9 ± 112.2*	2 090.1 ± 250.8	89.9 ± 12.2*	6 347.4 ± 919.8*
Week 5	3 428.9 ± 242.4	502.0 ± 97.4*	2 843.7 ± 144.2	83.3 ± 10.7*	1 077.1 ± 160.1*
Week 8	1 573.0 ± 82.1	0.0 ± 0.0	1 505.3 ± 82.3*	67.7 ± 5.6*	137.7 ± 14.1
Exposure	21 836.8 ± 1 273.6	3 644.2 ± 424.1*	17 524.7 ± 849.8*	667.9 ± 46.6*	19 944.7 ± 1 976.8*

Data are represented as mean ± S.E.M. ($n=8$–9). The data are also expressed as 'exposure' during the 8-week treatment phase. *$P<0.05$, as compared with the vehicle group using ANOVA and Dunnett's multiple comparison test.

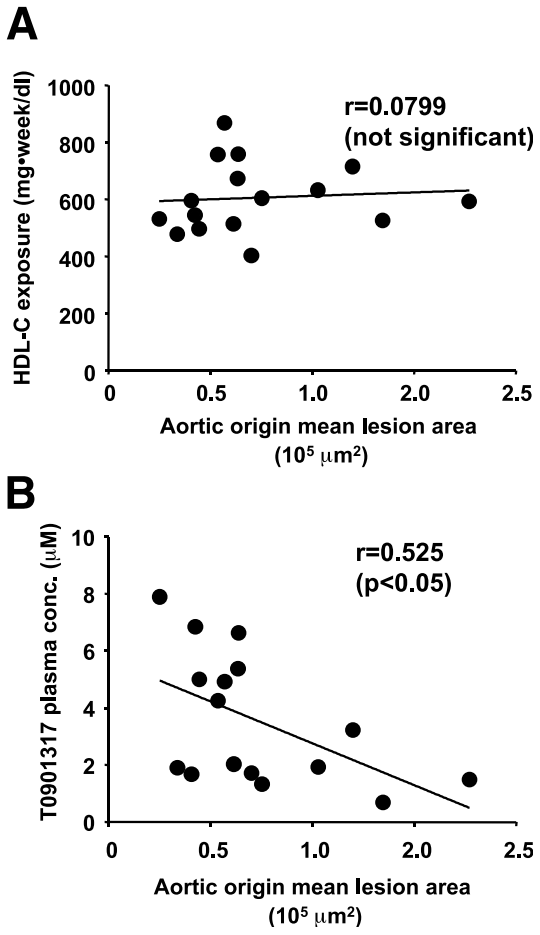

Fig. 4. Negative correlation between lesion area and plasma concentration of T-0901317. A: No significant correlation between lesion area and HDL-C. B: Significant negative correlation between lesion area and plasma concentration of T-0901317.

4. Discussion

Recent studies have identified LXRs as important regulators of cholesterol and lipid metabolism. It has been reported that LXR ligands increase expression of ABCA1 and induce apoA-I-dependent cholesterol efflux [7]. However, LXR ligands have also been shown to induce expression of genes involved in lipogenesis and elevate plasma TG levels [15,16]. These findings raised the question of whether the activation of LXRs promotes or inhibits atherosclerosis. In the present study, we demonstrated that T-0901317, a synthetic LXR ligand, dramatically inhibited the development of atherosclerosis in LDLR$^{-/-}$ mice fed an atherogenic diet.

We also observed that T-0901317 significantly increased HDL-C, although it did not change plasma TC. Another study indicated that the liver is a major site of HDL assembly [24]. Recent reports identifying mutations on the ABCA1 gene in patients with Tangier disease raised the possibility that ABCA1 participates in the synthesis of HDL [4–6]. However, T-0901317 did not change ABCA1 mRNA expression in liver (data not shown). This observation implies that other gene(s) regulated by LXRs in liver may participate in HDL increase. T-0901317 also increased plasma TG with the major lipoprotein peaks being VLDL and chylomicron, which is a TG-rich lipoprotein secreted from intestine. Although other studies investigated the effect of LXR agonists only in liver [15,16], our finding suggests that the action of an LXR agonist in intestine also contributed to TG elevation. However, TG elevation gradually declined during the administration period and completely recovered to the same level as the control group. SREBP-1c, FAS and SCD-1 mRNA levels in liver were hardly changed at week 8 (data not shown). Continuous increase in liver TG content might induce other regulatory pathway(s) for TG synthesis and metabolism, and as a result repress gene expression via LXRs.

The alteration of lipid profiles raised the question of whether T-0901317 affected macrophages in atherosclerotic lesions directly or indirectly. Interestingly, multiple regression analysis demonstrated that the extent of atherosclerosis negatively correlated with the plasma concentration of T-0901317, but not with that of HDL-C. Thus, the alteration of lipid profiles may minimally contribute to the anti-atherogenic effect. T-0901317 also induced expression of ABCA1 protein in the lesions, which co-localized with macrophages. In addition, T-0901317 significantly increased ABCA1 mRNA and enhanced HDL_3-dependent cholesterol efflux in vitro. These results strongly suggest that T-0901317 directly affected macrophages, enhanced cholesterol efflux via up-regulation of ABCA1 and resulted in significant prevention of atherosclerosis.

Cholesterol accumulation in macrophages is thought to be mediated primarily by uptake of modified LDL via scavenger receptors, scavenger receptor class A and CD36 being key players [25,26]. Unlike LDL receptors, scavenger receptors are not subject to negative regulation by cellular cholesterol. Thus, maintenance of cholesterol homeostasis under conditions of constitutive uptake of modified lipoproteins requires activation of the cholesterol efflux pathway. Other studies show that oxysterols, which exist abundantly in macrophages within atherosclerotic lesions, are the physiological ligands for LXRs [1,27,28]. In this study, we proved that an LXR agonist has a potent anti-atherogenic effect, showing direct induction of cholesterol efflux in macrophages. Recently Tangirala et al. [19] also demonstrated that LXRs in macrophages play a protective role in the development of atherosclerosis in a bone marrow transplantation study using LXR-deficient mice. These observations strongly support the idea that LXR ligands could be promising as therapeutic agents for the treatment of atherosclerosis.

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