Original Article

Cannabinoid 1 Receptor Blockade Reduces Atherosclerosis with Enhances Reverse Cholesterol Transport

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Aim: A recent clinical study using coronary intravascular ultrasound showed that rimonabant, a cannabinoid 1 (CB1) receptor antagonist, significantly reduced total atheroma volume, suggesting that CB1 receptor blockade could be beneficial in anti-atherogenic therapy. The reverse cholesterol transport (RCT) system plays important roles in atherogenesis. We investigated whether CB1 receptor blockade could modulate atherogenesis in mice.

Methods and Results: Oral administration of rimonabant (8 mg/kg/day) to apolipoprotein E-deficient mice for 3 months significantly reduced the relative area of atherosclerotic lesions in the aorta (vehicle: 12.6 ± 4.0% vs. rimonabant: 9.7 ± 2.3, n = 12 each, p < 0.05) with an increase in serum adiponectin levels (15.6 ± 2.3 μg/mL vs. 12.2 ± 2.1, n = 12 each, p < 0.001), without affecting body weight or serum cholesterol levels. Rimonabant tended to increase serum high-density lipoprotein cholesterol (HDL-C) (p = 0.05). The relative area of atherosclerotic lesions in the aorta correlated inversely with serum HDL-C levels (r = -0.45, n = 24, p < 0.05). Rimonabant upregulated the mRNA expression levels of various components of the RCT system on THP-1 cell-derived macrophages (scavenger receptor B1: 1.15 ± 0.12 fold, n = 6; p < 0.05, ATP-binding cassette [ABC] transporter G1: 1.23 ± 0.11 fold, n = 6; p < 0.01), but not ABCA1 (1.13 ± 0.20 fold, n = 6; p = 0.13).

Conclusion: CB1 receptor blockade reduced atherosclerosis in apoE-deficient mice through an increase in serum adiponectin levels and activation of the RCT system. CB1 receptor blockade may be therapeutically beneficial for atherogenesis by increasing the serum adiponectin level and enhancing of the RCT system.


Key words; Atherosclerosis, Obesity, Cannabinoid 1 receptor, Macrophages

Introduction

Overweight and obesity are quickly reaching pandemic levels¹. In particular, abdominal obesity is associated with increased cardiovascular risk factors, elevated blood pressure, increased triglycerides, fasting glucose, and decreased high-density lipoprotein cholesterol (HDL-C)². The endocannabinoid (EC) system has been implicated in the regulation of energy balance and food intake and has emerged recently as a therapeutic target for the modulation of multiple cardiometabolic risk factors related to obesity³.

Rimonabant was the first selective cannabinoid 1 (CB1) receptor antagonist used clinically. Treatment with this drug produced significant improvements in waist circumference, HDL-C, triglycerides, and insulin resistance, and the prevalence of metabolic syndrome⁴. A recent clinical study using intravascular coronary ultrasonography showed that CB1 receptor blockade significantly reduced total atheroma volume in coronary arteries, suggesting that CB1 receptor...
blockade may be potentially beneficial against atherosclerosis\(^5\).

The reverse cholesterol transport (RCT) is one of the major protective systems against atherosclerosis\(^6\),\(^7\). Recently, ATP-binding cassette (ABC) transporter G1 was found to induce an alternative cholesterol efflux pathway from macrophages and prevent cellular lipid accumulation\(^8\),\(^9\). Scavenger receptor B1 (SRB1) is also expressed on macrophages and can promote HDL-C-mediated cholesterol efflux\(^10\). The concept that promotion of macrophage RCT could prevent the progression or even induce regression of atherosclerosis is truly attractive\(^11\).

Both vascular inflammation and the RCT system play important roles in atherogenesis\(^11\),\(^12\). Inflammation may attenuate RCT related to human inflammatory pathophysiologies in chronic inflammatory states, including obesity, metabolic syndrome, and type 2 diabetes\(^13\). We have already demonstrated activation of the EC system in patients with coronary artery disease and the presence of high expression levels of CB1 receptor in coronary atheroma, as well as anti-inflammatory effects of CB1 receptor blockade on macrophages\(^14\). However, the correlation between the EC system and RCT system is still unknown. The aim of this study was to investigate whether CB1 receptor blockade could reduce atheroma in mice and whether any such effect is dependent on activation of the RCT system.

**Methods**

**Reagents**

Rimonabant was kindly provided by Sanofi-Aventis R&D (Chilly-Mazarin, France). Phorbol 12-myristate 13-acetate (PMA) was acquired from Sigma Aldrich Co. (St. Louis, MO). Recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) were purchased from R&D Systems, Inc. (Minneapolis, MN).

**Protocol**

Four-week-old apolipoprotein E (apoE)-deficient mice (C57BL/6.KOR-Apoeshl) were purchased from SLC (Shizuoka, Japan). Baseline data uniformity of these mice has been commercially established and confirmed. These mice were fed a high cholesterol diet (F2HFD1; ORIENTAL YEAST Co., Tokyo, Japan)\(^15\). All animal procedures were approved by the Animal Research Committee at Kumamoto University, and all procedures conformed to the Guide for the Care and Use of Laboratory Animals by the Institute of Laboratory Animal Resources. For mice in the rimonabant group, rimonabant was mixed with baked high cholesterol chow at a final concentration of 0.065\(\%\)\(^16\) and provided every day for 3 months. According to the Food and Drug Administration-recommended formula, this animal dose of 8 mg/kg/day is equal to a human dose of 0.33 mg/kg/day\(^17\). Twenty-four mice (12 controls and 12 receiving 8 mg/kg/day) were used for \textit{in vivo} evaluation. Blood samples were collected at 3 months. Serum cholesterol and triglyceride levels were determined by Liposearch (Skylight Biotech, Akita, Japan)\(^18\). Serum adiponectin levels were measured by the enzyme linked immunosorbent assay kit (Otsuka Co., Tokyo, Japan).

**In vivo Analysis of Anti-Atherosclerotic Effects of Rimonabant**

For analysis of atherosclerotic lesions, mice were fed a high-cholesterol diet mixed with or without rimonabant for 3 months and then sacrificed immediately after collection of blood samples. Whole aortas were collected and stained with Sudan IV. The luminal sides of the stained aortas were photographed. The extent of atherosclerosis was expressed as a percent of the lesion area extending from the ascending aorta to the abdominal bifurcation. Images were captured and analyzed using computer-assisted image analysis software (Lumina Vision, Mitani Co., Fukui, Japan).

**Cell Culture**

The human monocytic cell line, THP-1, was purchased from the American Type Culture Collection (Manassas, VA). Baseline data uniformity of these cells has been commercially established and confirmed. These cells were cultured in RPMI-1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10\% heat-inactivated fetal bovine serum (FBS), 10 ng/mL GM-CSF, 10 ng/mL M-CSF and 1.6 nmol/L phorbol 12-myristate 13-acetate (PMA) for differentiation into macrophages as described previously\(^14\),\(^19\). These cells were then used as “THP-1 cell-derived macrophages” after 4-day culture.

**Quantitative Real-Time RT-PCR**

For real-time RT-PCR, the primers and the TaqMan probe set for human 18S ribosomal RNA (Hs99999901), SRB1 (Hs00969818), ABCA1 (Hs01059122) and ABCG1 (Hs01555189) were purchased from Assays-on-Demand Gene Expression Products (Applied Biosystems, Foster City, CA). Real-time RT-PCR was carried out using a TaqMan Universal Master Mix kit with an ABI Prism 7900 sequence detection system (Applied Biosystems).
Western Blotting

Aliquots with equal protein contents (10 μg) from cultured macrophages were separated by standard electrophoresis and transferred onto membranes. These membranes were blocked using SuperBlock Blocking Buffer (Thermo Scientific, Rockford, IL). The membranes were incubated overnight with the primary antibodies (dilution 1:2000 anti-ABCG1 antibody and anti-SRB1; Epitomics Inc., Burlingame, CA) at 4°C, followed by incubation with the secondary antibody (GE Healthcare, Buckinghamshire, UK) for 1 hr at room temperature. Blots were incubated in chemiluminescence reagent and visualized by exposure to X-ray film. For evaluation of the total protein in each lane, all membranes were stripped and stained with anti-tubulin antibody (CALBIOCHEM, San Diego, CA).

Statistical Analysis

Data are presented as the mean ± SD of the indicated number of samples. Differences between two groups were analyzed using the unpaired Student’s t-test. Correlation between HDL-C levels and area of atherosclerotic lesion was examined by simple regression analysis. A p-value of < 0.05 was considered significant. All analyses were carried out using StatView-5.0 software (Tokyo, Japan).

Results

Rimonabant Increases Serum Adiponectin Levels in ApoE-Deficient Mice, but has no Effect on Body Weight and Serum Lipid Levels

As shown in Fig. 1A, the mean body weight gain did not change during the administration of rimonabant at 8 mg/kg/day for 3 months compared with the control. In our preliminary experiments, this dose of rimonabant significantly reduced the body weight of obse mouse strain (data not shown). While there were no significant differences in serum levels of triglyceride, LDL-C and total cholesterol between the rimonabant and control groups, HDL-C levels tended to increase after oral administration of rimonabant (control group, 36.4 ± 4.8 mg/dL, rimonabant group, 41.6 ± 7.3, n = 12 each, p = 0.05, Fig. 1B). This was associated with a decrease in the LDL-C/HDL-C ratio in the rimonabant group compared with the control group (control, 9.07 ± 0.69; rimonabant, 8.62 ± 0.73, n = 12 each, p = 0.13). In addition, rimonabant significantly increased serum levels of adiponectin (control, 12.2 ± 2.1 μg/mL, rimonabant, 15.6 ± 2.3, n = 12 each, p < 0.001, Fig. 1C).

Rimonabant Reduces Atherogenesis in Aorta of ApoE-Deficient Mice

In both the control and rimonabant groups, atherosclerotic lesions were found from the ascending to abdominal aorta; however, the extent of atherosclerotic lesions was smaller in the rimonabant group than the control, especially from the descending aorta to abdominal aorta (Fig. 2A). The relative surface area of the atherosclerotic lesions was significantly less in the rimonabant group than the control group (control; 12.6 ± 4.0%; rimonabant; 9.7 ± 2.3%, n = 12 each, p < 0.05; Fig. 2B). Furthermore, there was a significant inverse relationship between the relative surface area of atherosclerotic lesions in the aorta and serum HDL-C levels (r = −0.45, n = 24, p < 0.05; Fig. 3).
After differentiation to macrophages, the medium containing THP-1 cell-derived macrophages was changed to RPMI-1640 containing 2% FBS, and rimonabant (to a concentration of 1.0 μmol/L) or vehicle and the cells were further incubated for 24 hours. Real-time RT-PCR analysis indicated that rimonabant significantly increased the mRNA expression levels of RCT system-related molecules on THP-1 cell-derived macrophages (SR-B1: 1.15 ± 0.12 fold, n = 6; p < 0.05, ABCG1: 1.23 ± 0.11 fold, n = 6; p < 0.01, Fig. 4A, 4B), except for ABCA1 (1.13 ± 0.20 fold, n = 6; p = 0.13, Fig. 4C). Similarly, Western blot analysis of these cells showed increased levels of ABCG1 and SRB1 proteins (Fig. 4D).

**Discussion**

The present study showed that CB1 receptor blockade reduced atherosclerosis in mice, in association with an increase in serum adiponectin levels and activation of the RCT system. These results suggest that CB1 receptor blockade has beneficial effects on atherogenesis by increasing serum adiponectin and activating the RCT system, especially by enhancing ABCG1 and SRB1 on macrophages and contributing to HDL-C-mediated cholesterol efflux.

Our results showed that rimonabant enhances the expression levels of ABCG1 and SR-B1 but not ABCA1 in cultured human macrophages. In addition, the relative surface area of the atherosclerotic lesions correlated inversely and significantly with serum HDL-C levels. Although a report demonstrated that rimonabant did not increase HDL-C in a mouse model, rimonabant significantly increased HDL-C in human trials, which was confirmed by other large clinical trials. We thought that this difference was caused by the modality of mice and their diet. While the exact biochemi-

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**Fig. 2.** Rimonabant reduces atherosclerosis in the aorta of apoE-deficient mice.

Pinned-out aortas showing Sudan-IV-positive lesions of apoE-deficient mice without rimonabant treatment (A, left panel) and with rimonabant treatment (8 mg/kg/day) (A, right panel). Original magnification: ×2.5. Quantitative evaluations of surface atherosclerotic lesions in whole aorta (B). Open circles: control group; closed circles: rimonabant-treated group (control; 12.6 ± 4.0%; rimonabant; 9.7 ± 2.3%, n = 12 each, p < 0.05). Bars represent the mean ± SD.

**Fig. 3.** Inverse correlation between the relative area of atherosclerotic lesions in aorta and serum HDL-C levels.

Note the significant correlation between serum HDL-C levels and the relative area of atherosclerotic lesions in the aortas of all mice. Open circles: control group; closed circles: rimonabant-treated group (r = −0.45, n = 24, p < 0.05).

**Fig. 4.** Rimonabant increases the expression levels of ABCG1 and SR-B1 in cultured human macrophages.

A) Atherosclerotic area %

B) Aortic surface lesion area (%)

C) HDL-C levels

D) Western blot analysis of ABCG1 and SR-B1 proteins.
cal mechanism and in vivo relevance of this effect remain to be determined, this relationship suggests that rimonabant increases HDL-C generation by upregulating the expressions of ABCG1 and SR-B1 on macrophages. On the other hand, Tsubakio et al.\textsuperscript{24} postulated that adiponectin could accelerate the RCT system and reduce atherosclerosis by enhancing apoA-I-mediated cholesterol efflux through ABCA1 in macrophages. In our study, rimonabant upregulated ABCG1 and SR-B1, but not ABCA1. ABCA1 also showed an upward trend and fold change appeared similar to SR-B1, but was not statistically significant compared with ABCG1 and SR-B1. However, we did not prove that rimonabant could directly increase HDL-C-mediated cholesterol efflux in the present study, but these results suggest that the effects of rimonabant on ABCG1 and SR-B1 could be mediated through different pathways from its effect on adipo-
nectin. In the present study, rimonabant also increased serum adiponectin levels, and in part could produce mutually potentiating effects on macrophages in vivo. Furthermore, in a recently reported trial, rimonabant produced a significantly increase in HDL particle sizes, HDL2 and HDL3, with a decrease in small LDL. This may suggest that rimonabant promotes HDL-C-mediated cholesterol efflux in humans. We showed that rimonabant increases the expressions of ABCG1 and SR-B1, but further investigations are still required to elucidate the detailed molecule mechanisms.

In the present study, administration of rimonabant increased serum adiponectin levels but did not result in significant changes in body weight and serum lipid levels in apoE-deficient mice. Rimonabant is reported to increase plasma levels of adiponectin and enhance adiponectin production in cultured adipocytes. That the administration of rimonabant at the dose used in this study up-regulated serum adiponectin levels suggests that this drug acted peripherally, such as on adipocytes, which are known to secrete adiponectin in apoE-deficient mice. Another study reported that the effects of rimonabant are, at least in part, independent of body weight changes. Indeed, based on human clinical trials, the beneficial effects of rimonabant on cardiovascular risk factors were greater than those expected from its weight loss alone.

Our results indicated that rimonabant reduced atherosclerosis in aortas of apoE-deficient mice without any changes in body weight; therefore, it is conceivable that the anti-atherosclerotic effect of rimonabant is independent of body weight change. Dol-Gleizes et al. also reported that rimonabant had dose-dependent anti-atherosclerotic effects on LDLR-/- mice fed a Western-type diet. Whereas lower doses significantly decreased atherosclerosis without any effect on serum cholesterol levels, administration of rimonabant reduced body weight in their study. In addition, they suggested that the anti-atherogenic effects of rimonabant are probably mediated, at least in part, through a decrease in inflammation. We strongly agree with their suggestion, because our previous report demonstrated that rimonabant has anti-inflammatory effects on human macrophages. Several clinical studies demonstrated an inverse relationship between plasma adiponectin levels and several inflammatory markers, including C-reactive protein. Thus, one possible mechanism of the anti-inflammatory effects of rimonabant is an increase in plasma adiponectin levels, which in turn acts through its anti-inflammatory actions. We propose that local inflammation at atherosclerotic sites is also important, similar to systemic inflammation. In this regard, Wang et al. reported that C-reactive protein, a marker of systemic inflammation, inhibits cholesterol efflux from human foam cells derived from THP-1 and peripheral blood mononuclear cells in vitro through the down-regulation of intracellular cholesterol transport molecules ABCA1 and ABCG1.

In conclusion, the results of the present study demonstrated that CB1 receptor blockade reduced atherosclerosis without body weight reduction in apoE-deficient mice. The anti-atherosclerotic properties of CB1 receptor blockade seem to be mediated through modulation of the RCT system by enhancing ABCG1 and SRB1 expression and also by increasing serum adiponectin levels. These results suggest that CB1 receptor blockade is potentially therapeutically beneficial in patients with atherosclerosis and that such action is independent of weight reduction.

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References

21) Pi-Sunyer FX, Aronne LJ, Heshmati HM, Devin J, Rosenstock J RIO-North America Study Group: Effect of rimonabant, a cannabinoid-1 receptor blocker, on weight and cardiometabolic risk factors in overweight or obese patients: RIO-North America: a randomized controlled trial. JAMA, 2006; 295: 761-775
27) Bennetzen MF, Nielsen MP, Richelsen B, Pedersen SB: Effects on food intake and blood lipids of cannabinoid receptor 1 antagonist treatment in lean rats. Obesity (Silver Spring), 2008; 16: 2451-2455