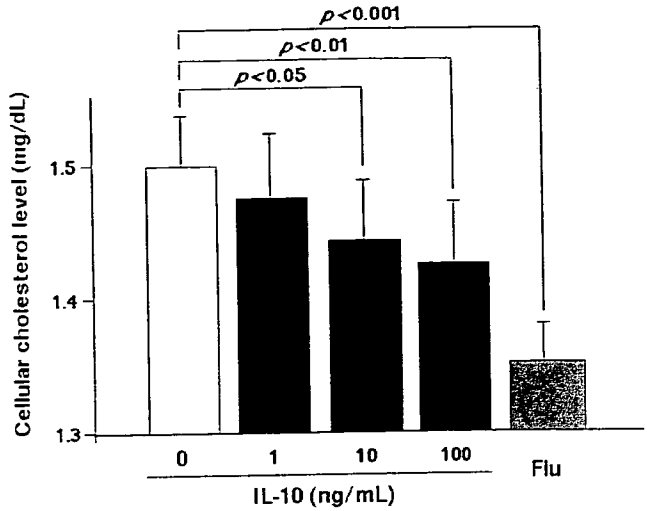


*Figure 7 Cellular cholesterol level of HepG2 cells incubated in the absence of LDL cholesterol. Recombinant human IL-10 (1–100 ng/ml) or fluvastatin ($10^{-5}$ mol/l) was added to the culture for 48 h. Data are means ± s.e.m. (n = 4).*

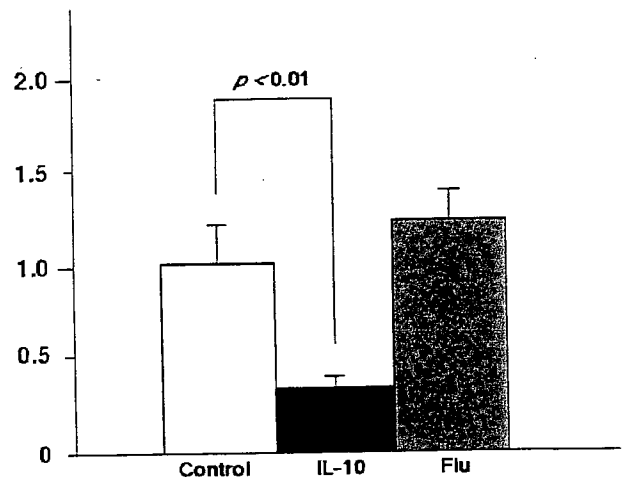


*Figure 8 Relative HMG-CoA reductase expression in HepG2 treated with recombinant IL-10 as analyzed by quantitative PCR. The relative expression of the HMG-CoA reductase mRNA was determined as the ratio of the expression in HepG2 treated with IL-10 or fluvastatin to that in HepG2 incubated normally (control). Data are means ± s.e.m. (n = 3–5).*

geous in investigating the effects of transgenes on chronic disease processes. Previous reports demonstrated that there was a significant difference in transgene expression by various AAV serotypes transduced into rodent muscle.[18–20] The AAV1 and AAV5 serotypes were shown to produce 100- to 1000-fold higher serum levels of transgene products, as compared to the AAV2. We selected AAV5 for use in *in vivo* experiments, since we also observed that gene transfer with AAV5 promoted a much higher level of expression of IL-10, as compared to AAV2-mediated transfer (Figure 2). However, AAV1 is reported to more efficiently transduce muscle than AAV5. Therefore, we anticipate that the use of AAV1 in future experiments would allow for a minimized vector dosage.

We focused on the effect of IL-10 on *in vivo* MCP-1 expression. MCP-1, one of the most studied members of the C–C family of chemokines, is expressed in several cell types in the arterial wall, including vascular endothelial cells, smooth muscle cells, and monocytes/macrophages, under numerous inflammatory conditions. Therefore, MCP-1 is thought to play an important role in the ongoing recruitment of monocyte–macrophages into developing lesions *in vivo*.[21] Previously, we[22] and others[21,23] reported enhanced MCP-1 expression in experimental and human atherosclerotic lesions. A role for MCP-1 in the initiation of atherogenesis was demonstrated by knockout mice in which the MCP-1 gene itself or its receptor CCR2 was inactivated.[24,25] In our study, we demonstrated that serum MCP-1 concentrations are significantly lower in IL-10-transduced mice than in control mice. In addition, we observed enhanced MCP-1 expression in the vascular wall of ApoE-deficient mice, as reported previously,[26] which was significantly inhibited by IL-10 gene transfer. Moreover, the extent of atherosclerotic lesion formation was significantly decreased in AAV5-mIL10-transduced mice, and positively correlated with serum MCP-1 concentration. These results suggest that the observed antiatherogenic effect of IL-10 is partly mediated by the inhibition of systemic and local MCP-1 expression.

We also investigated the effect of IL-10 on serum cholesterol levels. Nonlinear regression of serum cholesterol levels relative to serum IL-10 levels revealed a close correlation to a dose–effect model with an $EC_{50}$ (5.3 ng/ml). It is well known that high cholesterol levels lead to atherogenesis in both humans and mice. Atherosclerotic lesion surface area also correlated with the serum cholesterol concentration in our study. van Exel *et al*[12] recently found a negative correlation between a low IL-10 production capacity of whole blood and total cholesterol level in humans. They speculated that high levels of IL-10 counteract the effects of TNF-α and IL-6 on lipid metabolism. This clinical observation corresponds well with our observation that the serum IL-10 concentration negatively correlates with the reduction in serum cholesterol.

Pinderski *et al*[8] found that IL-10-transgenic mice showed a decrease in atherosclerotic lesions compared to control mice. However, they found no difference in plasma cholesterol levels between the transgenic and control mice. On the other hand, Von Der Thusen *et al*[13] showed that prolonged hepatic expression of IL-10 could be achieved following intravenous adenovirus-mediated IL-10 gene transfer to LDL receptor-deficient mice. Administration of IL-10 resulted in reductions in atherosclerotic lumen stenosis and of serum cholesterol, although the cholesterol-lowering mechanism was not clarified.

We analyzed the intracellular cholesterol level of HepG2 cells cultured with acetic acid, a cholesterol substrate, in the absence of lipoprotein. This assay can measure *de novo* cholesterol synthesis, but not cholesterol uptake, in these cells. We also used fluvastatin at a dose of $10^{-5}$ mol/l to inhibit *de novo* cholesterol synthesis almost completely.[27] The results of these experiments suggested that IL-10, like fluvastatin, significantly inhibits the hepatic production of cholesterol. It was reported that TNF-α and IL-1 increase the levels of HMG-CoA reductase mRNA without affecting the levels of LDL receptor mRNA.[28] In other words, the increase in serum cholesterol levels observed after TNF-α and IL-1 administration is caused by an increase in hepatic cholesterol synthesis rather than a decrease in the

clearance of circulating cholesterol. These observations suggest that IL-10 might have direct effects on cholesterol metabolism through the HMG-CoA reductase pathway. We evaluated the expression of HMG-CoA reductase using quantitative PCR. As is expected, IL-10 modulated HMG-CoA reductase expression, whereas fluvastatin did not. These data suggest that the use of IL-10 in combination with statin may improve cholesterol-lowering effects and benefit the patients with atherosclerotic diseases.

Finally, we evaluated whether the downregulation of the MCP-1 was affected by the cholesterol-lowering effects of IL-10 or not. Stepwise regression analysis revealed that not only serum cholesterol but also MCP-1 concentration might be significant independent predictors of atherosclerotic lesion area. Therefore, we speculated that anti-inflammatory effect of IL-10 plays an important role in anti-atherogenic effect as well as its cholesterol-lowering effects.

In summary, AAV5-mediated IL-10-gene transfer into the skeletal muscle could introduce efficient and stable IL-10 expression, resulting in a significant antiatherogenic effects in ApoE-deficient mice. It was suggested that this effect was mediated through the anti-inflammatory and cholesterol-lowering effects of IL-10. These results indicate the presence of complex interactions between inflammation and lipid metabolism, as well as the effectiveness of anticytokine therapy using IL-10 in the treatment of atherosclerotic disease.

## Materials and methods

### Production of AAV vectors

Two recombinant AAV serotypes, type 2 and type 5, were used in these experiments. AAV2 and AAV5 expressing the *Escherichia coli* β-galactosidase gene under the control of the cytomegalovirus promoter (AAV2-LacZ and AAV5-LacZ) were generated with the proviral plasmids pAAV-LacZ and pAAV5-RNL, respectively. To create AAV2 and AAV5 derivatives expressing murine IL-10 (AAV2-mIL10 and AAV5-mIL10), murine IL-10 cDNA (RIKEN DNA Bank, RDB-1476) was cloned into the *Bam*HI site of pCMV to form pCMVmIL10. The IL-10 expression cassette in pCMVmIL10 was ligated as a *Not*I fragment to *Not*I-digested pAAV-LacZ and pAAV5-RNL to form the proviral plasmids pAAV2-mIL10 and pAAV5-mIL10, respectively.

AAV viral stocks were prepared according to the previously described three-plasmid transfection adenovirus-free protocol.[29] Briefly, 60% confluent 293 cells were cotransfected with the proviral plasmid, an AAV helper plasmid (pHLP19[30] for AAV2 or pAAV5RepCap[31] for AAV5), and the adenoviral helper plasmid pAdeno. The crude viral lysate was purified by two rounds of CsCl two-tier centrifugation.[32] The viral stock was titered by dot-blot hybridization with plasmid standards.

pAAV5-RNL and pAAV5-RepCap (identical to 5RepCapB) were kindly provided by Dr JA Chiorini, and pAAV-LacZ, pHLP19 and pAdeno were obtained from Avigen, Inc. (Alameda, CA, USA).

### In vitro IL-10 gene transfer

The C2C12 cells were cultured in six-well plates with 2 ml Dulbecco's minimal essential medium (DMEM) containing 5% horse serum. At 8 days after plating, differentiated C2C12 cells were transduced with AAV2-mIL10 at various vector doses. The expression of IL-10 was detected by Western blot analysis after immunoprecipitation of conditioned medium and cell lysates. IL-10 concentrations were measured by ELISA (R&D Systems). To investigate the biological activities of IL-10, cultured medium from transduced C2C12 cells was added to cultured J774 cells for 30 min (final IL-10 concentration, 10 ng/ml). J774 cells were then treated with 100 ng/ml LPS and incubated for an additional 24 h. The supernatants were harvested and the concentrations of IL-6, TNF-α, and MCP-1 were quantified by ELISA (R&D Systems).

### In vivo IL-10 gene transfer

All animal experiments were performed in accordance with the *Jichi Medical School Guide for Laboratory Animals*, 1993. Male ApoE-deficient mice in the C57BL/6J background (a kind gift of Dr N Maeda[33,34]) were fed on a Western-type diet containing 21% fat and 0.15% cholesterol (Harlan Teklad) from 8 weeks of age. Water and food were given *ad libitum*, and the mice were maintained on a 12-h light–dark cycle. At 8 weeks of age, ApoE-deficient mice were injected with AAV2-mIL10 ($1 \times 10^{13}$ genome copies/body), AAV5-mIL10 ($1 \times 10^{11}$–$10^{13}$ genome copies/body), or AAV5-LacZ ($1 \times 10^{13}$ genome copies/body) as 50 µl in total into two distinct sites of the anterior tibial muscle. The serum concentrations of IL-10 and MCP-1 were measured by ELISA as described above. The serum concentrations of total cholesterol were measured by HDAOS methods (Wako Pure Chemicals).

### Morphometric analysis

At 8 weeks after the AAV5-mIL10 or -LacZ inoculation, the ascending aortas were removed after perfusion fixation with 4% paraformaldehyde at physiological pressure, embedded in OCT compound (Tissue-Tek), and frozen in liquid nitrogen. Atherosclerotic lesions in the aortic sinus were examined at five locations separated by 80 µm, with the most proximal site at the point where the aortic valves first appeared, and were stained with oil red-O. To quantify the aortic lesions, each image was digitized and analyzed under a microscope (Olympus) with National Institutes of Health Image software (ver. 1.61). Oil red-O-positive areas were analyzed in comparison with the total cross-sectional vessel wall area. The average value for five locations in each animal was determined.

### Immunohistochemical analysis

Arterial sections were prepared from 8-week-old mice transduced with AAV5-IL-10 or AAV5-LacZ at 4 weeks old. These sections were incubated with a primary goat polyclonal antibody against mouse MCP-1 (dilution 1/250, Santa Cruz Biotechnology). Nonspecific IgG was used as a negative control. Sections were incubated with biotinylated anti-mouse secondary antibody and treated with peroxidase-conjugated streptavidin, with 3′, 3′-diaminobenzidine tetrahydrochloride as the enzyme substrate, and counterstained with hematoxylin.

### *Measurement of cellular cholesterol*

HepG2 cells (human hepatocytes) were maintained in a 12-well plate with 1 ml of MEM containing 10% fetal bovine serum, 1% nonessential amino acids (NEAA; ICN), and 1 mmol/l sodium pyruvate at 37°C in a 5% $CO_2$ incubator for 5 days. The medium was then replaced with 1 ml of MEM containing 10% lipoprotein-deficient serum (LPDS; ICN), 1% NEAA, and 1 mmol/l sodium pyruvate. After 2 days later, the cells were incubated with 1 ml of MEM containing 10% LPDS, 1% NEAA and 1 mmol/l sodium pyruvate, and various concentrations (0–100 ng/ml) of recombinant human IL-10 (PeproTech) or $10^{-5}$ mol/l fluvastatin (Tanabe) were added to the culture. At 2 days after the treatment, the cells were incubated with 0.5 ml of MEM containing 10% LPDS, 1% NEAA, 1 mmol/l sodium pyruvate, and 2 mmol/l acetic acid for 16 h. The cellular cholesterol level was measured by an enzymatic method using Determiner TC-555 (Kyowa Medics).

### *mRNA analysis of HMG-CoA reductase*

HepG2 cells were incubated with either recombinant human IL-10 (10 ng/ml) or fluvastatin ($10^{-5}$ mol/l) in 10% LPDS medium for 24 h. Total RNA was isolated from the cell culture using an RNeasy Mini kit (QIAGEN) and reverse-transcribed into a single-stranded cDNA using SuperScript Preamplification System (GIBCO BRL). To estimate the expression of HMG-CoA reductase in HepG2, quantitative PCR analysis was conducted by using ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The reaction was performed using the primer pairs specific for the *HMG-CoA reductase* (HMGCR-5′: GGCCCA GTTGTGCGTCTTCC and HMGCR-3′: GTTTGCTGC ATGGGCGTTGTAG) and *GAPDH* (GAPDH-5′: CGCG GGGCTCTCCAGAACATCAT and GAPDH-3′: CCAGCC CCAGCGTCAAAGGTG). Quantitative values were obtained from the threshold cycle ($C_t$) number that indicated exponential amplification of the PCR product. To normalize each sample, we also quantified the expression of the *GAPDH* gene. The relative target gene expression was also normalized with a calibrator (HepG2 cells without treatment of IL-10 or fluvastatin). The final result, expressed as *N*-fold differences in target gene expression relative to the *GAPDH* gene and the calibrator, was determined by the following formula: $N_{\text{target}} = 2^{\text{corrected}\Delta C_t\,(\text{GAPDH}-\text{target gene})}$. $C_t$ values of the sample were determined by subtracting the average $C_t$ value of the target gene from the average $C_t$ value of the *GAPDH* gene.

### *Statistical analysis*

Student's *t*-test or ANOVA combined with Scheffe's test was used to compare individual groups, and the Pearson's correlation test was employed to measure the linear association between two variables by using StatView (Abacus Concepts, Inc). Data are presented as means ± s.e.m. A value of $P<0.05$ was considered significant.

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