J. Biochem. 129, 717–724 (2001)

Effects of Recombinant Human Macrophage Colony-Stimulating Factor on Atherosclerotic Lesions Established in the Aorta of High Cholesterol-Fed Rabbits

Hiroyuki Irie, Hiroshi Koshiba, Mamoru Koyama, Eiji Asakura, Hiroshi Shibata, Kazuyuki Kimura, Kazuhide Naito, Takeshi Yamauchi, Kouji Yada, Takuji Hanamura, Shuichi Hanada, Syun-ichi Abe, and Norifumi Nakamura[1]

Pharmaceutical Research Division, Welfide Corporation, 2-25-1, Shodai-Ohtani, Hirakata, Osaka 573-1153

Received January 11, 2001; accepted February 9, 2001

Anti-atherosclerotic effects of human macrophage colony-stimulating factor were investigated using rabbits fed a high cholesterol diet. Rabbits fed a diet containing 2% cholesterol for 59 days developed hyperlipidemia and atheromatous aortic plaques. They were then administered 80 μg/kg/day of either macrophage colony-stimulating factor or human serum albumin, as a control, for the next 12 weeks. Compared with the control group, rabbits treated with macrophage colony-stimulating factor had significantly fewer plaques on the inner surface of the thoracic and abdominal aortae, and half the sectional area of thickened intima in the aortic arch, as well as in the thoracic and abdominal aortae. Macrophage colony-stimulating factor also decreased the cholesterol content of the atherosclerotic lesions. Serobiochemical analyses revealed that macrophage colony-stimulating factor increased the levels of high density lipoprotein-cholesterol significantly, without influencing other lipid parameters such as the level of low density lipoproteins. The effects of macrophage colony-stimulating factor were evident until the fourth week of drug injection, at which time anti-human macrophage colony-stimulating factor antibodies were clearly induced in the serum. These results indicate that exogenously administered macrophage colony-stimulating factor suppresses atherosclerotic lesions induced by a high cholesterol diet by activating lipid metabolism *in vivo*.

Key words: atherosclerosis, cholesterol, HDL, M-CSF, reverse cholesterol transport.

Macrophage colony-stimulating factor (M-CSF), a glycoprotein with a molecular mass of 85 kDa (*1*), has been found not only to be a hematopoietic factor that stimulates the production of monocytes/macrophages in bone marrow (*2*, *3*), but also to have a wide range of biological activities (*4–9*). M-CSF is produced in the intima of atherosclerotic lesions (*10*), which may be caused by oxidized serum components (*11*), and is thought to be associated with disease progression. On the other hand, systemic administration of human urinary M-CSF has been found to reduce total serum cholesterol in both clinical trials in leukocytopenia patients (*12*), and in experimental animals (*13*, *14*). The cholesterol-lowering activity of M-CSF has been shown in patients with familial hypercholesterolemia (*15*). Further experiments using Watanabe heritable hyperlipidemic (WHHL) rabbits, hereditary atherogenic rabbits without functional low density lipoprotein (LDL) receptors, have revealed that serial injections of M-CSF arrest the progression of atheroma formation (*16*). *In vitro* studies indicate that M-CSF-treated macrophages experience increased uptake of acetyl-LDL and enhanced cholesterol esterification (*17*, *18*). M-CSF also regulates scavenger receptor gene expression in cultured monocytes, and stimulates apolipoprotein E (Apo E) and lipoprotein lipase gene expression *in vitro* (*19*, *20*), consistent with an enhancement of reverse cholesterol transport (*21*).

Recent studies using M-CSF-deficient mice (*op/op*), however, give paradoxical results, demonstrating that M-CSF plays a crucial role in atherogenesis. Atherosclerosis induced in either LDL receptor-null or Apo E-null mice was significantly suppressed by M-CSF-deficiency (*22–24*). Heterozygous mice (*op/+*) were also resistant to atherogenesis (*24*). These results clearly show that M-CSF at physiological levels supports atheroma formation caused by hereditary hyperlipidemic disease. On the other hand, a therapeutic dose of M-CSF suppresses the advancement of lesions established in WHHL rabbits (*25*). The hyperlipoproteinemia induced by an atherogenic diet increases the levels of very low-density lipoproteins (VLDL) as well as LDL, and is different from conditions in WHHL rabbits, in which LDL-cholesterol levels are more purely elevated. This pattern may resemble postprandial hyperlipoproteinemia and diabetic dyslipidemia more closely than conditions in WHHL rabbits. Thus, we have been interested in the

[1] To whom correspondence should be addressed. 2-6-9, Hirano-cho, Chuo-ku, Osaka 541-0046. Tel: +81-6-6201-1600, Fax: +81-6-6233-8814, E-mail: ac45560@welfide.co.jp

Abbreviations: Apo E, apolipoprotein E; ELISA, enzyme-linked immunosorbent assay; HDL, high density lipoprotein; HSA, human serum albumin; LDL, low density lipoprotein; M-CSF, macrophage colony-stimulating factor; VLDL, very low density lipoprotein; WHHL rabbit, Watanabe heritable hyperlipidemic rabbit.

effects of M-CSF on atherosclerosis established in dietary animal models, rather than in hereditary models. In the present study, we performed a sustained administration of M-CSF to high cholesterol fed-rabbits, and demonstrated its anti-atherosclerotic effects, which are probably elicited by up-regulating the serum level of high density lipoprotein (HDL)-cholesterol.

MATERIALS AND METHODS

Atherogenic Rabbits—Twelve-week-old male NZW rabbits (2.4–3.0 kg) were purchased from Kitayama Labes, and kept at 21°C and 55% humidity with light for 12 h/day (7:00–19:00). After quarantine for 6 days, animals confirmed to be normal were fed commercial solid food containing 2% cholesterol (RC-4, Oriental Yeast) for 59 days. Autopsies were performed on two animals to confirm the formation of atherosclerotic lesions in the aortic arch, thoracic and abdominal aortae. All experimental procedures met the ethical standards outlined in the Helsinki Declaration.

Drug Treatment—Atherogenic rabbits without jaundice were returned to a normal diet, and daily treatment with recombinant human M-CSF (Genetics Institute) was initiated (n = 11). Human serum albumin (HSA) (Alpha Therapeutic), another foreign protein, was used as a control drug (n = 11) (*16*). Drugs were dissolved in saline solution (Otsuka Pharmaceutical Factory), and injected into the femoral muscle at a dose of 80 μg/0.2 ml/kg/day for 12 consecutive weeks. The daily dose of M-CSF was determined to be below the critical limit for thrombocytopenia (*14*).

Pathological Examination of Aorta—Upon completion of drug-treatment, all animals were anesthetized with thiopental sodium (RAVONAL®, Tanabe Pharmaceutical) and sacrificed by bleeding *via* the carotid artery. Aortae from the arch to the abdominal region were excised and cut length-wise along the longitudinal muscle. Aortic strips were fixed in PBS containing 1% formalin for more than 12 h at 4°C, then sectioned at three sites: the first intercostal bifurcation, then 4 cm and 8 cm distally therefrom (Fig. 1). The resulting four parts represented the aortic arch, the proximal thoracic aorta, the distal thoracic aorta and the abdominal aorta. Atheromatous plaques in each aortic section were traced by a xerographic method (*26*). The total intimal surface area of the aorta and the area of plaques in the inner vessel wall were measured using a real-time image analyzer (LUZEX IID, Nireco). The atheromatous plaque formation ratio was calculated by the following equation:

$$(\%) = (\text{plaque area}) \div (\text{total aortic inner surface area}) \times 100$$

The aortic arch and thoracic aorta were then bisected longitudinally. From the left half of the bisected specimens, lipids were extracted by homogenization with a Potter-Elvehjem-type teflon homogenizer in a 20-fold volume of a 2:1 (v/v) mixture of chloroform and methanol (*27*). Total- and free-cholesterol contents were measured using commercially available kits (Wako Pure Chemical). Cholesterol ester content was calculated by subtracting free cholesterol from total cholesterol.

Histological Analyses—The remaining right half of the longitudinally bisected specimens and abdominal aorta were fixed in formalin-calcium fixative (*28*), and divided into 10 × 4-mm slices (Fig. 1). The specimens were then embedded in paraffin using a routine technique, alternating five tissue samples between osmium (*28*) and hematoxylin-eosin stain. Using an image analyzer, the intimal (hypertrophied) and medial areas of each specimen were measured.

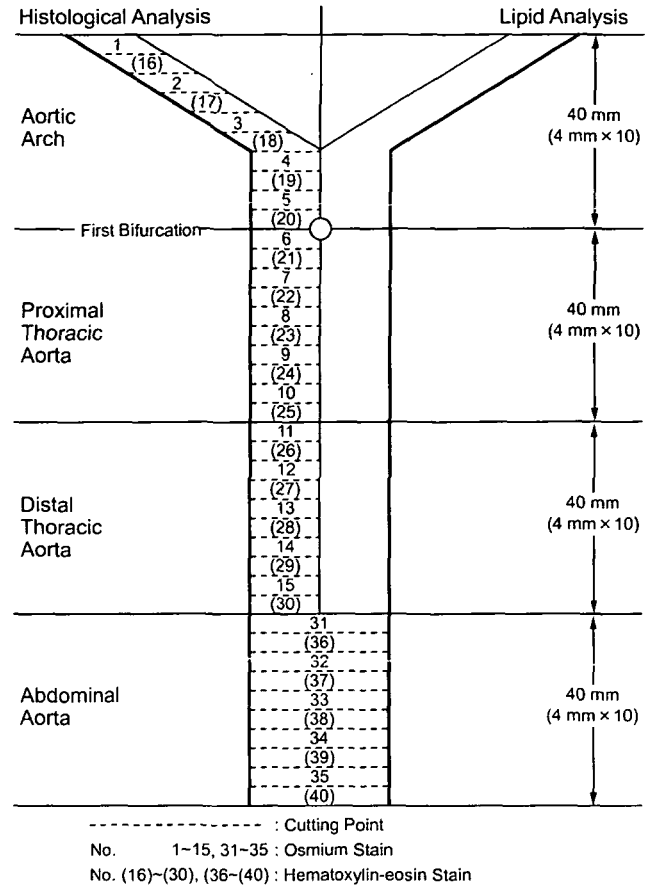

Fig. 1. **Schematic diagram of atherosclerotic lesions.** Aortae, from the arch to the abdominal area, of atherosclerotic rabbits were excised and processed for histological (left) and lipid (right) analyses. Forty sectional specimens were prepared from each aorta, and stained with osmium (for lipid-staining) or hematoxylin-eosin.

Immunohistochemical Analyses—Intimal cell number on the aortic arch specimens was counted using an image analyzer. The aortic arch specimens were further immunostained for macrophages (RAM11, DAKO) using the streptoavidine-biotin method (HISTOFINE SAB-PO kit, NICHIREI). The immunostained specimens were projected onto an image analyzer to measure positively stained areas.

Serobiochemical Analyses—Blood was sampled from the auricular vein before and every 2 weeks during the drug-treatment period. Sera were analyzed using commercially available kits for total cholesterol (Wako Pure Chemical), lipoprotein fractions (LDL, VLDL, and chylomicron) (Eiken Chemical), and HDL-cholesterol (KYOWA-Medecs).

Anti-Human M-CSF Antibody Titration—Recombinant human M-CSF in PBS (100 μg/ml) was dispensed at 100 μl/well into 96-well plates, and left for 3 h. The wells were washed twice with washing buffer (0.01% Tween 20 in saline), and incubated with blocking buffer (10% normal horse serum, 0.1% BSA, 0.5% gelatin, 0.02% NaN_3 in PBS)

at 4°C overnight. Rabbit serum was diluted 5 to 5 × 10^5-fold in blocking buffer, and dispensed into the M-CSF–coated wells. Plates were incubated for 2 h, and washed 4 times. Horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Bio-Rad) was diluted 2 × 10^5-fold in blocking buffer, and added to the wells for 2 h. After 6 washes, the *o*-phenylene diamine substrate was dispensed. H_2SO_4 (2 N) was added to the wells after 30 min, and $A_{492/690}$ was measured. ED_{50} was calculated as the dilution rate giving the half maximal value of $A_{492/690}$.

The neutralizing activity of the test serum was assayed to inhibit hematopoietic colony formation elicited by recombinant human M-CSF. M-CSF at 10^4 U/ml and serially diluted serum were mixed, and incubated for 1 h. This mixture was assayed for colony-formation using murine bone marrow cells (*1*). The neutralizing titer in the sample was determined to be 5,000 U/ml, when the colony number decreased by half.

Statistical Analysis—Unpaired, two-tailed *t*-test was used to compare M-CSF– and HSA-treated groups.

RESULTS

Atherogenic Rabbits—Within 59 days on the 2% cholesterol diet, four out of 28 rabbits developed jaundice, two of which died around the end of the dietary period. Two rabbits without jaundice were examined anatomically for the progression of atherosclerosis. Advanced atheromatous plaques were observed on the whole surface from the aortic arch to abdominal aorta in both rabbits. Serobiochemical analyses revealed remarkably elevated lipid parameters, compared with data obtained from normal NZW rabbits (*29*); total cholesterol: 2,150 ± 200, LDL: 2,280 ± 200, VLDL: 1,630 ± 160, chylomicron: 1,700 ± 280 (mean ± SE, n = 17). In contrast, the serum level of HDL-cholesterol in the atherogenic rabbits (43.6 ± 11.4 mg/dl) was twice that in normal rabbits (*29*). Based on these results, we returned the animals to a normal diet, and initiated daily M-CSF or HSA treatments for animals without jaundice (n = 11). Two rabbits developed jaundice within one month of M-CSF treatment, one of which eventually died. Four rabbits in the HSA-treated group died due to deterioration caused by jaundice.

Atheromatous Aortic Plaques—Autopsies carried out at the end of the drug-treatment period revealed the formation of a large number of atheromatous plaques on the inner aortic surfaces of all animals. The area most affected was the aortic arch, with the plaques gradually decreasing in frequency toward the abdominal region (Fig. 2). Treatment with M-CSF improved the severity of plaque lesions in both the thoracic and abdominal regions. The ratio of plaque area to the aortic inner surface was calculated for all animals, and compared between the M-CSF– and HSA-treated groups (Fig. 3). From these results, it was found that M-CSF removed plaques covering approximately 30% of the surface area in the thoracic and abdominal regions.

Intimal Thickness of Aorta—Aortic specimens ranging from the aortic arch to the abdominal area were stained with hematoxylin-eosin (Fig. 4). In contrast to other specimens, little trace of intimal lesions was observed in distal thoracic and abdominal sections from M-CSF–treated rabbits. The sectional areas of both thickened intimal lesions and vascular media of all animals examined were measured and compared between the M-CSF– and HSA-treated group (Fig. 5). Although there was no difference in the medial sectional area of the aorta, the intimal area (hypertrophied area) was significantly reduced by M-CSF treatment. The intimal area was halved by M-CSF treatment, and the effect was observed not only in the thoracic and abdominal regions, but in the aortic arch, where no effect of M-CSF had been observed by plaque analysis (Fig. 3).

Cholesterol Content of Aorta—Cholesterol was extracted from aortic lesions and measured as the free or esterified form. Table I shows the cholesterol content in the aortic

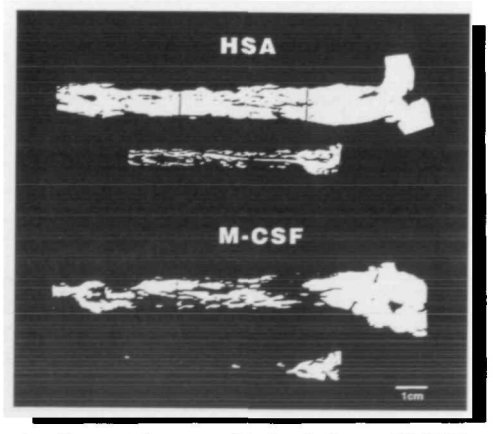

Fig. 2. **Aortic specimens obtained from M-CSF– or HSA-treated rabbits.** Atherosclerotic rabbits were treated with 80 μg/kg/day of either HSA (above) or M-CSF (below) for 12 weeks. Aortic lesions were excised, and atheromatous plaques were visualized. Aortae from the arch to the thoracic aorta are shown above the abdominal aorta. One representative specimen from 10 (M-CSF) or 7 (HSA) animals is shown.

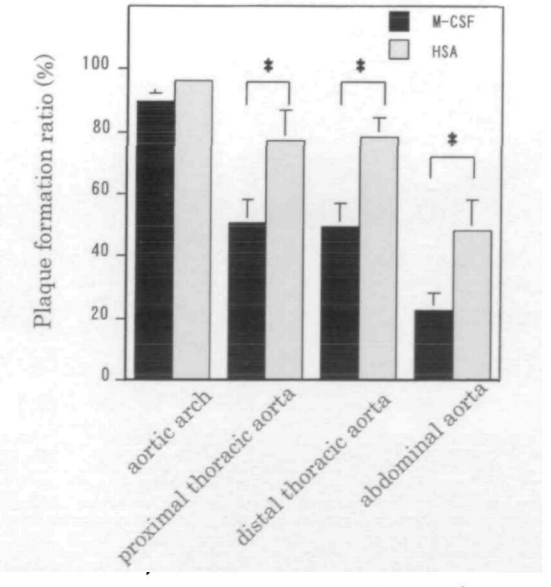

Fig. 3. **Atheromatous plaques reduced by M-CSF treatment.** Atheromatous plaques, as shown in Fig. 2, were measured and the ratio to total aortic inner surface area was calculated separately for the aortic arch, proximal thoracic aorta, distal thoracic aorta and abdominal aorta. Data are expressed as the mean ± SEM of 10 (M-CSF) and 7 (HSA) animals. Significant differences were found between the HSA- and M-CSF–treated groups ($^*p < 0.05$).

arch, proximal, and distal thoracic aorta. According to the severity of atherosclerosis, the cholesterol content was higher in the aortic arch than in the thoracic aorta. Total cholesterol content in the aorta was lower in the M-CSF–treated group than in the HSA-treated group, most significantly in the thoracic region. M-CSF removed not only cholesterol ester, but also free cholesterol that accumulated in the lesion.

Macrophages in the Lesions—Cellularity in the aortic lesions did not differ between M-CSF–treated and control rabbits [0.143 ± 0.013 cells/μm^2 (n = 10), 0.128 ± 0.027 (7), respectively (mean ± SE)]. To investigate the effect of M-CSF on macrophage infiltration into atherosclerotic lesions, specimens of aortic arch were stained for macrophages using RAM11 monoclonal antibody (Fig. 6). Macrophages were detected deep in the intima, where cholesterol deposits and foam cells were occasionally observed. The positively stained areas were measured and compared between

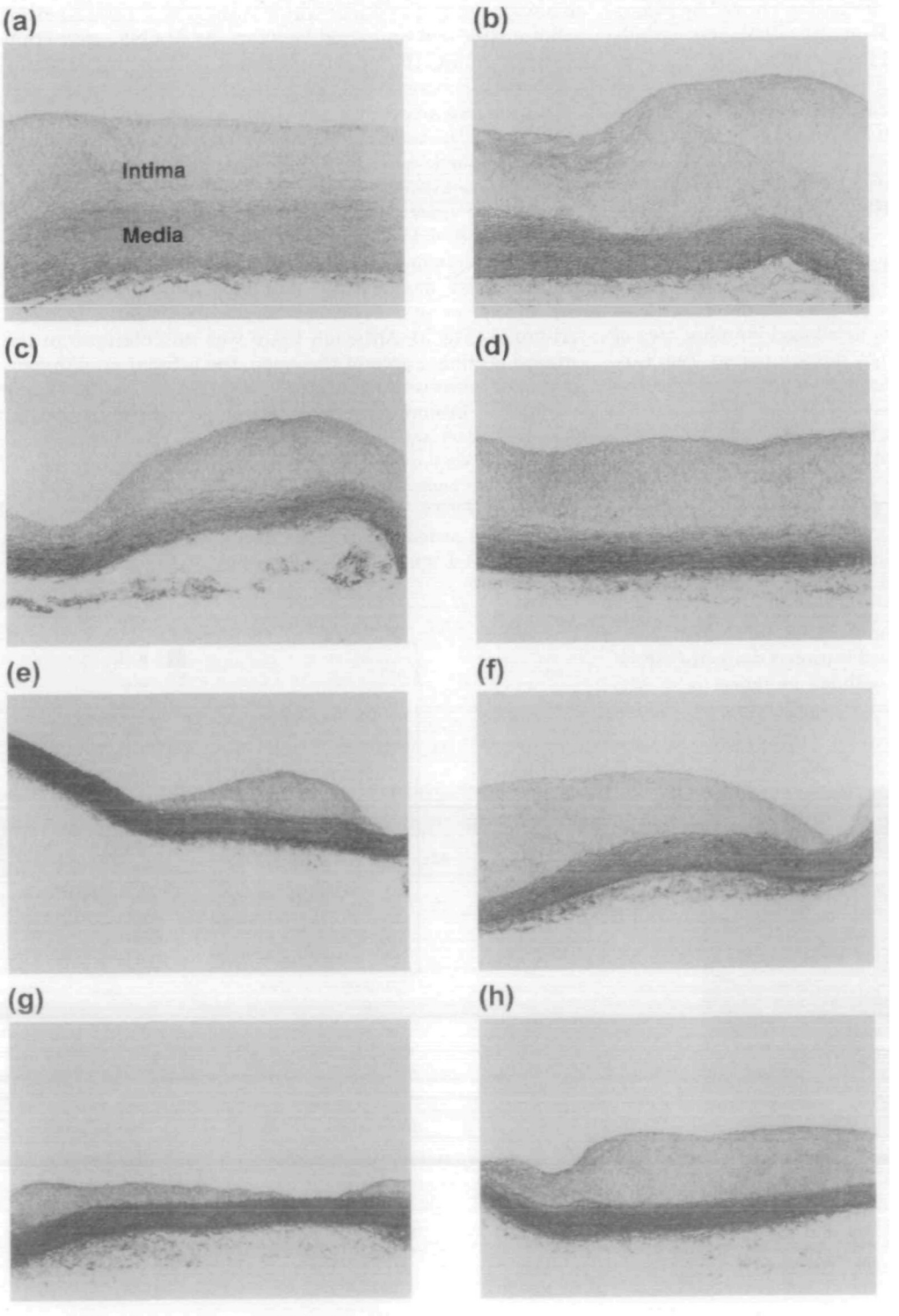

Fig. 4. **Light micrographs of atherosclerotic lesions.** Atherosclerotic rabbits were treated for 12 weeks with 80 μg/kg/day of either M-CSF (a, c, e, g) or HSA (b, d, f, h). Aortic lesions were excised, and specimens were stained at the aortic arch (a, b), proximal thoracic aorta (c, d), distal thoracic aorta (e, f), and abdominal aorta (g, h). One representative specimen of 10 (M-CSF) or 7 (HSA) is shown.

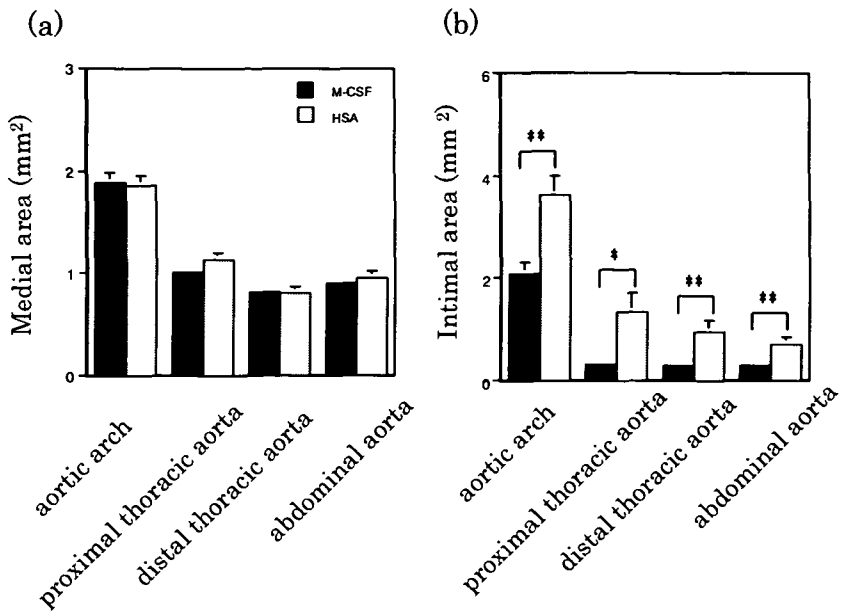

Fig. 5. **Cross-sectional intimal area reduced by M-CSF treatment.** Cross-sectional intimal (a) and medial (b) areas were measured separately at the aortic arch, proximal thoracic aorta, distal thoracic aorta, and abdominal aorta. Data are expressed as the mean ± SEM of 10 (M-CSF) or 7 (HSA) animals. Significant differences were found between the HSA- and M-CSF–treated groups ($^{*}p < 0.05$, $^{**}p < 0.01$).

TABLE I. **Aortic cholesterol content is reduced by M-CSF-treatment.**[a]

Cholesterol	Drug	Cholesterol (mg/4 cm aortic vessels)		
		Aortic arch	Proximal thoracic aorta	Distal thoracic aorta
Free	M-CSF	7.5 ± 0.9**	0.4 ± 0.4*	0.0 ± 0.0**
	HSA	13.6 ± 2.0	3.0 ± 1.2	2.1 ± 0.8
Esterified	M-CSF	11.1 ± 0.9	3.4 ± 0.4**	3.4 ± 0.1**
	HSA	14.2 ± 2.0	8.6 ± 1.0	5.3 ± 0.6
Total	M-CSF	18.6 ± 1.5	3.7 ± 0.5**	3.4 ± 0.1**
	HSA	27.8 ± 3.6	11.6 ± 1.8	7.4 ± 1.1

[a]Data expressed as the mean ± SEM of 10 (M-CSF) and 7 (HSA) animals. *, **Significant difference between M-CSF– and HSA-treated groups at $p < 0.05$, < 0.01, respectively.

the M-CSF–treated and control group. The difference was not significant [M-CSF; 48 ± 7 (n = 10), HSA; 43 ± 8% (7) (mean ± SE)].

Serum Levels of Lipid Parameters—After the change to a normal diet, serum levels of cholesterol continued to decrease during the experimental period regardless of the drug administered (Fig. 7). Chylomicron levels decreased rapidly, while the decrease in VLDL and LDL was gradual. Serum levels of total cholesterol, LDL, VLDL, and chylomicrons were lower in the M-CSF–treated than HSA-treated group, but the differences were not significant. HDL-cholesterol levels in M-CSF–treated rabbits increased up to the fourth week of treatment, while those in control rabbits decreased. These results, together with the decrease in the cholesterol content of the lesions, seem to indicate that M-CSF activates reverse cholesterol transport by HDL in atherosclerotic rabbits.

Anti-Human M-CSF Antibody Titer—The effect of M-CSF on the increase in the serum level of HDL-cholesterol was evident until the fourth week of treatment. Thus, the possibility was investigated that an antibody against human M-CSF was induced, leading to the inactivation of exogenous M-CSF. Antibody titers against human M-CSF in the sera of experimental animals were assayed by enzyme-linked immunosorbent assay (ELISA), and the results are presented as the dilution rate giving half-maximal absorbance (ED_{50}) (Fig. 8). No antibody was detected before M-CSF-injection in any animal, and induced after 2 weeks. The ED_{50} value reached a plateau after 4 weeks of injection, and this continued to the end of drug administration. Furthermore, the neutralizing ability to inhibit colony-formation by human M-CSF was examined in the serum of an M-CSF–treated rabbit, and shown to increase in parallel with the ED_{50} value. From these results, it is plausible that the effect of M-CSF was masked to some degree by antibody induction in this model.

DISCUSSION

Anti-Atherosclerotic Effect of M-CSF—This study demonstrates that the sustained injection of M-CSF diminishes atheromatous lesions in atherogenic rabbits fed a high cholesterol diet, a non-hereditary atherosclerotic model. Inoue *et al.* also demonstrated suppressive effects of M-CSF on the development and progression of atherosclerosis in WHHL rabbits (*16*). Watanabe *et al.* reported that repeated injections of M-CSF into 11-month-old WHHL rabbits reduced the intimal thickness of advanced lesions significantly (*25*). Therefore, exogenous M-CSF would seem to have some effect on advanced atherosclerosis due to both hereditary (LDL receptor deficiency) and dietary causes.

Effect of M-CSF on Lipid Parameters—The anti-atherosclerotic effects of M-CSF are thought to be caused by the cholesterol lowering activity of M-CSF (*12–15*), which results from functional modulation of macrophages including the expression of LDL- and scavenger-receptors (*13, 30*), the activity of cholesterol-metabolic enzymes (*31*) and the production of ApoE and lipoprotein lipase (*19, 20*). Our study using a dietary model shows a tendency for decreases in serum cholesterol, LDL, chylomicrons, and VLDL, but the effect of M-CSF was not significant. Since these lipid parameters decreased rapidly after the removal of the high

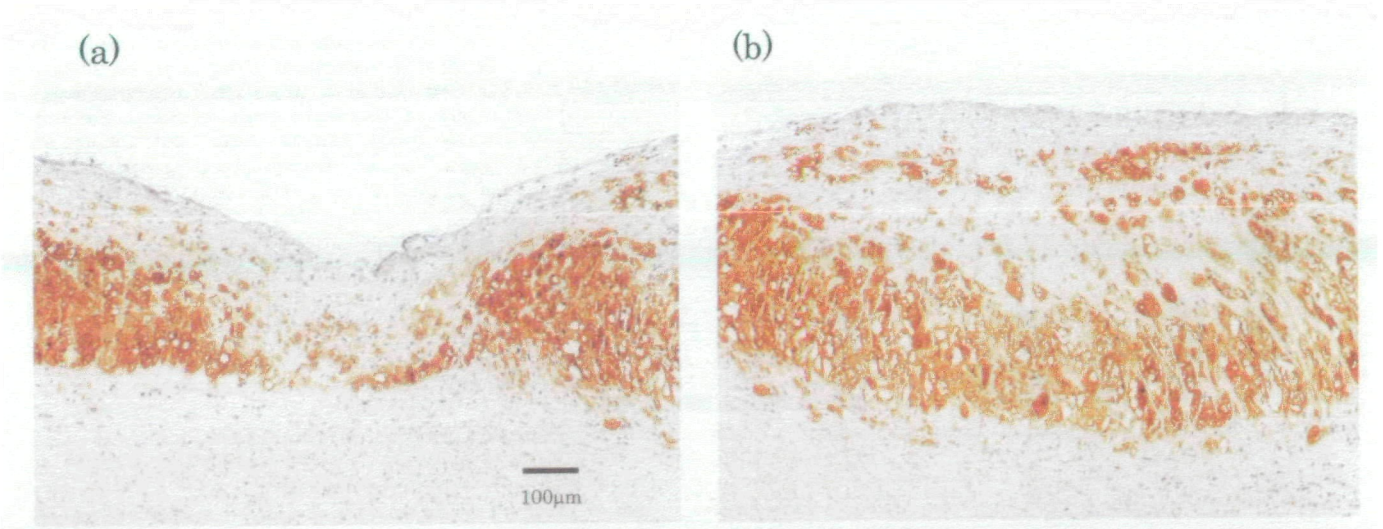

Fig. 6. **Effect of M-CSF on macrophages infiltrating the aortic lesion.** Specimens of aortic arch from M-CSF (a)– and HSA (b)-treated rabbits were immunostained for macrophages using RAM11 monodonal antibody. One representative specimen of 10 (M-CSF) or 7 (HSA) is shown. Bars indicate 100 μm.

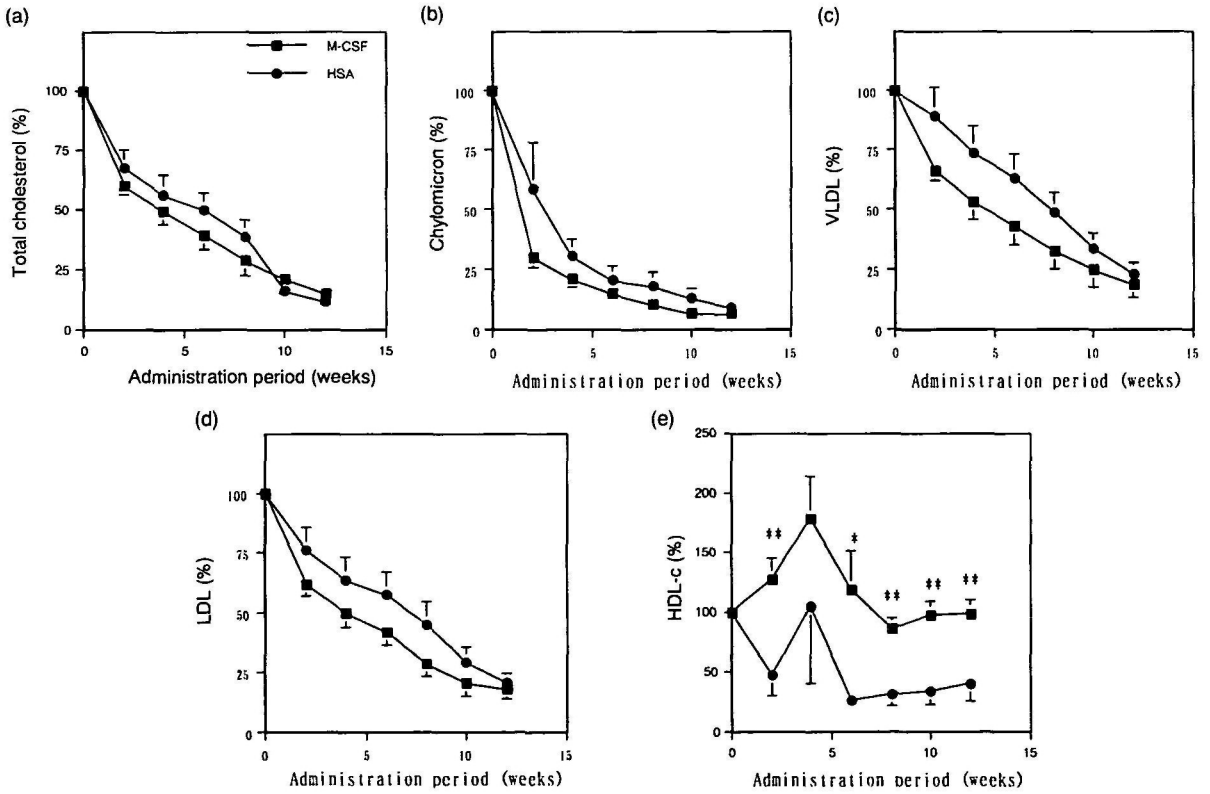

Fig. 7. **Effect of M-CSF on serum lipid parameters in atherosclerotic rabbits.** Atherosclerotic rabbits were treated for 12 weeks with 80 μg/kg/day of either M-CSF (n = 10) or HSA (n = 7). Serum levels of total cholesterol (a), chylomicrons (b), VLDL (c), LDL (d), and HDL-cholesterol (e) were assayed during treatment. Data are normalized to the levels at the initiation of treatment, and expressed as mean ± SEM. Significant differences are found between the two groups ($^{*}p < 0.05$, $^{**}p < 0.01$).

cholesterol diet, the cholesterol lowering activity of M-CSF might have been overcome. However, M-CSF increased the serum level of HDL-cholesterol significantly in our atherogenic rabbits. This is consistent with the experimental results of Stoudemire and Garnick in which M-CSF was found to up-regulate serum HDL cholesterol levels in WHHL rabbits (*14*). However, there are reports that M-CSF has little effect on HDL-cholesterol levels (*16*, *21*, *25*). The highly atherogenic conditions in our rabbit study may have elicited a rise in serum HDL-cholesterol caused by M-CSF.

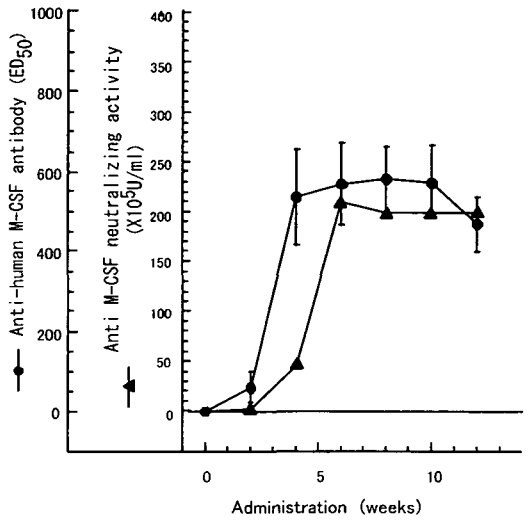

Fig. 8. **Anti-human M-CSF antibody induced in atherosclerotic rabbits.** Atherosclerotic rabbits were treated for 12 weeks with 80 μg/kg/day of M-CSF (n = 10). The serum titer of anti-human M-CSF antibody was assayed by ELISA, and the data are expressed as mean ± SEM. Neutralizing activities in one rabbit were measured by a colony-formation assay with recombinant human M-CSF.

Underlying Mechanism of the Anti-Atherosclerotic Action of M-CSF—HDL functions as a reverse cholesterol transporter from the periphery to the liver, and might remove accumulated cholesterol in atherosclerotic lesions, probably leading to regression of the lesions. Watanabe *et al.* reported that M-CSF has little effect on cholesterol ester content in advanced atherosclerotic lesions in WHHL rabbits, and that plasma HDL-cholesterol levels do not differ between M-CSF–treated and control animals (*25*). These results, taken together with our data, may indicate that the removal of cholesterol from atherosclerotic lesions is associated with up-regulation of serum HDL-cholesterol level by M-CSF. M-CSF reduces cholesterol ester incorporation from VLDL fractions, and enhances cholesterol-release by macrophages prepared from rabbits fed a high cholesterol diet (*32*), while M-CSF accelerates both the cellular incorporation and release of cholesterol derived from LDL and modified LDL (*17*). Furthermore, VLDL-induced cholesterol ester deposition in macrophages is suppressed by M-CSF when M-CSF acts at the stage of monocytes (*33*). The serum level of VLDL is higher in our hyperlipidemic rabbits than in WHHL. So, differences in the effects of M-CSF in the two atherosclerotic models may result from a modification of macrophages in our dietary model, making it more difficult for them to deposit cholesterol ester in response to an elevated serum VLDL fraction. Although M-CSF did not increase the number of macrophages infiltrating the lesions, we have suggested that M-CSF induces the production of MMP-1, MMP-9, and a urokinase-type plasminogen activator by macrophages, leading to the degradation of the extracellular matrix accumulated in the lesions (*34*). Thus, M-CSF may perform anti-atherosclerotic actions by mechanisms other than the augmentation of lipid metabolism.

Thrombocytopenia and Antibody Induction—In our dietary model of atherosclerosis, neither monocytosis nor thrombocytopenia was observed in hematological analyses during the period of M-CSF treatment (data not shown). A transient decrease in the platelet count is caused by the continuous intravenous injection of greater than 100 μg/kg/day of M-CSF into WHHL rabbits (*14*, *35*). Thus, the present study clearly shows that the M-CSF-dosage for anti-atherosclerotic action is lower than that which causes hematological abnormalities. Antibodies against human M-CSF are induced 2 to 3 weeks after continuous infusion, leading to a disappearance of the cholesterol-lowering and platelet-decreasing activity of M-CSF (*35*). Our results also demonstrate antibody-induction in this dietary atherosclerotic model, which might mask the anti-atherosclerotic effect of M-CSF to some degree. These results suggest that recombinant human M-CSF is more efficient against atherosclerosis in humans.

REFERENCES

1. Motoyoshi, K., Takaku, F., Mizoguchi, H., and Miura, Y. (1978) Purification and some properties of colony-stimulating factor from normal human urine. *Blood* **52**, 1012–1020
2. Clark, S.C. and Kamen, R. (1987) The human hematopoietic colony-stimulating factors. *Science* **236**, 1229–1237
3. Motoyoshi, K., Yoshida, K., Hatake, K., Saito, M., Miura, Y., Yanai, N., Yamada, M., Kawashima, T., Wong, G.G., Temple, P.A., Leary, A.C., Witek-Giannoti, J.S., Fujisawa, M., You, A., Okabe, T., and Takaku, F. (1989) Recombinant and native human urinary colony-stimulating factor directly augments granulocytic and granulocyte-macrophage colony-stimulating factor production of human peripheral blood monocytes. *Exp. Hematol.* **17**, 68–71
4. Munn, D.H. and Cheung, N.K.V. (1989) Antibody-dependent antitumor cytotoxicity by human monocytes cultured with recombinant macrophage colony-stimulating factor. *J. Exp. Med.* **170**, 511–526
5. Yoshida, H., Hayashi, S., Kunisada, T., Ogawa, M., Nishikawa, S., Okamura, H., Sudo, T., Shultz, L.D., and Nishikawa, S. (1990) The murine mutation osteopetrosis is in the coding region of macrophage colony-stimulating factor gene. *Nature* **345**, 442–444
6. Bock, S.N., Cameron, R.B., Kragel, P., Mul, J.J., and Rosenberg, S.A. (1991) Biological and antitumor effects of recombinant human macrophage colony-stimulating factor in mice. *Cancer Res.* **51**, 2649–2654
7. Saito, S., Saito, M., Motoyoshi, K., and Ichijo, M. (1991) Enhancing effects of human macrophage colony-stimulating factor on the secretion of human chorionic gonadotropin by human chorionic villous cells and tPA-30 cells. *Biochem. Biophys. Res. Commun.* **178**, 1099–1104
8. Gregory, S.H., Wing, E.J., Tweardy, D.J., Shadduck, R.K., and Lin, H.S. (1992) Primary listerial infections are exacerbated in mice administered neutralizing antibody to macrophage colony-stimulating factor. *J. Immunol.* **149**, 188–193
9. Asakura, E., Hanamura, T., Umemura, K., Yada, K., Yamauchi, T., and Tanabe, T. (1996) Effects of macrophage colony-stimulating factor (M-CSF) on lipopolysaccharide (LPS)-induced mediator production from monocytes in vitro. *Immunobiology* **195**, 300–313
10. Rosenfeld, M.E., Yla-Herttuala, S., Lipton, B.A., Ord, V.A., Witztum, J.L., and Steinberg, D. (1992) Macrophage colony-stimulating factor mRNA and protein in atherosclerotic lesions of rabbits and human. *Am. J. Pathol.* **140**, 291–300
11. Asakura, E., Tojo, N., and Tanabe, T. (1999) Monocyte proliferation by modified serum is associated with endogenous M-CSF production: An evidence for involvement of signaling pathway via scavenger receptor. *Cell Proliferat.* **32**, 185–194
12. Motoyoshi, K. and Takaku, F. (1989) Serum cholesterol-lowering activity of human monocytic colony-stimulating factor. *Lancet* **2**, 326–327
13. Shimano, H., Yamada, N., Ishibashi, S., Harada, K., Matsu-

moto, A., Mori, N., Inaba, T., Motoyoshi, K., Itakura, H., and Takaku, F. (1990) Human monocyte colony-stimulating factor enhances the clearance of lipoproteins containing apolipoprotein B-100 via both low density lipoprotein receptor-dependent and -independent pathways in rabbits. *J. Biol. Chem.* **265**, 12869–12875
14. Stoudemire, J.B. and Garnick, M.B. (1991) Effects of recombinant human macrophage colony-stimulating factor on plasma cholesterol levels. *Blood* **77**, 750–755
15. Shimano, H., Yamada, N., Motoyoshi, K., Matsumoto, A., Ishibashi, S., Mori, N., and Takaku, F. (1990) Plasma cholesterol-lowering activity of monocyte colony-stimulating factor (M-CSF). *Ann. N.Y. Acad. Sci.* **587**, 362–370
16. Inoue, I., Inaba, T., Motoyoshi, K., Harada, K., Shimano, H., Kawamura, M., Gotoda, T., Oka, T., Shiomi, M., Watanabe, Y., Tsukada, T., Yazaki, Y., Takaku, F., and Yamada, N. (1992) Macrophage colony-stimulating factor prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbits. *Atherosclerosis* **93**, 245–254
17. Ishibashi, S., Inaba, T., Shimano, H., Harada, K., Inoue, I., Mokuno, H., Mori, N., Gotoda, T., Takaku, F., and Yamada, N. (1990) Monocyte colony-stimulating factor enhances uptake and degradation of acetylated LDL cholesterol esterification in human monocyte-derived macrophages. *J. Biol. Chem.* **265**, 14109–14117
18. Ishibashi, S., Yamada, N., Shimano, H., Inaba, H., Mori, N., and Takaku, F. (1990) Effect of monocyte colony stimulating factor (M-CSF) on lipoprotein metabolism. *Ann. N.Y. Acad. Sci.* **598**, 556–557
19. Clinton, S.K., Underwood, R., Hayes, L., Sherman, M.L., Kufe, D.W., and Libby, P. (1992) Macrophage colony-stimulating factor gene expression in vascular cells and in experimental and human atherosclerosis. *Am. J. Pathol.* **140**, 301–316
20. Mori, N., Gotoda, T., Ishibashi, S., Shimano, H., Harada, K., Inaba, T., Takaku, F., Yazaki, Y., and Yamada, N. (1991) Effects of human recombinant macrophage colony-stimulating factor on the secretion of lipoprotein lipase from macrophages. *Arterioscler. Thromb.* **11**, 1315–1321
21. Yamada, N., Ishibashi, S., Shimano, H., Inaba, T., Gotoda, T., Harada, K., Shimada, M., Shiomi, M., Watanabe, Y., Kawakami, M., Yazaki, Y., and Takaku, F. (1992) Role of monocyte colony-stimulating factor in foam cell generation. *Proc. Soc. Exp. Biol. Med.* **200**, 240–244
22. Smith, J.D., Trogan, E., Ginsberg, M., Grigaux, C., Tian, J., and Miyata, M. (1995) Decreased atherosclerosis in mice deficient in both macrophage colony-stimulating factor (op) and apolipoprotein E. *Proc. Natl. Acad. Sci. USA* **92**, 8264–8268
23. Qiao, J.H., Tripathi, J., Mishra, N.K., Cai, Y., Tripathi, S., Wang, X.P., Imes, S., Fishbein, M.C., Clinton, S.K., Libby, P., Luis, A.J., and Rajavashisth, T.B. (1997) Role of macrophage colony-stimulating factor in atherosclerosis: Studies of osteopetrotic mice. *Am. J. Pathol.* **150**, 1687–1699
24. Rajavashisth, T., Qiao, J.H., Tripathi, S., Tripathi, J., Mishra, N., Hua, M., Wang, X.P., Loussararian, A., Clinton, S., and Libby, L.A. (1998) Heterozygous osteopetrotic (op) mutation reduces atherosclerosis in LDL receptor-deficient mice. *J. Clin. Invest.* **101**, 2702–2710
25. Watanabe, Y., Inaba, T., Shimano, H., Gotoda, T., Kawamura, M., Shiomi, M., Yazaki, Y., and Yamada, N. (1997) Effect of macrophage colony-stimulating factor on the advanced atherosclerosis in Watanabe heritable hyperlipidemic rabbits. *Horm. Metab. Res.* **29**, 507–509
26. Hata, Y., Shigematsu, H., Tsushima, M., and Aihara, K. (1978) A xerographic method for the quantitative assessment of atherosclerotic lesions. *Atherosclerosis* **29**, 251–258
27. Folch, J., Lees, M., and Stanley, G.H.S. (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**, 497–503
28. Baker, J.R. (1958) *Principles of Biological Microtechnique*, John Wiley and Sons, New York
29. Pescador, R. (1978) Rabbit strain differences in plasma lipoprotein pattern and in responsiveness to hypercholesterolemia. *Life Sci.* **23**, 1851–1861
30. Villiers, W.J.S., Fraster, I.P., Hughes, D.A., Doyle, A.G., and Gordon, S. (1994) Macrophage-colony-stimulating factor selectively enhances macrophage scavenger receptor expression and function. *J. Exp. Med.* **180**, 705–709
31. Inaba, T., Shimano, H., Gotoda, T., Harada, K., Shimada, M., Kawamura, M., Yazaki, Y., and Yamada, N. (1993) Macrophage colony-stimulating factor regulates both activities of neutral and acidic cholesteryl ester hydrolases in human monocyte-derived macrophages. *J. Clin. Invest.* **92**, 750–757
32. Ishii, I., Kimuro, T., Saito, Y., and Hirose, S. (1995) Cholesterol metabolism in monocyte-derived macrophages from macrophage colony-stimulating factor administered rabbits. *Biophys. Biochim. Acta* **1254**, 51–55
33. Ishii, I., Yanagimachi, M., Shirai, K., Saito, Y., and Hirose, S. (1994) Impact of monocyte colony-stimulating factor upon β-very low density lipoprotein (β-VLDL) cholesterol metabolism in tetradecanoyl phorbol acetate-derived THP-1 cells. *Biophys. Biochim. Acta* **1212**, 278–284
34. Tojo, N., Asakura, E., Koyama, M., Tanabe, T., and Nakamura, N. (1999) Effects of macrophage colony-stimulating factor (M-CSF) on protease production from monocyte, macrophage and foam cell in vitro: A possible mechanism for anti-atherosclerotic effect of M-CSF. *Biophys. Biochim. Acta* **1452**, 275–284
35. Schaub, R., Bree, M.P., Hayes, L.L., Rudd, M.A., Rabbani, L.R., Loscalzo, J., and Clinton, S.K. (1994) Recombinant human macrophage colony-stimulating factor reduces plasma cholesterol and carrageenan granuloma foam cell formation in Watanabe heritable hyperlipidemic rabbits. *Arterioscler. Thromb.* **14**, 70–76