Recombinant Human Macrophage Colony-Stimulating Factor Reduces Plasma Cholesterol and Carrageenan Granuloma Foam Cell Formation in Watanabe Heritable Hyperlipidemic Rabbits

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Abstract Previous studies have demonstrated that short-term administration of recombinant human macrophage colony-stimulating factor (rhM-CSF) reduces plasma cholesterol in rabbits, nonhuman primates, and human subjects. This study extended the dose schedule of rhM-CSF to 8 weeks of continuous intravenous infusion (CIVI) in the Watanabe heritable hyperlipidemic (WHHL) rabbit and expanded the scope to include an assessment of macrophage-derived foam cell development. Ten male WHHL rabbits were injected subcutaneously with 1% carrageenan to promote formation of a macrophage-rich foam cell granuloma. rabbits were infused with either vehicle or rhM-CSF at 100 µg/kg per day (weeks 1 through 5) followed by 300 µg/kg per day (weeks 6 through 8). rhM-CSF (100 µg/kg per day) decreased total plasma cholesterol by 45% at 2 weeks compared with controls. The gradual return of plasma cholesterol toward control concentrations over the subsequent 3 weeks correlated with the appearance of circulating antibodies specific to rhM-CSF. Granulomas from rabbits treated with rhM-CSF contained lower concentrations of cholesterol (2.0±0.7 versus 6.1±1.5 µg/mg, P<.03) and cholesteryl ester (0.7±0.4 versus 3.9±1.2 µg/mg, P<.03) than controls. Histological evaluation revealed that granulomas from the rhM-CSF-treated rabbits were more fibrous and contained fewer foam cells than those from controls. Immunohistochemical localization of endogenous macrophage colony-stimulating factor (M-CSF) in granuloma tissue from vehicle-treated rabbits revealed a diffuse, faint staining pattern. Granuloma tissue from rhM-CSF-treated rabbits exhibited staining for M-CSF that was more intense than in tissue from control rabbits. This study showed that the CIVI of rhM-CSF produced a significant reduction in plasma cholesterol and granuloma foam cell formation in WHHL rabbits. The increase in immunoreactive M-CSF in granulomas from rhM-CSF-treated rabbits suggested that M-CSF may have enhanced the viability of macrophages and modulated their function in a manner that stimulated the processing of cholesterol and its mobilization from the granuloma.

Key Words • foam cells • recombinant human macrophage colony-stimulating factor • cholesterol • WHHL rabbits • recombinant proteins • macrophages

M acrophage colony-stimulating factor (M-CSF) is a hematopoietic growth factor that promotes the growth and differentiation of monocytes and macrophages and is necessary for the survival of mature cells. The cloning and expression of the recombinant human M-CSF (rhM-CSF) gene provides quantities of recombinant material sufficient for both experimental and clinical studies of its pharmacological activity. Several studies have demonstrated that both purified human urinary M-CSF and rhM-CSF induce rapid and significant decreases in plasma cholesterol in normal rabbits and primates, Watanabe heritable hyperlipidemic (WHHL) rabbits, and humans with familial hypercholesterolemia. Decreases in low-density lipoprotein (LDL) and increases in high-density lipoprotein (HDL) are most commonly associated with rhM-CSF or M-CSF treatment. M-CSF–treated rabbits exhibit an increase in the clearance of lipoproteins via LDL receptor–dependent and LDL receptor–independent pathways. In addition, increased HDL particle size and enhanced cholesterol efflux from tissue have been observed during rhM-CSF treatment in rabbits, suggesting an increase in reverse cholesterol transport. A recent study in WHHL rabbits addressed the effect of rhM-CSF on the development of atherosclerotic lesions. rhM-CSF treatment (an intravenous 300-µg bolus three times weekly) for 8.5 months reduced aortic cholesterol content and the surface area of aortic lesions. These observations have stimulated interest in a role for rhM-CSF in the pharmacological management of human hyperlipidemias and atherosclerosis.

The circulating monocytes are the major source of the lipid-filled foam cells in a developing fatty streak and the more advanced atherosclerotic lesion. Monocyte adhesion and infiltration of arterial tissue are some of the earliest events in human and experimental atherogenesis. M-CSF mRNA and protein have been observed in atheromatous lesions. M-CSF may be one
cytokine that modulates monocyte migration across the endothelium and also supports survival of these cells within the subendothelial space. In vitro studies indicate that M-CSF–treated macrophages exhibit increased uptake of acetyl-LDL and enhanced cholesterol esterification.\textsuperscript{13,16} M-CSF also regulates scavenger receptor gene expression in cultured monocytes.\textsuperscript{13} In addition, M-CSF stimulates apolipoprotein (apo) E gene expression in vitro,\textsuperscript{13} which is consistent with an enhancement of reverse cholesterol transport.\textsuperscript{7,9} The purpose of the present study was to investigate the effect of rhM-CSF on foam cell formation in vivo. The subcutaneous injection of carrageenan, a long-chain sulfated polysaccharide, results in the formation of a granuloma composed of macrophages that develop into foam cells in hypercholesterolemic rabbits.\textsuperscript{17,18} We used this experimental model of foam cell formation in the WHHL rabbit to define the effect of intravenous rhM-CSF infusions, at doses that reduce plasma cholesterol, on the size and cholesterol content of foam cell lesions as well as their microscopic appearance.

\textbf{Methods}

\textbf{Animal Surgery and Protocol}

Ten male Japanese White WHHL rabbits (National Institutes of Health, Bethesda, Md) averaging 6 to 7 months of age and weighing 2.5 to 3.8 kg were acclimated for a minimum of 2 weeks and subjected to standard health screens. Blood samples for baseline hematologic parameters and plasma cholesterol concentrations were evaluated twice before assigning the rabbits to control (n=5) or rhM-CSF (n=5) groups based on matched plasma total cholesterol values. Five days before the start of dosing on day 0, a single-lumen, indwelling venous catheter (0.40-mm outer diameter, 0.25-mm inner diameter; Micro-Renathane Type MRF, Braintree Scientific, Inc, Braintree, Mass) was surgically implanted into the jugular vein under aseptic conditions. At the time of the catheter placement, 15 mL of a 1% carrageenan solution (RE-95/52 lot RE6595-65, FMC Corp, Marine Colloids Division, Rockland, Me) prepared in sterile water was injected subcutaneously into the midabdominal region.\textsuperscript{17,18} Immediately after surgery, animals were fitted with a nylon equipment vest (Alice King Chatham, Los Angeles, Calif), and externalized catheters were connected to a micropump ambulatory medication infuser (model 2004, Parker Biomedical, Irvine, Calif). Rabbits received intravenous saline for the first 5 days after surgery.

\textbf{Blood Samples and Counting Assays}

Rabbits were tranquilized with 5 mg subcutaneous acepromazine maleate (PromAce, 10 mg/mL, Aveco Co, Inc, Fort Dodge, Iowa), and blood was collected from the central auricular artery. Circulating immunoreactive M-CSF concentration was measured by asymmetric sandwich enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody (HM7/5.9.13, Genetics Institute, Department of Immunology) for capture and a biotinylated polyclonal rabbit anti–rhM-CSF immunoglobulin G (IgG; RAB 263, Genetics Institute, Department of Immunology) for detection. The standard and calibrator for the assay was rhM-CSF, lot No. A04P01 (1.9 mg/mL). The following values constituted the range for this assay: limit of detection, 0.2 ng/mL; ED\textsubscript{50}, 1.5 ng/mL; and upper limit, 10 ng/mL. The coefficient of variation was 18%.

\textbf{Tissue Weights and Cholesterol Measurement}

The rabbits were euthanatized on the first day of week 9. Representative samples of spleen, liver, and granuloma were harvested and weighed for lipid analysis. The aorta of each animal from the arch to the femoral bifurcation was placed in a solution of 1% formaldehyde in phosphate-buffered saline and stored on ice overnight. All adventitial adipose tissue was carefully removed from the aortas to ensure accurate lipid analysis. Additional granuloma tissue samples were placed in 10% formaldehyde for microscopic evaluation.

Aorta, granuloma, liver, and spleen tissue samples were minced and transferred to duplicate preweighed homogenizing chambers for weight measurement. From the minced tissues, 0.5 mg of each sample was used per tissue cholesterol assay. Ethanol (9 mL of 95%) and 3 mL diethyl ether were added to one sample for determination of free cholesterol. A 4.7-mL volume of 95% ethanol and 0.3 mL of 33% (weight per volume) potassium hydroxide were added to the duplicate sample for total cholesterol determination. Each sample was homogenized for approximately 15 minutes.

Homogenates for analysis of free cholesterol were incubated at room temperature for 5 minutes and centrifuged at 55g for 10 minutes. Homogenates for determining total cholesterol were incubated for 15 minutes in a 55°C water bath. The supernatants were removed and dried with nitrogen until the solvent was completely removed or evaporated. The samples were tightly sealed and stored at −20°C until analysis. Samples were resuspended in 400 μL chloroform and analyzed by using a Hewlett Packard 5890 gas chromatograph (Hewlett Packard, Palo Alto, Calif). A cholesterol standard curve (the area under the curve versus concentration) was generated by using cholesterol purchased from Nu Chek Prep, Inc, Elysian, Minn, before running samples. Free cholesterol concentration was determined from the standard curve. The total amount of free cholesterol in tissues was calculated by multiplying the concentration and volume of resuspended sample and was expressed per milligram of tissue. Total cholesterol was similarly calculated. We determined a correction factor to compensate for the inefficient recovery of cholesteryl ester by similarly processing a known amount of cholesteryl oleate and linoleate (Sigma Chemical Co, Inc, St Louis, Mo). Cholesteryl ester content of tissue was calculated as the difference between total and free cholesterol per milligram of tissue.

\textbf{Histology and Immunohistochemistry}

Sections of granulomas were cut at 5 μm, floated onto chromium potassium sulfate–coated slides, and baked at 58°C overnight before immunohistochemical staining for macrophages and M-CSF. Macrophages were stained using RAM 11
**Statistical Analysis**

Multiple group comparisons were analyzed by using one-way ANOVA followed by a Newman-Keuls evaluation. Unpaired t tests were used for comparison between treated and untreated groups.

**Results**

**Plasma rhM-CSF and Antibody Titer Measurement**

The plasma concentration of immunoreactive M-CSF in control animals was less than the level of detection (0.2 ng/mL) during the entire study period (Fig 1). The rhM-CSF–treated rabbits had 6.0 ng/mL of circulating immunoreactive M-CSF after 1 week of CIVI at 100 μg/kg per day, and their concentrations remained between 2.0 and 5.0 ng/mL through week 5 (Fig 1). An increase in the dose of rhM-CSF to 300 μg/kg per day after 5 weeks produced a slight elevation of plasma immunoreactive M-CSF to 9.0 ng/mL during week 7. However, by week 8, the plasma immunoreactive M-CSF had decreased to 2 ng/mL. Antibody specific to rhM-CSF was detected in treated rabbits by week 3 (Fig 2). The initial antibody titer was modest, representing approximately 1000 ppm of total IgG. However, 2 weeks after the increase in dose to 300 μg/kg per day, the proportion of circulating IgG directed against rhM-CSF increased 120-fold.

**Plasma Cholesterol and Blood Counts**

Baseline plasma cholesterol values were 522±47 and 535±80 mg/dL for control and rhM-CSF–treated rabbits, respectively. Vehicle-treated rabbits had no significant change in plasma cholesterol during the first 5 weeks. However, during the last 3 weeks, there was a gradual decline in plasma cholesterol that was probably secondary to the prolonged stress of continuous intravenous catheterization. Plasma cholesterol decreased by 30% after 1 week of rhM-CSF CIVI (Fig 3) and continued to decrease during the second week, reaching a nadir of 45% compared with the pretreatment plasma concentration (Fig 3). During weeks 3 through 5, there was a gradual rise in the plasma cholesterol of rhM-CSF–treated rabbits, a trend that was stabilized briefly after the increase in the dose to 300 μg/kg per day.

A transient decrease in platelets was observed for the first week after the initiation of treatment at both doses of rhM-CSF (Fig 4). There was a transient increase in total white blood cell (WBC) count in control and rhM-CSF–treated groups during week 1 (Fig 5). The WBC count in the control group returned to baseline at week 2, whereas the rhM-CSF–treated animals developed a sustained decrease in the WBC count that was primarily due to a decrease in circulating neutrophils (data not shown). Peripheral monocyte, eosinophil, and basophil counts were not altered by rhM-CSF treatment.
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Fig 3. Line graph showing that plasma cholesterol decreased by 30% after 1 week of recombinant human macrophage colony-stimulating factor treatment and continued to decrease by 45% after 2 weeks. A gradual increase in plasma cholesterol was observed from weeks 3 through 6 in rhM-CSF-treated rabbits and was stabilized after initiation of the 300 μg/kg per day dose. Control animals showed a decrease in plasma cholesterol during the last 3 weeks of the experiment. Values represent mean±SEM. Plasma cholesterol was significantly less (P<.05) in rhM-CSF-treated rabbits compared with controls during weeks 1 through 4.

Tissue Weights and Cholesterol Content

Vehicle-treated rabbits had large (7.1±1.6 g, mean±SEM) granulomas with a white appearance typical of lipid accumulation in these tissues (Fig 6). Granulomas from rhM-CSF-treated rabbits were smaller (2.8±0.7 g, P<.05) and more fibrotic in gross appearance than those of controls. Granuloma total cholesterol in control rabbits was 6.1±1.5 μg/mg; rhM-CSF decreased this value to 2.0±0.7 μg/kg in treated rabbits (P<.03).

Cholesteryl ester levels were also significantly decreased in the granulomas of rabbits treated with rhM-CSF (3.9±1.2 versus 0.7±0.4 μg/mg, P<.03). Examination of the aortic luminal surface from control and rhM-CSF-treated rabbits did not reveal any noticeable differences in the surface area covered by atherosclerotic lesions. Aortic cholesteryl ester concentrations were significantly less in rhM-CSF-treated rabbits compared with controls (0.2±0.3 versus 1.0±0.1 μg/mg, P<.05), although no statistically significant differences were observed for aortic total cholesterol concentrations (1.6±0.3 versus 2.1±0.8 μg/mg, P>.05). rhM-CSF-treated and control rabbits exhibited no significant differences in cholesterol content of the spleen (2.1±0.5 versus 2.0±0.4 μg/mg, P<.05) or liver (1.6±0.3 versus 2.1±0.8 μg/mg, P>.05). Additionally, rhM-CSF-treated and control rabbits exhibited no significant differences in liver weight (32.85±1.21 versus 30.84±1.67 g/kg) or spleen weight (0.53±0.04 versus 0.48±0.06 g/kg).

Histology and Immunohistochemistry

Granulomas from vehicle-treated animals contained large numbers of macrophage-derived foam cells (Fig 7a) that exhibited positive staining with RAM 11 (Fig 7b). Granulomas from rhM-CSF-treated rabbits were qualitatively different. Small portions of the granulomas from rhM-CSF-treated rabbits showed macrophage accumulations similar to those seen in controls (Fig 7c). The remainder of the granuloma tissues from rhM-CSF-treated rabbits consisted of fibroblast-like cells as well as connective tissue and skeletal muscle fibers that had become integrated into the lesion (Fig 7d). Vehicle-treated animals exhibited some immunoreactive M-CSF in granulomas that was primarily observed in the extracellular space between foam cells (Fig 7e). Few macrophages stained positively for immunoreactive M-CSF.
Granuloma tissue of rhM-CSF-treated rabbits exhibited more intense extracellular and intracellular staining than did vehicle-treated animals (Fig 7f).

Discussion

The continuous infusion of rhM-CSF in rabbits resulted in measurable increases in the plasma concentrations of immunoreactive M-CSF. The slight decline in plasma concentration of rhM-CSF seen after the first week of treatment may have been due to an overall increase in the population of monocytes/macrophages that exhibit the receptor for M-CSF called c-fms. An expanded population of cells expressing c-fms accelerates M-CSF clearance. The decline in plasma rhM-CSF concentration observed during week 7 of the study may have been due to the rising anti-rhM-CSF antibody titer observed after initiation of the higher dose of rhM-CSF after week 5. The production of neutralizing antibodies to recombinant human proteins in laboratory animals makes it difficult to complete and interpret prolonged studies designed to evaluate the effects of these proteins on chronic diseases such as atherosclerosis. A definitive evaluation of the effects of pharmacological dosages of M-CSF on experimental atherogenesis will require the use of an autologous recombinant protein that does not induce the formation of neutralizing antibodies.

Total plasma cholesterol concentrations were decreased by rhM-CSF infusion as previously reported. The progressive recovery of cholesterol levels after week 2 correlated with the appearance of circulating anti-rhM-CSF antibody. Although plasma cholesterol declined after the week-5 increase of rhM-CSF to 300 μg/kg per day, the associated elevation of anti-rhM-CSF antibody limited the effectiveness of continued drug administration and was the basis for our decision to terminate treatment after 56 days. A reduction in plasma cholesterol within the range achieved by rhM-CSF in this study has been demonstrated to reduce the rate of progression and promote regression of athero-}

![Granuloma tissue of rhM-CSF-treated rabbits exhibiting more intense extracellular and intracellular staining than did vehicle-treated animals (Fig 7f).](image)

![Fig 6. Bar graphs showing that granulomas from recombinant human macrophage colony-stimulating factor from Watanabe heritable hyperlipidemic rabbits were smaller (P <.05) and contained lower concentrations of cholesterol (P <.03) and cholesteryl esters (P <.03) than granulomas from control animals. Values represent mean±SEM.](image)
recent study showing that rhM-CSF enhances the mobilization of cholesterol from tissues to the circulation. The importance of endogenous cytokines, growth factors, and immunomodulators in atherogenesis is now appreciated but not well understood. The possibility must be considered that M-CSF produced endogenously by cells of the vessel wall may modulate the production of many of these factors within the lesion and thus alter the atherogenic process. Although M-CSF has been reported to enhance the production of interleukin-1 and to prime monocytes for the synthesis of tumor necrosis factor–α in vitro, other studies suggest that M-CSF may be a negative modulator of inflammation. For example, rhM-CSF–treated macrophages were found to produce less interleukin-1 and prostaglandin E2 in response to endotoxin stimulation. rhM-CSF has been shown to stimulate synthesis of interferon alfa and beta by bone marrow–derived macrophages. Interferon activity has been demonstrated in atherosclerotic lesions, and administration inhibits atherogenesis in cholesterol-fed rabbits. Furthermore, aortic lesions from interferon-treated rabbits contained...
significantly less cholesterol and fewer macrophages. 

At the present time, our knowledge is incomplete concerning the ability of endogenously produced or exogenously administered M-CSF to alter the relative balance between proatherogenic and antiatherogenic mediators within the developing foam cell lesion. However, this study did support the hypothesis that exogenous M-CSF will reduce cholesterol accumulation by macrophages and inhibit atherogenesis.

In summary, administration of rhM-CSF reduced serum cholesterol and inhibited the formation of foam cell lesions in the WHHL-carrageenan model. Although the pathophysiological mechanisms whereby M-CSF mediates this process have not been clearly defined, future studies to investigate a therapeutic role for exogenously administered rhM-CSF in atherogenesis are warranted.

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