Effects of Recombinant Human Macrophage Colony-Stimulating Factor on Plasma Cholesterol Levels

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Recombinant human macrophage colony-stimulating factor (rhM-CSF) is a hematopoietic growth factor that stimulates the growth, differentiation, proliferation, and activation of cells of the monocyte/macrophage lineage. rhM-CSF was administered to rabbits and nonhuman primates to evaluate effects on cholesterol homeostasis. Decreases in plasma cholesterol concentrations were observed during rhM-CSF administration. The observed mean (±SD) decreases over a range of doses in nonhuman primates receiving rhM-CSF by continuous intravenous infusion (CIVI) or intravenous bolus (IVB) injection were approximately 16% ± 8% and 43% ± 10%, respectively. Low-density lipoprotein (LDL) cholesterol levels decreased 55% ± 9% from pretreatment baseline values in the animals receiving rhM-CSF by IVB. Normocholesterolemic New Zealand white rabbits receiving rhM-CSF over a range of doses by CIVI showed a decrease from baseline in total cholesterol of approximately 28% ± 17%, with LDL cholesterol levels decreasing by approximately 72% ± 33%, while high-density lipoprotein levels showed variable changes, including increased values. A decrease of 36% ± 26% in total plasma cholesterol was observed in Watanabe Heritable Hyperlipidemic rabbits receiving rhM-CSF by CIVI for 7 days. This decrease was attributable almost entirely to decreases in LDL cholesterol, which fell approximately 34% ± 24% from baseline. Although the mechanism of this cholesterol-lowering effect is unknown, these results strongly suggest that rhM-CSF may provide a novel treatment for hypercholesterolemia and may be useful in investigations into the mechanisms of cholesterol homeostasis and atherogenesis.

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THE NATIVE LOW-DENSITY lipoprotein (LDL) receptor is expressed on mouse peritoneal macrophages, human peripheral blood monocytes, and monocyte-derived macrophages. However, in vitro studies have identified additional receptors on monocytes and macrophages that mediate the uptake of chemically modified LDL. The modifications that convert native LDL into the form recognized by this receptor include acetylation and oxidation. Additional studies have shown that LDL incubated with endothelial cells, smooth muscle cells, or monocytes/macrophages can undergo chemical modification into a form that is also recognized by this receptor and another receptor. These receptors have collectively been referred to as scavenger receptors. Recently, two of these receptors have been cloned from bovine lung and from familial hypercholesterolemic homozygotes, are isolated from the reticuloendothelial system (RES) by ethyl oleate. This treatment, which induced a significant reduction of LDL receptor-independent LDL catabolism, implicated the RES in LDL clearance by this pathway.

Additional evidence of the role of the macrophage in cholesterol homeostasis comes from evaluations of LDL cholesterol metabolism in patients suffering from myeloproliferative disorders (MPD). In these patients there are low levels of LDL cholesterol, which are associated with increased nonreceptor-mediated catabolism of LDL. It has also been noted in MPD patients that monocyte/macrophages were increased in number and showed increased signs of activity as suggested by enhanced removal of intravenously administered heat-aggregated albumin.

In a phase I/II human clinical trial in which patients received human granulocyte-macrophage colony-stimulating factor (GM-CSF), which stimulates the production and activation of cells of monocytic lineage, there were profound decreases in serum cholesterol concentration during treatment. Preliminary findings showed that the cholesterol decreases were caused by decreases in LDL cholesterol with variable changes in high-density lipoprotein (HDL) cholesterol.

Further evidence of the role of the monocyte/macrophage system in the receptor-independent catabolism of LDL comes from the observation that macrophages, isolated from familial hypercholesterolemic homozygotes, are replete with cholesteryl ester. Because these cells do not possess a specific receptor for native LDL, it is presumed that the accumulation of cholesteryl ester is a result of LDL uptake by receptor-independent mechanisms or the scavenger pathway.

The results of these studies and observations suggest that increasing the number or activation of monocytes and macrophages could increase the rate of LDL uptake and degradation through LDL receptor-mediated and receptor-independent processes or the scavenger pathway, and thereby lead to a decrease in total plasma cholesterol.

Recombinant human macrophage CSF (rhM-CSF) is a hematopoietic growth factor that stimulates the growth, differentiation, proliferation, and activation of cells of the monocyte/macrophage lineage. The genes for this protein have been successfully cloned and expressed. Administration of rhM-CSF to rodents, rabbits, and nonhuman primates has been associated with a nondose-related increase in circulating peripheral blood monocytes and large macrophage-like cells. These cells may account for up to 70% of the white blood cell differential. A variety of biologic
activities have been induced by rhM-CSF. Peripheral blood monocytes obtained from rhM-CSF-treated monkeys were found to have increased expression of IA antigen, LFA3, CD16, and CD14 antigen, and were able to mediate antibody-dependent cell-mediated cytolysis against melanoma and neuroblastoma targets in in vitro assays. In preliminary studies in our laboratories, we noted a marked reduction in the plasma cholesterol of primates receiving rhM-CSF alone or in combination with rhGM-CSF.

In the current study we have investigated the effects of parenterally administered rhM-CSF on plasma cholesterol levels in normocholesterolemic rabbits, nonhuman primates, and Watanabe heritable hyperlipidemic (WHHL) rabbits.

MATERIALS AND METHODS

Recombinant protein. rhM-CSF was cloned and expressed in Chinese hamster ovary cells at Genetics Institute (Cambridge, MA). rhM-CSF is a 90-Kd homodimer consisting of two 25-Kd disulphide-bonded monomers of 223 amino acids, each with 2 N-linked glycosylation sites and numerous O-linked glycosylation sites. The specific activity was greater than 5 x 10^6 U/mg as measured by an in vitro bone marrow stimulation assay. The purified protein was shown to be sterile and pyrogen and endotoxin free.

Animals. Female New Zealand White (NZW) and WHHL rabbits, weighing 2.5 to 3.5 kg, and male cynomolgus monkeys, weighing 3.6 to 7 kg, were used in all experiments. The animals were unfasted during evaluations and fed Purina laboratory chows (Ralston Purina, St Louis, MO) and given water ad libitum. The animals were housed and cared for under the National Institutes of Health guidelines.

Procedures. rhM-CSF was administered as an intravenous bolus injection (IVB), subcutaneous (SC) injection, or continuous intravenous infusion (CIVI). For CIVI, a catheter was surgically implanted into the iliac vein of the nonhuman primate, or into the external jugular vein of the rabbit. A Broviac access catheter (6.6 F; Evermed, Cranston, RI) was used for primates, and Micro- Renathane tubing (Braintree Scientific, Braintree, MA) was used for rabbits. The catheter was exteriorized and connected to an ambulatory infusion pump (Parker Micro-pump, Model 2004; Parker, Irvine, CA), which was held in a jacket (Alice King Chatham Medical Arts, Los Angeles, CA) worn by the animal. This setup allowed a continuous IV delivery of the protein at a selected dosage rate over specified time intervals. The protein in these studies was administered at a dose of 10 to 300 μg/kg/d, administered by a CIVI for one cycle of 7 to 14 days, followed by a break period for up to 14 days. The rabbits received a single cycle of dosing; the nonhuman primates received one to three cycles of treatment. The protein was also administered by daily IVB to primates at a dose of 100 or 1,000 μg/kg/d for 14 consecutive days, and by daily SC injections to NZW rabbits for 7 to 14 days at dose levels of 10 to 300 μg/kg/d.

Hematology parameters, including complete blood cell count, differential, platelet count, and plasma cholesterol concentrations were evaluated before dosing, and at intervals during and after the dosing period. Plasma cholesterol concentrations were measured by an automated method (Technicon Auto-Analyzer, Terrytown, NY) in some studies and by an enzymatic method (Stanbio, San Antonio, TX) in others. In selected studies, HDL and LDL cholesterol concentrations were determined by an enzymatic method (Stanbio), following a combination of ultracentrifugal and chemical (MgCl₂-dextran sulfate) separation of the apolipoprotein B containing lipoprotein fractions. The cholesterol concentrations reported in these studies represent the nadir concentrations with respect to baseline observed during the treatment period, and the peak cholesterol concentrations after the treatment period. Acute-phase reactant proteins, including α1-antitrypsin, transferrin, C3, fibrinogen, and orosomucoid were measured in primates receiving one to three cycles of treatment by CIVI.

Isolation of LDL. LDL was prepared from fresh NZW rabbit plasma by sequential ultracentrifugation at a background density of 1.025 to 1.055 g/mL. The LDL was recentrifuged once at a density of 1.055 g/mL and dialyzed overnight at 4°C against 0.15 mol/L NaCl, 0.01% EDTA, pH 7.4. The dialyzed LDL was iodinated by the technique of Bilheimer et al. Unreacted iodine was removed by chromatography on Sephadex G-10 (Pharmacia, Uppsala, Sweden), followed by dialysis overnight at 4°C against 0.15 mol/L NaCl. Lipid labeling was uniformly less than 8%, and more than 98% of the label was protein bound as shown by precipitation with 10% trichloroacetic acid (TCA).

LDL turnover. The plasma clearance of LDL was evaluated in untreated NZW rabbits and treated animals receiving rhM-CSF by CIVI at a dose of 100 μg/kg/d for 7 consecutive days. The evaluations of the treated animals were performed 7 to 21 days following the treatment with rhM-CSF. In these studies, radioiodinated LDL was injected into a marginal ear vein and blood samples were collected at intervals from the opposite ear vein. Plasma was isolated and assayed for TCA precipitable radioactivity. Plasma radioactivity time curves were constructed from these data and the fractional catabolic rates (FCR) were derived from the best fit of these tracer data.

RESULTS

Primate, CIVI. In normocholesterolemic primates (n = 6) receiving rhM-CSF by CIVI at dose levels of 25 to 175 μg/kg/d for one to three cycles of treatment, dose-related decreases in plasma cholesterol concentrations were seen in five of six primates during the first cycle of treatment, in three of four of these animals in the second cycle treatment, and in two of two animals receiving a third cycle of treatment. The mean (±SD) decrease from baseline LDL cholesterol was 16% ± 8% with a range of 3% to 30%. The mean (±SD) decreases were 22% ± 7%, 8% ± 4%, and 12% for cycles one, two, and three, respectively. The pretreatment, treatment, and posttreatment plasma cholesterol values (all cycles) were 132 ± 12, 114 ± 15, and 132 ± 8 mg/dL, respectively (Fig 1).

Primate, IVB. In primates (n = 6) receiving rhM-CSF by IVB at a dose of 1 mg/kg/d for 14 consecutive days, the mean plasma cholesterol concentration decreased 43% from a pretreatment baseline value of 153 ± 24 mg/dL to a mean value of 89 ± 25 mg/dL during the treatment period. The LDL decreased 55% from a baseline pretreatment value of 67 ± 15 mg/dL to a treatment value of 30 ± 8 mg/dL. In this study, the HDL cholesterol also decreased 33% from a pretreatment baseline value (mean ± SD) of 85 ± 13 mg/dL to 57 ± 22 mg/dL.

NZW Rabbit, CIVI or SC. At dose levels of 50 to 300 μg/kg/d administered by CIVI or SC injection, 14 of 16 animals showed a decrease in plasma cholesterol concentration from a mean pretreatment value of 83 ± 26 to a nadir treatment value of 63 ± 23 mg/dL. (Fig 2). LDL and HDL cholesterol were measured in 8 of the 16 animals. LDL

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Fig 1. Effects of rhM-CSF on plasma cholesterol levels of cynomolgus monkeys. rhM-CSF was administered by CIVI for one to three cycles of 14 days on and 14 days off at doses of 25 to 175 μg/kg/d. Plasma cholesterol concentrations were measured pretreatment, during treatment, and after treatment. (A) Mean (±SD) of plasma cholesterol levels for all cycles. (B) Individual plasma cholesterol values for animals for all cycles.

decreased in seven of the animals (72% ± 33%) and increased (6%) in one animal. HDL levels were increased (33% ± 7%) in three animals, decreased (32% ± 16%) in four animals, and were unchanged in one animal. No consistent decreases in plasma cholesterol concentrations were observed in normocholesterolemic NZW rabbits receiving rhM-CSF by CIVI or SC injection at lower dose levels of 10 to 30 μg/kg/d for 14 consecutive days.

WHHL rabbit. WHHL rabbits receiving rhM-CSF by CIVI for 14 consecutive days showed a decrease in plasma cholesterol from a pretreatment mean (±SD) value of 606 ± 166 mg/dL to a mean treatment value of 363 ± 64 mg/dL, a decrease of almost 40%. The decrease in cholesterol was attributable to a decrease in LDL cholesterol, which fell from 475 ± 147 mg/dL pretreatment to 292 ± 37 mg/dL during treatment, a decrease of approximately 39%. However, HDL cholesterol increased almost 400% from a mean pretreatment value of 6 ± 1 mg/dL to 25 ± 7 mg/dL during treatment.

LDL clearance. 125I-labeled LDL was cleared more rapidly from the plasma of NZW rabbits receiving rhM-CSF by CIVI at a dose level of 100 μg/kg/d, as compared with control NZW rabbits. The mean FCR in treated rabbits (n = 3) was 3.75 pools per day, as compared with a mean FCR of 2.07 pools per day in untreated rabbits (n = 3).

Hematologic effects. The most consistent hematologic effect noted in the animals receiving rhM-CSF was a nondose-related increase in circulating blood monocytes and large vacuolated macrophage-like cells (Fig 3). Up to 35% to 70% of the circulating white blood cell differential consisted of these macrophage-like cells. When protein was discontinued, the monocyte numbers returned to pretreatment values. A dose-related decrease in platelet count was also noted at doses greater than 100 μg/kg/d. This effect was rapidly reversible after rhM-CSF discontinuation.

Histopathologic findings. Liver and spleen biopsies were taken from primates receiving rhM-CSF by IVB at a dose of 1,000 μg/kg/d for 14 consecutive days. The most notable histopathologic finding in these tissues was an infiltration of macrophages into these organs, with evidence of extra- medullary hematopoiesis.

DISCUSSION

In these studies, we have shown that the administration of rhM-CSF to normocholesterolemic rabbits and nonhuman primates and WHHL rabbits resulted in decreases in both total plasma cholesterol levels, and, in some cases,
EFFECTS rhM-CSF ON PLASMA CHOLESTEROL LEVELS

Fig 3. Peripheral blood response in NZW rabbits to rhM-CSF. rhM-CSF was administered to NZW rabbits by CIVI for 7 to 14 days at dose levels of 100 μg/kg/d. This figure shows large macrophage-like cells in a peripheral blood smear. These cells can be one of the preponderant cell types in the differential.

decreases in LDL cholesterol levels. Also noted in animals receiving rhM-CSF were variable increases in the number of circulating monocytes and macrophage-like cells and an increased infiltration of macrophages into the liver and spleen. Although our observations do not clearly indicate the mechanism by which plasma cholesterol concentrations were lowered, a possible mechanism would be through an enhancement of monocyte/macrophage-mediated clearance of plasma cholesterol. Macrophages have been shown to take up LDL through receptor-mediated endocytosis using functional LDL receptors or through scavenger pathways, which include a high-affinity receptor-mediated uptake of a modified LDL, and through nonreceptor-mediated processes, including bulk pinocytosis. These pathways are not regulated by LDL uptake and could account for a continued removal of LDL cholesterol from the plasma and interstitial fluids. Therefore, rhM-CSF-mediated increases in the number and function of cells of the monocytic lineage could lead to enhanced LDL clearance. The increased fractional catabolic rate of LDL seen in these studies argues that the decrease in plasma cholesterol is mediated through increased clearance, rather than through a decrease in production of LDL.

Goldstein et al suggested that LDL cholesterol could be lowered by pharmacologic methods in the absence of LDL receptors. These methods included decreasing LDL production or stimulation of an alternative LDL receptor-independent catabolic pathway. Increasing the number of monocytes/macrophages in an animal could lead to a decreased rate of production of LDL by an increased catabolism of LDL precursors, or through increased uptake and catabolism of LDL by the scavenger pathway. There is evidence that the modified forms of LDL cleared by this pathway exist in vivo and that this modified LDL is a physiologic ligand for the scavenger receptor. However, these biologically modified forms are only thought to be present in tissue microenvironments rather than the circulation. Any circulating modified LDL would be rapidly cleared by tissue macrophages. M-CSF stimulates the activation of monocytes and macrophages. These activated cells will produce superoxide anions which, in vitro, have been shown to oxidize LDL. Increasing the number and activation of monocytes/macrophages in vivo may lead to oxidation of LDL with subsequent removal by the scavenger receptor.

In the present study, the increases in peripheral blood monocytes were variable and no direct correlation could be made between monocyte number and cholesterol lowering. However, there were increases in the spleen and liver weights of the nonhuman primates receiving rhM-CSF. The
organ weights were increased as a result of an infiltration of macrophages. This observation is consistent with macrophage-mediated enhancement of LDL catabolism in these animals.

Another potential explanation for the observed decrease in plasma cholesterol concentrations could be an effect related to an acute-phase response. The acute-phase response, which accompanies infection or trauma, has been associated with decreases in total plasma cholesterol and LDL and HDL cholesterol. Evaluations of the effects of rhM-CSF on clinical laboratory parameters in primates have shown no evidence for an acute-phase response; these results argue against this effect as a mechanism of cholesterol lowering.

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Enhancement of LDL uptake by macrophages mediated by rhM-CSF could theoretically cause a progression of plaque development. However, it has been shown that macrophages can excrete cholesterol when exogenous cholesterol acceptors are present and that macrophages simultaneously synthesize and excrete large amounts of apolipoprotein E (ApoE). The secreted ApoE and cholesterol could associate with HDL to produce HDL. The HDL could be rapidly taken up by the liver, and thereby facilitate “reverse cholesterol transport.” Recent studies by Mahley et al have suggested that increased amounts of ApoE in the plasma may decrease plasma cholesterol concentrations by accelerating the clearance of lipoprotein remnants. Therefore, this mechanism could promote regression of the atherosclerotic plaque. Measurements of serum ApoE and HDL levels in rhM-CSF-treated animals and evaluations of plaque development in these animals could help elucidate the potential effect rhM-CSF on atherogenesis.

To more fully evaluate the role of the monocyte/macrophage in cholesterol homoeostasis, additional studies are currently underway. These studies include evaluations of both receptor- and nonreceptor-mediated clearance of LDL in rhM-CSF–treated animals, the evaluation of LDL uptake and degradation by peripheral blood monocytes and macrophage-like cells recovered from animals treated with rhM-CSF, and additional prospective evaluations of changes in lipoprotein cholesterol concentrations in response to rhM-CSF dosing in both normal and hypercholesterolemic animals.

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