Role of Monocyte Colony-Stimulating Factor in Foam Cell Generation (43427)

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Abstract. **Recently, we reported that human monocyte colony-stimulating factor (M-CSF) stimulates the clearance of lipoproteins containing apoB100 via both low density lipoprotein receptor-dependent and -independent pathways in target cells of M-CSF, and reduces plasma cholesterol level (Journal of Biological Chemistry, 265:12869–12875, 1990). This suggests a linkage of cytokines to the metabolic regulation of plasma cholesterol. Furthermore, we found a significant role of M-CSF in cholesterol metabolism of human monocyte-derived macrophages. M-CSF enhanced not only the uptake of acetylated low density lipoprotein and oxidized low density lipoprotein in macrophages, but also the efflux of cholesterol from cholesterol-loaded macrophages. To elucidate *in vivo* effects of M-CSF on cholesterol efflux from tissues, we administered an intravenous injection of ^{3}H-cholesterol (150 μCi) into WHHL rabbits 1 month before starting M-CSF treatment. We observed an increased cholesterol efflux from tissues to plasma high density lipoprotein after M-CSF treatment when cholesterol efflux was estimated as the change in specific radioactivity of plasma high density lipoprotein-cholesterol. This result suggests that M-CSF can enhance the excretion of cholesterol from target cells of M-CSF, such as cholesterol-loaded macrophages in the arterial wall, and reduce the rate of atherogenesis.**

[P.S.E.B.M. 1992, Vol 200]

The human monocyte-specific colony-stimulating factor (CSF1, M-CSF) is an 85-kDa glycoprotein that stimulates the proliferation and differentiation of monocytic progenitor cells (1, 2). Besides its actions as a growth factor, M-CSF stimulates a number of functions of mature macrophages (3–13). Macrophages endocytose a variety of lipoproteins, including β-very low density lipoprotein, large very low density lipoprotein, and low density lipoprotein (LDL) through the LDL receptor (14–16) and acetylated LDL and oxidized LDL through the scavenger receptor (17, 18), resulting in the accumulation of cholesterol and the generation of foam cells *in vitro*. However, the precise mechanism of the generation of foam cells and the role of macrophages in the arterial wall is not fully understood. In the arterial wall, various cytokines, such as interleukin 1, interleukin 6, tumor necrosis factor-α, γ-interferon, and colony-stimulating factors, have been reported to be synthesized and secreted by vascular cell components, including endothelial cells, monocyte-macrophages, smooth muscle cells, and lymphocytes (19–22). These cytokines are presumed to play important roles in the process of atherosclerosis. Among cytokines, M-CSF specifically regulates macrophage activity and it is well known that macrophage is one of major cells in atherosclerotic plaque. Thus, it is very possible that M-CSF plays a significant role in atherogenesis.

Recently, we demonstrated that M-CSF enhanced both uptake and efflux of cholesterol in human monocyte-derived macrophages (23). Thus, M-CSF can modulate the atherosclerotic process involving macrophages in the arterial wall; however, it is difficult to estimate the amount of modified LDL generated in the arterial wall. In the present study, we evaluated the cholesterol excretion from tissues to study the effects of M-CSF on the atherogenic process *in vivo*.

Materials and Methods

Materials. Sodium [^{125}I]iodide (629 TBq/μg) and [1-^{14}C]oleic acid (1.11–2.22 GBq/mmol) were pur-

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0037-9727/92/2002-0240$3.00/0

chased from ICN Radiochemicals. Cholesteryl [^{3}H] oleate (1.9 GBq/mmol) was purchased from New England Nuclear. 1,2-n[^{3}H]-Cholesterol was purchased from Amersham International. Recombinant human M-CSF was a purified product obtained from Morinaga Milk Industry Co., Ltd. (24). Recombinant human granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor were purified products obtained from Kirin Brewery Co., Ltd. Culture dishes and plates were obtained from Corning. Lymphoprep was purchased from Daiichi Pharmaceutical Co. RPMI 1640 culture medium, penicillin-streptomycin, and L-glutamine were obtained from GIBCO. All other chemicals were of analytical grade.

Cells. Human monocyte-derived macrophages were prepared by culturing human peripheral monocytes. Monocytes were isolated from peripheral blood of a normolipidemic healthy donor using the Ficoll-Hypaque gradient method, as described previously (25). The cells were suspended in RPMI 1640 and plated at 3×10^6 cells/16 mm wells in 12-well plastic plates. After 2 hr of incubation at 37°C in 5% CO_2 and 95% air, nonadherent cells were removed by three washes with phosphate-buffered saline. The cells were then placed in fresh medium containing 10% autologous serum and the medium was changed twice weekly. Monocyte-derived macrophages were used within 10 days after plating.

Lipoproteins. LDL (d = 1.019–1.063 g/ml) were prepared from human plasma containing 0.1% EDTA, 0.02% sodium azide, and 0.5 mg/ml benzamidine, of fasted normolipidemic volunteers (26). The nonlipoprotein fraction after removing HDL was used as lipoprotein-deficient serum (LPDS). LDL were acetylated by repetitive additions of acetic anhydride, as described previously (27). LDL (50–150 μg/ml) containing no EDTA were incubated with 50 μM cupric sulfate in RPMI 1640 at 37°C for 24 hr to oxidize LDL. Lipoproteins were radioiodinated with Na^{125}I using the iodine monochloride method (28). All the lipoprotein preparations were sterilized by filtration (0.45 μm) and used within 1 week.

Degradation of ^{125}I-Labeled Lipoproteins. After preincubation in a medium containing 5 mg protein/ml of LPDS for 24 hr, the macrophage monolayers were incubated with a medium containing the indicated amounts of ^{125}I-labeled lipoproteins (μg protein/ml) and 5 mg/ml of LPDS at 37°C for 24 hr, and lipoprotein degradation was measured. Cell-degraded ^{125}I-labeled lipoproteins were measured according to the method of Goldstein *et al.* (27). The cellular protein was measured by the method of Lowry (29) and the cellular DNA was measured by the method of Labarca and Palgan (30).

Cellular Cholesterol Esterification. After 24 hr of preincubation with LPDS, the cells were incubated with 0.1 mM [1-^{14}C]oleate bound to bovine serum albumin and various concentrations of lipoproteins (μg protein/ml) for 24 hr at 37°C, and radioactivity incorporated into cholesteryl oleate was determined as cellular cholesterol esterification (31).

Cholesterol Excretion. Four rabbits received an intravenous injection of ^{3}H-cholesterol (150 μCi) which was first dissolved in ethanol, then mixed with 2 ml of autologous plasma 1 month before starting M-CSF treatment (32). M-CSF (300 μg) was intravenously injected once a day for 7 consecutive days. Blood samples were obtained at intervals before and after the M-CSF treatment for determination of the concentration and specific activity of total and HDL cholesterol in plasma. HDL was isolated by the precipitation method, as described by Kano *et al.* (32). Radioactivity in plasma or HDL fraction was determined in a liquid scintillation counter.

Results

Uptake and Degradation of ^{125}I-Labeled Lipoproteins by Macrophages. We determined the effects of M-CSF on the uptake of lipoproteins. Both ^{125}I-acetyl-LDL and ^{125}I-oxidized LDL were taken up and degraded by macrophages by a saturable high affinity process (Fig. 1). A 50-fold excess of unlabeled lipoproteins displaced the degradation of ^{125}I-lipoproteins by more than 90%, both in the presence and in the absence of M-CSF. In this experiment, M-CSF augmented the

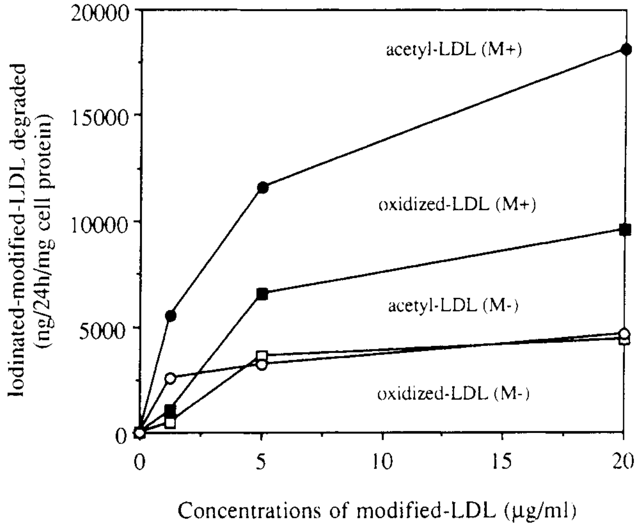

Figure 1. Effects of M-CSF on the uptake and degradation of either ^{125}I-acetyl LDL or ^{125}I-oxidized-LDL of varying concentrations by macrophages. Macrophages were incubated with medium containing 10% autologous serum with or without 128 ng/ml of M-CSF. At the ninth day, the medium was removed and the cells were washed three times with phosphate-buffered saline and preincubated with medium containing 5 mg/ml of LPDS with and without M-CSF for 24 hr. After washing with phosphate-buffered saline three times, each monolayer received 1 ml of medium containing 5 mg/ml of LPDS and varying concentrations of either ^{125}I-acetyl-LDL or ^{125}I-oxidized-LDL with or without a 50-fold excess of the respective unlabeled lipoproteins. After 24 hr at 37°C, the amounts of degraded ^{125}I-labeled lipoproteins were determined. The values are the means of duplicate wells.

B_{max} 5-fold for specific degradation of ^{125}I-acetyl-LDL and 2-fold for ^{125}I-oxidized LDL and did not change the K_m of degradation significantly for both ^{125}I-acetyl-LDL and oxidized LDL.

Effects of Cytokines on Cholesterol Esterification. In addition to M-CSF, there are several cytokines that modulate the function and growth of macrophages and are available in highly pure forms (33). When the stimulatory effects were compared at the same protein concentration of each cytokine (10 ng/ml) (Table I), M-CSF stimulated acetyl-LDL-cholesterol esterification 3.7-fold, and granulocyte-macrophage colony-stimulating factor stimulated acetyl-LDL-cholesterol esterification 3.6-fold. Interleukin 1 also showed significant stimulatory effects on acetyl-LDL-cholesterol esterification (1.8-fold). On the other hand, granulocyte colony-stimulating factor and tumor necrosis factor did not have any significant effects on acetyl-LDL-cholesterol esterification.

Cholesterol Efflux. It is possible that macrophages excrete cholesterol that has been taken up. After preincubation with 10 μg protein/ml of acetyl-LDL for 24 hr, cells were incubated with LPDS-containing medium without lipoproteins for 8 hr. The amounts of cellular cholesteryl ester decreased as a function of time (Fig. 2). Both net and relative decreases in cellular cholesteryl ester were greater in M-CSF-treated cells than in nontreated cells. Within 8 hr, almost 50% of the cholesteryl ester previously accumulated within the cells had disappeared.

Table I. Effects of Various Cytokines on Cholesterol Ester Formation in Macrophages Incubated with Acetyl-LDL[a]

Cytokine	Concentrations (ng/ml)	Cholesterol ester formation (n*M*/24 hr/μg cell DNA)
No addition	—	0.726 ± 0.117
M-CSF	10	1.796 ± 0.086[b]
M-CSF	100	2.714 ± 0.157[b]
GM-CSF	1	2.476 ± 0.071[b]
GM-CSF	10	2.623 ± 0.051[b]
G-CSF	1	0.862 ± 0.081
G-CSF	10	0.847 ± 0.056
IL-1	10	1.329 ± 0.213[c]
TNF	10	0.604 ± 0.030

[a] Macrophages were grown for 9 days in media containing 10% autologous serum and for 1 additional day in media containing 5 mg/ml of LPDS in the presence or absence of various cytokines. After being washed with phosphate-buffered saline three times, the cells were incubated with media containing 5 mg/ml of LPDS and 0.5 m*M* [^{14}C]oleate/albumin with either 10 μg/ml of acetyl-LDL in the presence or absence of the cytokines. After 24 hr at 37°C, the amounts of cholesteryl [^{14}C]oleate were determined. Each value represents the mean ± SD of quadruplicate wells.

[b] Significantly different from the control value at $P < 0.001$.

[c] Significantly different from the control value at $P < 0.01$.

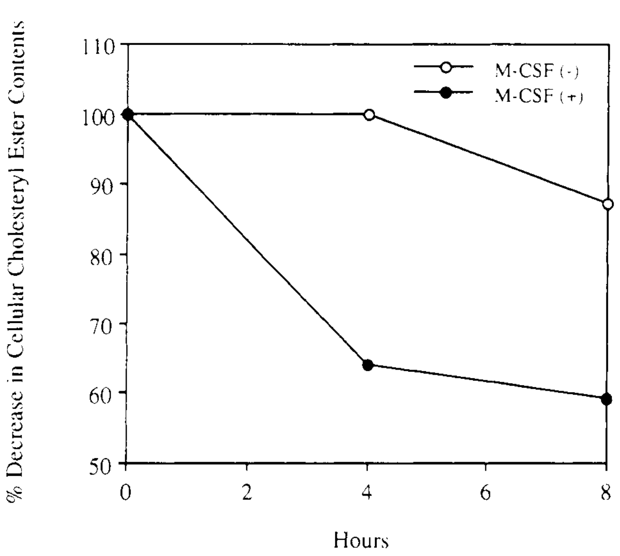

Figure 2. Effects of M-CSF on cholesterol efflux from cholesterol-loaded macrophages. Macrophages were grown for 9 days in media containing 10% autologous serum and 1 additional day in 5 mg/ml of LPDS with 10 μg/ml of acetyl-LDL in the presence or absence of M-CSF (128 ng/ml). After the indicated time of incubation with medium containing only 5 mg/ml of LPDS, the cells were chilled to 4°C and the amounts of cholesteryl ester in the cells were determined. Each value represents the mean ± SD of quadruplicate wells.

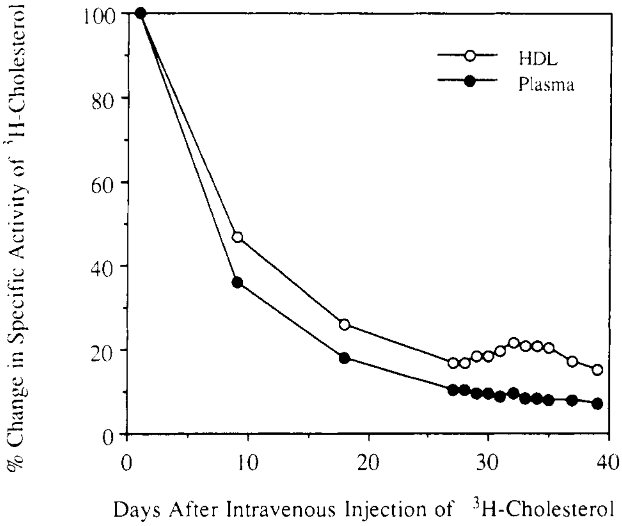

Figure 3. Percentage of change in specific activity in plasma cholesterol and HDL-cholesterol after the injection of ^{3}H-cholesterol. ^{3}H-Cholesterol (150 μCi) dissolved in 2 ml of autologous plasma was intravenously injected into four WHHL rabbits 1 month before starting M-CSF treatment, in which 300 mg of M-CSF were intravenously injected once a day for 7 consecutive days. Specific activities of plasma cholesterol and HDL-cholesterol were 100.9 × 10^3 and 84.1 × 10^3 dpm/mg 1 day after the injection of ^{3}H-cholesterol.

The effects of M-CSF treatment on the specific activities of plasma cholesterol 1 month after injection of ^{3}H-cholesterol are shown as percentage of change in specific activities of plasma cholesterol in Figure 3. Specific activities of plasma cholesterol and HDL-cholesterol were 100.9 × 10^3 and 84.1 × 10^3 dpm/mg 1 day after the injection of ^{3}H-cholesterol. After the M-CSF treatment, an increase in the specific radioactivity of HDL-cholesterol (1.4 ± 0.1-fold, mean ± SD) was demonstrated in four WHHL rabbits as compared with that before the treatment. During the M-CSF treatment, plasma cholesterol levels were decreased from 657 ± 86 mg/dl to 522 ± 48 mg/dl and HDL-cholesterol levels were not changed (8.2 ± 2.9–8.0 ± 3.1 mg/dl). Kano *et al.* (32) reported that specific activities in tissues other than brain and arteries will be higher than that in plasma or HDL by waiting long enough (more than 21 days) after the injection of ^{3}H-cholesterol. Under these circumstances, any subsequent increase in plasma specific activity must indicate movement of cholesterol from peripheral tissues to plasma, mainly due to net transfer. In the control experiment, in which saline was injected, no increase in specific activity of HDL-cholesterol was observed.

Discussion

M-CSF is widely produced by cells such as fibroblasts, lymphocytes, and macrophages and has many physiological functions (1–13). Thus, M-CSF may play an important role in the uptake of lipoproteins in macrophages, which are major target cells of M-CSF. We previously reported the stimulatory effects of M-CSF on the uptake of acetyl-LDL and LDL by macrophages (23). Here, we found that M-CSF enhances the uptake of oxidized LDL as well as acetyl-LDL by increasing the number of receptors. There are several candidates of scavenger receptor for modified proteins such as acetyl-LDL, oxidized LDL, and maleylalbumin (34–38). It has been postulated that oxidized LDL binds to the acetyl-LDL receptor and to a receptor specific for oxidized LDL (37, 38). Recently, two closely related but distinct molecules for the bovine acetyl-LDL receptor were cloned (18). Although the receptor specific for oxidized LDL has not been isolated, the current study demonstrating that M-CSF enhanced the uptake of oxidized LDL to a lesser extent than that of acetyl-LDL suggests the presence of a distinct receptor for oxidized LDL whose regulation is different from that of acetyl-LDL.

Although the overall effect of M-CSF is unknown in the atherosclerotic process *in vivo*, M-CSF-treated macrophages released substantial amounts of cholesterol into the medium (Fig. 2). Furthermore, we observed an increased cholesterol efflux from tissues to plasma high density lipoprotein after M-CSF treatment *in vivo* when cholesterol efflux was estimated as the change in specific radioactivity of plasma HDL-cholesterol. This result suggests that M-CSF can enhance the excretion of cholesterol from target cells of M-CSF, such as macrophages. M-CSF may cause the removal of cholesterol-loaded macrophages from the arterial wall, although a 20% reduction in plasma cholesterol levels was observed during the experiment and this may influence the efflux of cholesterol. Cellular cholesterol has been proposed to be excreted via cell surface HDL receptors (39). The increased binding of HDL to macrophages by the addition of M-CSF is a possible mechanism to explain the enhanced cholesterol efflux from macrophages in response to M-CSF that we reported previously (23).

Increased cholesterol uptake by M-CSF-treated macrophages *in vitro* (23) may be understood as a positive action to remove extracholesterol from the extracellular matrix; then, extracholesterol may be excreted from macrophages and transported to the liver via a reverse cholesterol transport system involving HDL (16).

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