Macrophages (MØ) serve as the principal inflammatory cell in the atheromatous plaque microenvironment. The macrophage scavenger receptor (MSR) mediates modified lipoprotein-cholesterol uptake and subsequent cholesteryl ester accumulation and foam cell formation (1) and the regulation of MSR expression and activity therefore represents an important determinant of the extent of atherogenesis.

Regulation of macrophage scavenger receptor (MSR) activity may be an important determinant of the extent of atherogenesis. The effect of macrophage-colony-stimulating factor (M-CSF) on this pathway was studied using a recently developed monoclonal antibody to murine MSR. M-CSF markedly and selectively increased MSR synthesis in murine macrophages: posttranslationally, the receptor appeared more stable and shifted to a predominantly surface distribution. Functionally, M-CSF enhanced modified lipoprotein uptake and increased divalent cation-independent adhesion in vitro. These results suggest a plausible mechanism whereby M-CSF production in the atheromatous plaque microenvironment could promote the recruitment and retention of mononuclear phagocytes and subsequent foam cell formation.

Materials and Methods

Elicited peritoneal MØ populations were obtained from 5–7-wk-old Balb/c mice from this department by injecting Bio-Gel P100 polyacrylamide beads (Bio-Rad, Richmond, CA) (Biomold) 4–5 d before harvesting by sterile PBS peritoneal lavage. RPMI 1640 (GIBCO BRL, Paisley, UK) was supplemented with 2 mM glutamine, 50 mg/ml streptomycin, 50 IU/ml penicillin G, 20 mM Hepes (pH 7.3), and 10% FCS (Advanced Protein Products, Brierley Hill, UK).

Antibodies and Cytokines. The following primary rat mAbs were used: 2F8 (IgG2b, anti-MSR), F4/80 (IgG2b, anti-160 kD M(2) membrane antigen), 5C6 (IgG2b, anti-CR3), FA/11 (IgG2a, antimacrosialin) (all from this laboratory); TIB120 (IgG2b, anti-class II MHC, from American Type Culture Collection, Rockville, MD) and CAMPATH-1G (IgG2b, anti-human CDw52 but unreactive to murine antigens, provided by Dr. G. Hale, Cambridge University, Cambridge, UK). Neutralizing murine monoclonal anti-human M-CSF antibody SH4 was from Dr. J. Schreurs (Chiron Corporation, Emeryville, CA) and goat anti-L cell M-CSF and M-CSF receptor antisera as well as rabbit anti-mouse macrophage mannose receptor (MMR) antiserum were from Drs. E. Richard Stanley (Albert Einstein College of Medicine, Bronx, NY) and Phillip Stahl, (Washington University, St. Louis, MO) respectively. As second antibodies the following were used: goat anti-rat IgG (alkaline phosphatase- and peroxidase-conjugated) and rabbit anti–goat IgG (peroxidase-conjugated) antiserum from Sigma Chemical Co. (St. Louis, MO) and mouse anti–rat IgG (FITC-conjugated F(ab')2) from Jackson ImmunoResearch Laboratories (West Grove, PA).
Cytokines were used at the final concentrations indicated. Purified recombinant human M-CSF (1,000 U/ml) from Dr. S. Aukerman (Cetus Corp., Berkeley, CA) is known to act on murine cells (12). Purified recombinant murine GM-CSF (1,000 U/ml) was a gift from Dr. A. Bernard (Glaxo Institute for Molecular Biology, Geneva, Switzerland). Murine M-CSF reagents used were either L cell conditioned medium (15% of RPMI 1640 incubation medium) as a rich source of murine M-CSF (13) or murine M-CSF (1,000 U/ml) as supernatant from transfected insect cells obtained from Dr. J. Schreurs.

**Immunoblotting and Immunoprecipitation.** Cells were plated at a density of 5 x 10⁴/well in 35-mm tissue culture plastic dishes in RPMI plus 10% FCS and washed after 1-h incubation at 37°C. Remaining adherent cells were treated with cytokines for 48 h. Metabolic labeling and immunoprecipitation was carried out as described (11). Protein concentrations of lysates were measured using the bicinchoninic acid Protein Assay Reagent Kit (Pierce, Rockford, IL). Protein separation was by nonreducing (for immunoblotting) or reducing (for immunoprecipitation) 5-10% linear gradient SDS-PAGE. Equal amounts of total cellular protein (20 μg) were loaded. First, antibody was diluted to 10 μg/ml followed by incubation with peroxidase-conjugated anti-rat IgG antibody (1:1,000) and detection by chemiluminescence (ECL; Amersham International, Amersham, Bucks, UK).

Cell surface distribution of MSR was investigated by labeling BgPMO overnight with 100 μCi/ml Trans3S-label™ (ICN Biochemicals, Irvine, CA). Cells were subsequently washed and subjected to surface biotinylation using sulfo-NHS-SS-biotin (Pierce) as described (14). Quantification of protein bands on dried gels was performed in the presence of 0.1% saponin in PBS with 1% FCS and 1% normal mouse serum (NMS) for 30 min. All subsequent steps were performed in the presence of 0.1% saponin in PBS with 1% FCS and 1% NMS.

**Flow Cytometry.** Cells were stained with primary and FITC-conjugated secondary antibodies before flow cytometry (FACScan®, Becton Dickinson & Co., Mountain View, CA). Cells and debris having low forward scatter were routinely gated out of the analysis. For visualization of intracellular antigen, cells were permeabilized with 1% (wt/vol) saponin in PBS with 1% FCS and 1% normal mouse serum (NMS) for 30 min. All subsequent steps were performed in the presence of 0.1% saponin in PBS with 1% FCS and 1% NMS.

**Reverse Transcription (RT)-PCR Analysis.** Total cellular RNA was extracted with RNAsol solution (Cinna/Biotex Laboratories, Houston, TX) and reverse transcribed using Moloney murine leukemia virus reverse transcriptase (BRL/GIBCO). The cDNA obtained served as a template for PCR using oligonucleotide pairs for type II receptor (5' primer: 5'-TGGTGCTCCAGGAATAAG-3'; 3' primer: 5'-ACCAACGACCTCAGACTGAA-3'; size of amplified fragment: 236 bp); (b) specific for type I receptor (5' primer: 5'-ACCAACGACCTCAGACTGAA-3'; 3' primer: 5'-TAGACTCGCAGACAACTT-3'; size of amplified fragment: 299 bp); Ethidium bromide-stained PCR products were visualized on a UV transilluminator after electrophoresis on 2% agarose gels. Specificity of the amplified bands was validated by their predicted size and restriction enzyme digests. Differences in intensity of PCR bands were confirmed by 10-fold serial dilutions of cDNA samples to ensure comparability and that the plateau phase of amplification had not been reached. The results shown are representative of three independent experiments.

**Cell Adhesion Assays.** BgPMO were cultured in RPMI plus 10% FCS on tissue culture plastic (TCP) surfaces in the presence or absence of recombinant human M-CSF. Divalent cation-independent adhesion to TCP was assayed as described (11).

**Quantification of AcLDL Uptake.** BgPMO in 24-well TCP plates (3 x 10⁴ cells/well) with M-CSF treatment in triplicate were exposed to 10 μg/ml AcLDL labeled with DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) (Biogenesia, Bournemouth, UK). Uptake of DiIAcLDL was measured by flow cytometry as described (16).

**Results and Discussion**

To investigate the effects of M-CSF on MSR expression and function, we treated primary murine MO with recombinant human M-CSF which is known to act on murine cells (12). M-CSF treatment for 48 h markedly upregulated the expression of MSR protein on immunoblot analysis (Fig. 1, A and B). Recombinant murine GM-CSF, which decreases serum cholesterol to a lesser extent when infused as an adjunct to chemotherapy (17), caused a noticeable, but less marked MSR increase (Fig. 1 B). To exclude effects due to growth factor–induced proliferation, loading of lysates per well was corrected for total cellular protein concentration.

In addition, it has previously been shown that M-CSF treatment of human monocyte–derived MO increases cell size rather than cell number (2). The observed increase in MSR protein would therefore be even more significant at a single cell level. Conditioned media from mouse L cells (a rich source of murine M-CSF [13]) or insect cells transfected with murine M-CSF cDNA similarly increased MSR expression, and this effect was abrogated by the addition of species-specific antibodies to M-CSF (data not shown). The increase in MSR expression was dose dependent and plateaued at concentrations of 500–1,000 U/ml M-CSF (data not shown).

Immunoprecipitation of MSR from metabolically labeled cells confirmed the upregulation by M-CSF (Fig. 2). The mAb 2F8 detects both type I and II isoforms of the MSR, and the MSR is seen in its reduced monomeric form. M-CSF increased MSR protein synthesis 2.3-fold (PhosphorImager quantification) even after 48 h of treatment, as is evident from Figure 1. M-CSF increases MSR protein expression. For immunoblot analysis with mAb 2F8, BgPMO were treated for 48 h with human M-CSF (1,000 U/ml) and murine GM-CSF (1,000 U/ml). Results (A and B) are from two independent experiments. (<) 200-kD marker.
the 0-h chase point after a 30-min labeling period. The more impressive increases seen at 12 (4.5-fold) and 36 h (6-fold) signify that, in addition to increasing MSR synthesis, M-CSF also markedly prolonged the half-life of synthesized receptor.

Expression of MSR at a single cell level as determined by flow cytometry (Table 1 A) confirmed that M-CSF upregulated MO cell surface expression of the receptor. The greater than twofold increase in MSR surface expression was not paralleled by the more modest 18% increase seen when intracellular (internal) levels of antigen were assessed by permeabilizing the cells with saponin. It was therefore possible that M-CSF may have redistributed the substantial intracellular pool of MSR to the cell surface. Indirect immunofluorescence studies with the mAb 2F8, although difficult to quantify, supported this hypothesis (data not shown). It is interesting to note that GM-CSF had little effect on surface or internal MSR expression as assessed by flow cytometry, in keeping with the slight increase seen by immunoblotting.

The possible redistributive effect of M-CSF was investigated by biochemically quantifying the fraction of total MSR on the cell surface at steady state (Table 1 B). Cells were metabolically labeled to equilibrium followed by surface biotinylation, on ice, by the membrane-impermeant probe sulfo-NHS-SS-biotin (14). After cell lysis, total MSR was precipitated by mAb 2F8 and the resulting immunoprecipitate divided into two equal aliquots. Biotinylated “surface” MSR was precipitated from one aliquot using streptavidin-agarose and “total” MSR was precipitated from the other aliquot by a second round of mAb 2F8 immunoprecipitation. Total and biotinylated MSR were quantitated after SDS-PAGE by PhosphorImager analysis of dried gels. In these experiments M-CSF increased surface MSR expression 13-fold and total MSR protein synthesis nearly 5-fold relative to untreated controls. In addition, M-CSF treatment was associated with a shift of the cellular pool of MSR from a mainly intracellular location (69% of total) to a predominantly surface distribution (82% of total).

To exclude the possibility that the M-CSF effect on MSR expression represented a nonepithelial phenomenon of MO differentiation, we analyzed the action of M-CSF on several other MO integral membrane proteins (Table 1 B). The expression of these proteins was not measurably affected by M-CSF, in contrast to the substantial effect on MSR.

Table 1. M-CSF Selectively Increases MSR Surface Expression

| A | Treatment | Surface | Internal |
|---|-----------|---------|----------|
| Control | 100 | 100 |
| M-CSF | 232 | 118 |
| GM-CSF | 81 | 107 |

| B | Treatment | Total | Surface |
|---|-----------|-------|---------|
| Control | 23,100 | 7,100 |
| Percent surface/total | 31% |
| M-CSF | 115,400 | 94,900 |
| Percent surface/total | 82% |

| C | Surface | Internal |
|---|---------|----------|
| Treatment | Control | M-CSF | Control | M-CSF |
| MSR | 60 | 117 | 177 | 208 |
| F4/80 antigen | 221 | 212 | 325 | 343 |
| CR3 | 281 | 324 | 509 | 416 |
| Class II MHC | 43 | 36 | 44 | 41 |
| Macrosialin | 39 | 26 | 257 | 182 |

(A) Effect of M-CSF and GM-CSF on expression of MSR by flow cytometry. BgPMO were treated in vitro for 48 h as indicated and stained with either mAb 2F8 or CAMPATH-1G plus FITC-conjugated second antibody before flow cytometry. Results are depicted as percentage of control values and are the means of three experiments (each experiment in triplicate) in which specific fluorescence intensities were determined by subtracting geometric mean fluorescence obtained with CAMPATH-1G from that obtained with mAb 2F8. (B) Effect of M-CSF on cell surface distribution of MSR. BgPMO were metabolically labeled and surface biotinylated using sulfo-NHS-SS-biotin. Quantification of protein bands on dried gels was done by PhosphorImager and results expressed as a ratio of arbitrary APUs. (C) Effect of M-CSF on expression of MO differentiation antigens by flow cytometry. BgPMO were stained with the indicated mAb or its isotype control plus FITC-conjugated second antibody before analysis by flow cytometry. The specific mAbs used were F4/80, 5C6 (CR3), TIB120 (class II MHC), and FA-11 (macrosialin). Results show the mean values (single experiment in triplicate) of specific fluorescence intensities determined by subtracting geometric mean fluorescence obtained with the isotype control from that obtained with the specific mAb.

Figure 2. M-CSF increases MSR protein synthesis. BgPMO were treated in vitro for 48 h with human M-CSF (1,000 U/ml), metabolically labeled with Tran3SS-label and chased for the indicated periods. Precipitates were adjusted to represent equivalent total protein concentrations, and separated by SDS-PAGE in the presence of β-ME. MSR precursor and monomer migrate at ~64 and 90 Kd, respectively. Gels were fixed, impregnated with ENHANCE, dried, and exposed to film at -70°C. (►) Molecular weight standards in kilodaltons.
pression of surface and internal pools of F4/80 (a MØ-specific differentiation antigen), type 3 complement receptor, MHC class II antigen and macro-ialin (a MØ-specific late endosomal membrane molecule) were analyzed by flow cytometry. M-CSF treatment elicited slight increases only in surface CR3 (15%) and internal F4/80 (6%) levels. The other antigens were either unchanged or decreased. Immunoblots of M-CSF treated BgPMØ also showed no alteration in expression of MMR or class II MHC (data not shown). These results point to a selective effect of M-CSF on MSR expression in an elicited primary MØ population.

The point at which M-CSF mediates its upregulatory effect was investigated by semi-quantitative RT-PCR to detect MSR mRNA changes in response to M-CSF (Fig. 3). This assay provides a sensitive measure of mRNA in MØ, and control serial dilution experiments confirmed that for each set of primers the level of detection could be related to the initial cellular input used to prepare the cDNA. Two isoforms of MSR arise from alternative splicing of transcripts derived from a single gene. Both isoforms have been detected in atherosclerotic lesions and bind MSR ligands with equal affinities (18, 19). After M-CSF treatment for 48 h, there was a substantial increase in MSR mRNA (MSR-C). In addition, levels of mRNA specific for both type I and II MSR mRNA were increased, excluding a type-selective effect for M-CSF. Although type I MSR mRNA was present in untreated cells, type II MSR protein is more prevalent in vivo and in vitro (15). Detection of type I message in the control cells could, however, be explained by increased annealing affinity of the specific set of primers for the cDNA template. Similar results were obtained after 24 h of M-CSF incubation (data not shown). M-CSF therefore also upregulates MSR expression at mRNA level. Whether this is due to transcriptional up-regulation or posttranscriptional stabilization was not addressed.

As described, M-CSF increased MSR expression at several different levels: message, protein synthesis, protein stability, and shift of mature protein to the cell surface. The significance of these observed differences was tested by relating them to functional MSR studies. Traditionally, the MSR has been characterized as an endocytic receptor (20) with a broad ligand-binding specificity for a range of polyanions (including modified lipoproteins). The ability of treated BgPMØ to endocytose and accumulate fluorescent AcLDL (DiI-AcLDL) was therefore tested (Table 2). M-CSF-mediated upregulation of murine MSR was associated with an enhanced capacity (1.7–3-fold) of treated MØ to endocytose AcLDL. AcLDL uptake is regarded as a specific marker for MSR function, as the thrombospondin receptor (CD36) and the MØ receptor for the Fc region of IgG (FcγRII-B2; CD32) may mediate uptake of oxidized but not acetylated lipoprotein (6, 7).

A recently described novel function for the MSR is divalent cation-independent adhesion (11). The ability of M-CSF-treated BgPMØ to adhere to serum-treated TCP in an EDTA-independent manner was investigated. M-CSF increased adhesion at 60 min by 63% and at 90 min by 96%; this effect was completely abolished in the presence of mAb 2F8 (data not shown). The adherence of M-CSF-treated MØ in vitro was therefore markedly increased in a MSR-dependent (divalent cation-independent, 2F8-inhibitable) fashion. Atherogenesis is characterized by both the proliferation of smooth muscle cells, MØ, and lymphocytes in the artery wall and the accumulation of cholesterol and cholesteryl esters in the surrounding connective tissue matrix and associated cells. Besides acting as a growth factor for the proliferation and differentiation of monocyct progenitors, M-CSF is also required for the survival and activation of mature monocytes and MØ (21). The LDL receptor is primarily responsible for regulating plasma LDL homeostasis and LDL-cholesterol delivery to tissue and cells by clearing plasma-derived LDL-cholesterol. Hepatic LDL receptor function, as assayed by LDL binding, is however not markedly affected by M-CSF treatment. GM-CSF had a minimal effect on MSR activity and its cholesterol lowering effect could be mediated indirectly through stimulation of TNF and/or IL-1 secretion (22). In contrast, our evidence shows that M-CSF significantly enhances MSR expression and function in vitro. This provides a mechanism whereby production of M-CSF in the atheromatous plaque microenvironment could enhance the recruitment and retention of mononuclear phagocytes and subsequent accumulation of cholesteryl esters and foam cell formation.

Table 2. M-CSF Increases the Uptake of DiI-AcLDL by BgPMØ

| Treatment | Expt. 1 | Expt. 2 | Expt. 3 |
|-----------|--------|--------|--------|
| Control   | 314 ± 15 | 239 ± 14 | 166 ± 17 |
| M-CSF     | 541 ± 17 | 496 ± 34 | 492 ± 32 |
| Percent increase | 172% | 208% | 297% |

BgPMØ were treated for 48 h with human M-CSF and MSR-mediated uptake of DiI-AcLDL measured as described (11,16). Specific fluorescent intensity was calculated by subtracting autofluorescent intensity from fluorescent intensity of DiI-AcLDL-labeled cells using geometric means derived from data analysis by Lysys II software (Becton Dickinson). Results show the mean values ± standard deviation (three independent experiments in triplicate).
References

1. Krieger, M., S. Acton, J. Ashkenas, A. Pearson, M. Pennan, and D. Resnick. 1993. Molecular flypaper, host defense, and atherosclerosis. J. Biol. Chem. 268:4569.

2. Ishibashi, S., T. Inaba, H. Shimano, K. Harada, I. Inoue, H. Mokuno, N. Mori, T. Gotoda, F. Takaku, and N. Yamada. 1990. Monocyte colony-stimulating factor enhances uptake and degradation of acetylated low density lipoproteins and cholesterol esterification in human monocyte-derived macrophages. J. Biol. Chem. 265:14109.

3. Nagelkerke, J.F., K.P. Barto, and T.J.C. van Berkel. 1983. Acetylated low-density lipoprotein by rat liver endothelial, Kupffer, and parenchymal cells. J. Biol. Chem. 258:12221.

4. Kume, N., H. Arai, C. Kawai, and T. Kita. 1991. Receptors for modified low-density lipoproteins on human endothelial cells: different recognition for acetylated low-density lipoprotein and oxidized low-density lipoprotein. Biochim. Biophys. Acta. 1091:63.

5. Pitas, R.E. 1990. Expression of the acetyl low density lipoprotein receptors by rabbit fibroblasts and smooth muscle cells: upregulation by phorbol esters. J. Biol. Chem. 265:12722.

6. Endemann, G., L.W. Stanton, K.S. Madden, C.M. Bryant, R.T. White, and A.A. Protter. 1993. CD36 is a receptor for oxidized low density lipoprotein. J. Biol. Chem. 268:11811.

7. Stanton, L.W., R.T. White, C.M. Bryant, A.A. Protter, and G. Endemann. 1992. A macrophage Fc receptor for IgG is also a receptor for oxidized low density lipoprotein. J. Biol. Chem. 267:22446.

8. Clinton, S.K., R. Underwood, L. Hayes, M.L. Sherman, D.W. Kufe, and P. Libby. 1992. Macrophage colony-stimulating factor gene expression in vascular cells and in experimental and human atherosclerosis. Am. J. Pathol. 140:301.

9. Rosenfeld, M.E., S. Yla-Herttuala, B.A. Lipton, V.A. Ord, J.L. Witzum, and D. Steinberg. 1992. Macrophage colony-stimulating factor mRNA and protein in atherogenic lesions of rabbits and humans. Am. J. Pathol. 140:291.

10. Rajavashisth, T.B., A. Andalibi, M.C. Territo, J.A. Berliner, M. Navah, A.M. Fogelman, and A.J. Luus. 1990. Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low-density lipoproteins. Nature (Lond.). 344:254.

11. Fraser, I., D. Hughes, and S. Gordon. 1993. Divalent cation-independent macrophage adhesion inhibited by monoclonal antibody to murine scavenger receptor. Nature (Lond.). 365:343.

12. Ralph, P., M. Warren, M. Lee, J. Csejty, J. Weaver, H. Brommeyer, D. Williams, E. Stanley, and E. Kawasaki. 1986. Inducible production of human macrophage growth factor, CSF-1. Blood. 68:633.

13. Stanley, E.R., and P.M. Heard. 1977. Factors regulating macrophage production and growth. Purification and some properties of the colony stimulating factor from medium conditioned by mouse L-cells. J. Biol Chem. 252:4305.

14. Harter, C., and I. Mellman. 1992. Transport of the lysosomal membrane glycoprotein lgp120 (lgp-A) to lysosomes does not require appearance on the plasma membrane. J. Cell Biol. 117:311.

15. Ashkenas, J., M. Penman, E. Vasile, S. Acton, M. Freeman, and M. Krieger. 1993. Structures and high and low affinity ligand binding properties of murine type I and type II macrophage scavenger receptors. J. Lipid Res. 34:983.

16. Geng, Y.-j., and G.K. Hansson. 1992. Interferon-γ inhibits scavenger receptor expression and foam cell formation in human monocyte-derived macrophages. J. Clin. Invest. 89:1322.

17. Nimer, S.D., R.E. Champlin, and D.W. Golde. 1988. Serum cholesterol-lowering activity of granulocyte-macrophage colony-stimulating factor. JAMA. (J. Am. Med. Assoc.) 260:3297.

18. Emi, M., H. Assoka, A. Matsumoto, H. Itakura, Y. Kurihara, Y. Wada, H. Kanamori, Y. Yazaki, E.-i. Takahashi, M. Lepert, et al. 1993. Structure, organization, and chromosomal mapping of the human macrophage scavenger receptor gene. J. Biol. Chem. 268:2120.

19. Naito, M., H. Suzuki, T. Mori, A. Matsumoto, T. Kodama, and K. Takahashi. 1992. Coexpression of type I and II human macrophage scavenger receptors in macrophages of various organs and foam cells in atherosclerotic lesions. Am. J. Pathol. 141:591.

20. Goldstein, J.L., Y.K. Ho, S.K. Basu, and M.S. Brown. 1979. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein producing massive cholesterol deposition. Proc. Natl. Acad. Sci. USA. 76:333.

21. Hume, D.A., P. Pavli, R.E. Donahue, and I.J. Fidler. 1988. The effect of human recombinant macrophage colony-stimulating factor (CSF-1) on the murine mononuclear phagocyte system in vivo. J. Immunol. 141:3405.

22. Stopec, A.T., A.C. Nicholson, F.P. Mancini, and D.P. Hajjar. 1993. Cytokine regulation of low density lipoprotein receptor gene transcription in HepG2 cells. J. Biol. Chem. 268:17489.