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Rapid Communication

Macrophage Colony-stimulating Factor mRNA and Protein in Atherosclerotic Lesions of Rabbits and Humans

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In this study, the authors demonstrate the expression of mRNA and the presence of protein for macrophage colony-stimulating factor (MCSF) in atherosclerotic lesions from humans and rabbits. **In situ** ***hybridization of serial sections of human fatty streaks demonstrated expression of MCSF mRNA by cells dispersed throughout the lesions. Immunocytochemical staining with a panel of MCSF-specific antibodies showed extensive cell-associated staining of all of the cell types in the lesions. Immunocytochemical studies of atherosclerotic lesions from Watanabe heritable hyperlipidemic (WHHL) and cholesterol-fed rabbits demonstrated a similar cell-associated pattern of staining. There was no MCSF-specific staining of aortas from normal rabbits or of cultured aortic smooth muscle cells from either humans or rabbits. Macrophage-derived foam cells (MFC) were isolated from the aortas of ballooned, cholesterol-fed rabbits. A Northern blot demonstrated that RNA isolated from the MFC hybridized with a human cDNA probe for MCSF. RNA from alveolar macrophages isolated simultaneously from the same rabbits did not hybridize with the MCSF probe. Conditioned media from an 18- to 24-hour incubation of the MFC contained colony-stimulating activity as demonstrated in a mouse bone marrow culture assay. Most of this colony-stimulating activity was neutralized by preincubating the conditioned media with an MCSF-specific antibody. (Am J Pathol 1992, 140:291–300)***

Recent studies of arterial macrophages, isolated from atherosclerotic lesions or examined *in situ*, have demonstrated that these cells bind and degrade modified lipoproteins[1–3] and express the scavenger receptor.[4] The cells contain oxidation-specific epitopes characteristic of oxidized low-density lipoprotein (LDL), and are capable of oxidizing lipoproteins possibly via a 15-lipoxygenase-mediated process.[2–5] Arterial macrophages express and secrete monocyte-chemotactic protein-1,[6] and the BB homodimer of platelet-derived growth factor (PDGF).[7,8] The cells also express major hisotcompatability complex (MHC) and CD 11b, CD 14, CD 36, and CD 68 leukocyte differentiation antigens,[9,10] as well as proliferate.[11,12] Taken together, these data suggest that macrophages within atherosclerotic lesions are differentiated cells that are capable of responding to activating signals associated with inflammatory and immune events. However, it is still unclear which factors attract monocytes into the intima, and play important roles in stimulating the subsequent differentiation into macrophages.

Macrophage colony-stimulating factor (MCSF) is a glycoprotein expressed by a variety of cell types including monocytes and macrophages, endothelial cells, fibroblasts, and lymphocytes.[13–16] It promotes the growth and differentiation of bone marrow progenitor cells into mononuclear phagocytes[17] and stimulates the proliferation and immune activation of monocytes and mature macrophages.[18–20] Human MCSF exists as two distinct proteins containing 256 or 552 amino acids arising via alternative splicing from a single gene. The larger protein appears to be the secreted form of MCSF whereas the smaller protein is stably expressed as a membrane-bound, yet biologically active factor.[21,22]

Data from recent studies suggest that MCSF may play a role in the atherogenic process. Shimano et al.[23] have demonstrated that MCSF lowers plasma cholesterol levels in normal and hypercholesterolemic rabbits due to an

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enhancement of the clearance of apoprotein B-containing lipoproteins by both receptor-dependent and receptor-independent processes. MCSF also stimulates macrophage degradation of acetyl-LDL, increases efflux of cholesterol from lipid-loaded macrophages,[24] and stimulates monocyte chemotaxis.[25]

We present data that shows that macrophages, smooth muscle cells, and endothelial cells, resident within atherosclerotic lesions of both humans and rabbits, express MCSF mRNA and immunoreactive protein. In addition, we demonstrate that macrophage-derived foam cells, freshly isolated from rabbit atherosclerotic lesions, express and secrete biologically active MCSF.

Methods

Immunocytochemistry and In Situ *Hybridization*

New Zealand white (NZW) rabbits fed cholesterol (2%) for 10 weeks, and mature Watanabe heritable hyperlipidemic (WHHL) rabbits were perfusion-fixed with formal-sucrose containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 50 μM butylated hydroxyfoluene (BHT), as previously described.[26] Segments of the thoracic aorta and aortic arch containing atherosclerotic lesions were embedded in paraffin and serial 8–10 micron sections were used for both immunocytochemistry and *in situ* hybridization. Alternatively, segments of nonfixed aorta were snap frozen in OCT compound (Miles Laboratories Inc. Elkhart, IN) by immersion in isopentane cooled in liquid nitrogen. Frozen sections (12–15 μm) were cut for immunocytochemical staining. In addition, segments of liver, lung, spleen, and bone marrow from WHHL and normal NZW rabbits were paraffin embedded and used for immunocytochemical analysis of MCSF. Rabbit and human aortic smooth muscle cells were plated on eight-well chamber slides (Lab-Tek NVAC, Inc, Naperville, IL) at 25,000 cells/well, grown for 3 days, fixed with cold methanol, and studied for the presence of immunoreactive MCSF. Portions of human aortic atherosclerotic lesions were obtained from two organ donors during renal transplants and from two autopsies within 5 hours of death through the Department of Forensic Medicine, University of Oulu, Oulu, Finland. Tissues were immersion-fixed for 12 hours in formal-sucrose and paraffin embedded as described earlier.

Immunocytochemistry

Serial paraffin-embedded or frozen sections of rabbit and human tissue, as well as rabbit and human smooth muscle cell cultures, were immunostained for 1 hour at room temperature with primary antibodies against human or mouse MCSF, against a rabbit macrophage-specific protein (RAM-11) or a human macrophage-specific protein (HAM-56),[27] a muscle actin-specific antibody (HHF-35),[28] and an antibody specific for factor VIII-related antigen to mark endothelial cells (Incstar Corp., Stillwater, MN). In all, eight separate antibodies generated against MCSF were tested with the rabbit and human tissue and cells. Two of the antibodies were antisera generated against partially purified human urine MCSF or mouse L-cell MCSF and were gifts from E. Richard Stanley (Albert Einstein School of Medicine).[29] These antisera could not be used with paraffin-embedded tissue but exhibited positive reactivity with the frozen sections of the rabbit lesions (data not shown). The remaining antibodies were all mouse monoclonal antibodies generated against recombinant human MCSF and were gifts from the Genetics Institute Inc. (Cambridge, MA). Four of these antibodies exhibited positive staining with the formal sucrose-fixed, paraffin-embedded human and rabbit tissues; avidin-biotin horseradish peroxidase and alkaline-phosphatase systems (Vector Labs) were used to visualize the areas of staining. Nonimmune sera or irrelevant antibodies of the same class as the primary antibodies were used as negative controls.

In Situ *Hybridization*

Serial paraffin-embedded sections of the human tissue were hybridized with both hybridizing anti-sense and nonhybridizing sense probes as well as irrelevant nonhybridizing probes [i.e., retinoic acid receptor probe[5]]. Sections were also treated with RNase A before hybridization as an additional negative control. The specific details of the *in situ* hybridization procedure have been recently published.[4–6] The slides were deparaffinized, pretreated with proteinase K, and acetylated. The sections were dried in vacuo and hybridized for 14 hours at 50–52°C with an ^{35}S-labeled riboprobe (0.15 kb *Pst*1-*Eco*R1 fragment) derived from a mouse MCSF cDNA[30] (provided by A. J. Lusis, UCLA). After hybridization the slides were treated with RNase A. The final wash was with 0.1 × SSC at 55°C for 1 hour. The slides were dehydrated and dipped in autoradiographic emulsion (NTB-2 Kodak) and exposed for 3–10 weeks before development.

Isolation of Macrophage-derived Foam Cells

The cells were isolated as recently described.[2] Atherosclerotic lesions were induced by ballooning the entire

aorta and iliac arteries of NZW rabbits with a Fogarty embolectomy catheter (4F) and then feeding the animals a 2% cholesterol-rich diet for 12 weeks. The cells were released from finely minced tissue using collagenase, elastase digestion,[31] and purified using density gradient centrifugation through metrizamide. The cells were placed in culture with Opti-MEM I media (Gibco) either serum free or containing 0.5% fetal calf serum (FCS) for 18–24 hours postisolation. The purity of the cultures was verified immunocytochemically using RAM-11 and HHF-35. Previous immunocytochemical analysis of the lesions from which the foam cells are isolated demonstrated no T lymphocytes. After the 18–24 hour incubation, the conditioned medium was collected, filtered (0.45 micron), and assayed for hematopoietic activity.

Colony-stimulating Activity Assay

The colony-stimulating activity in the MFC conditioned media was assayed in mouse bone marrow cultures according to the method of Stanley.[29] Briefly, bone marrow was obtained from the femurs of Balb-C mice and incubated in agar (Bacto-agar) plus complete alpha-MEM (Gibco) containing 15% FCS and 50 μl of the MFC conditioned media or no-cell control media. Total colonies consisting of more than 50 cells were counted after 7 days of incubation. To determine how much of the colony-stimulating activity in the MFC was due to MCSF, an equal volume of the goat anti-mouse MCSF antibody[29] (Table 1) was mixed with the MFC conditioned medium before addition to the bone marrow cultures. Mouse L929 cell-conditioned medium was used as a positive control for stimulation of mouse monocytic colonies.

RNA Isolation and Northern Blot Analysis

Total RNA was extracted from the MFC as well as alveolar macrophages obtained via saline lavage from the same animals, immediately after the isolation procedure but before placing the cells in culture. The RNA was isolated from the cells using 4 M guanidium isothiocyanate, and harvested using gradient centrifugation through CsCl.[32] For Northern blot analysis, ethanol-precipitated total RNA (25 μg) was electrophoresed on 1% agarose/formaldehyde gels and transblotted to nylon membranes (Nytran, Schleichen and Schuell, Keene, NH). The membranes were hybridized overnight at 42°C with a randomly primed ^{32}P-CTP labeled human MCSF cDNA probe[21] (provided by A. J. Lusis, UCLA). The final wash was with 0.2 × (150 mM NaCl, 15 mM sodium citrate, pH 7.0, 0.2%) sodium dodecyl sulfate, (SDS) at 45°C for 30 minutes. The hybridizing signals were detected using autoradiography with XAR-5 film (Kodak).

Results

Figure 1 demonstrates both *in situ* hybridization and immunocytochemistry on serial sections of a fatty streak taken from a human abdominal aorta. The shoulder region of this particular fatty streak contained many HAM-56 reactive macrophages (Figure 1D) and an intact endothelium (Figure 1H). There was extensive expression of mRNA for MCSF, observed as a diffuse pattern of silver grains spread throughout the lateral margin of the lesion (Figure 1 A, B). In contrast, using the nonhybridizing sense probe (Figure 1C) the preparation was practically devoid of silver grains. When the lesion was immunocytochemically probed for the presence of MCSF protein, all of the cell types exhibited positive immunoreactivity

Table 1. *Colony-stimulating Activity in MFC-conditioned Media*

Addition	Colony-forming units		
	Exp 1	Exp 2	Exp 3
Control (NCM)	459.1 ± 27	226.8 ± 98	291.4 ± 25
L929 CM	2,319.7 ± 200	1,718.2 ± 175	2,087.8 ± 463
L929 CM + MCSF Ab***		367.7 ± 42	
MCSF Ab alone		232.5 ± 10	296.7 ± 9
Foam cell CM #1*	813.6 ± 121	532.6 ± 142	694.6 ± 99
Foam cell CM #1** + MCSF Ab		323.0 ± 83	443.1 ± 67
Foam cell CM #2*	672.7 ± 83	525.7 ± 166	630.7 ± 114
Foam cell CM #3*	995.4 ± 163	439.9 ± 57	
Foam cell CM #4*	1,100.0 ± 269	419.6 ± 73	

* $P < .01$ with respect to the associated nonconditioned medium.
** $P < .023$ with respect to FC CM#1 without antibody.
*** $P < .0001$ with respect to the L929 CM without antibody.
Total colonies of greater than 50 cells were counted in bone marrow cultures from Balb-c mice after 7 days of incubation. Colony-forming units equals the number of total colonies/ml conditioned media/10^5 bone marrow cells. CM = conditioned medium, NCM = nonconditioned medium, L929 CM = the 3-day conditioned media from mouse L cell cultures added at the same volume as the MFC conditioned media. The anti-MCSF antibody was a goat anti-mouse MCSF antibody used at a 1:50 dilution. The foam cell CM are conditioned media from an 18–24 hour incubation of four separate MFC preparations. Values including the standard deviations are the means of three to four wells.

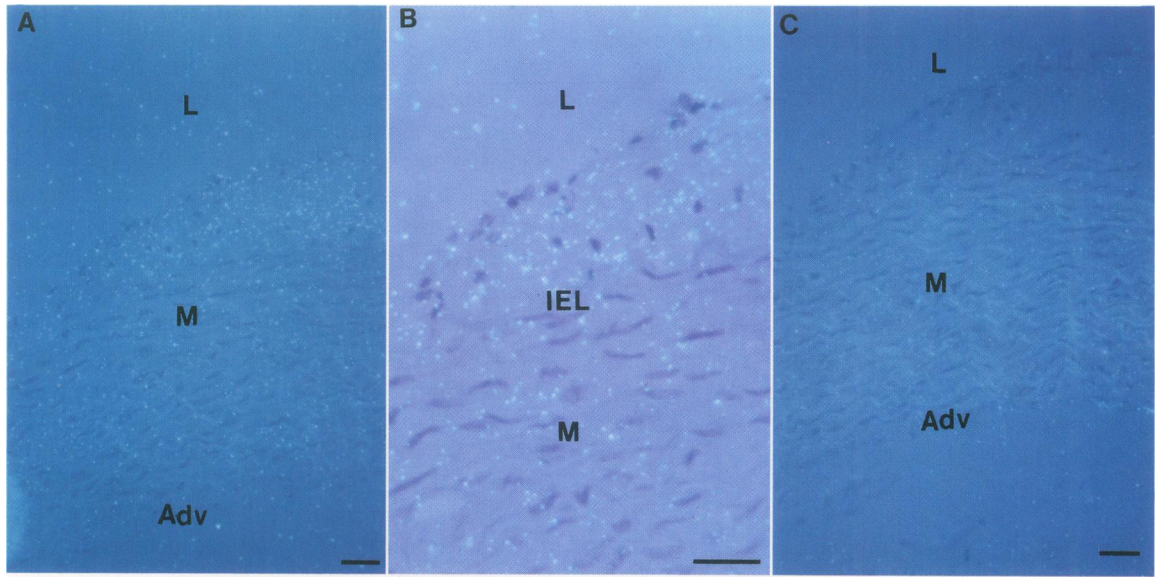

Figure 1. In situ *hybridization and immunocytochemical analysis of MCSF in macrophage-rich human fatty streak. Serial sections of a fatty streak from the lower abdominal aorta were immunostained or used for* in situ *hybridization as described in Methods.* **A, B:** In situ *hybridization with MCSF specific antisense riboprobe.* **C:** *Nonhybridizing MCSF sense probe.* **D:** *Macrophage specific antibody HAM-56 (dilution = 1:1,000).* **E:** *Nonimmune control.* **F:** *MCSF specific antibody HM7/41410 (dilution = 1:100).* **G:** *MCSF specific antibody HM7/7710 (dilution = 1:100).* **H:** *Endothelium specific anti factor VIII related antigen antiserum (used undiluted).* **I:** *Staining of endothelial cells with MCSF specific antibody HM7/7710.* **J:** *High magnification micrograph of macrophages staining with MCSF specific antibody HM7/41410.* **K:** *High magnification micrograph of smooth muscle cells staining with MCSF specific antibody HM7/41410.* **A–G:** *Bar = 100 microns.* **H–J:** *Bar = 10 microns, L = lumen, IEL = internal elastic lamina, M = media, Adv = adventitia, mp = macrophage, s = smooth muscle cell.*

with each of four different MCSF-specific monoclonal antibodies (Figures 1F, G, I–K). Interestingly, with all four antibodies the pattern of staining was identical as only cell-associated staining was observed; the extracellular matrix was devoid of any immunoreactive MCSF. The application of nonimmune serum (Figure 1E) or an irrelevant IgG (data not shown) did not yield any observable reactivity.

We also immunostained atherosclerotic lesions both from WHHL rabbits (Figure 2) and cholesterol-fed NZW rabbits (data not shown). In both cases, most of the cells in the rabbit lesions, as in the human lesions, exhibited positive immunoreactivity with the MCSF antibodies (Figure 2 C–F). The advanced fatty streaks in the rabbits were similar to the human fatty streaks in that they also contained large numbers of both macrophages and smooth muscle cells (Figure 2 A, B). Sections of aorta from normal rabbits (Figure 2G) did not stain with any of the MCSF specific antibodies, and cultured rabbit or human aortic smooth muscle cells (data not shown) also did not stain with any MCSF specific antibodies. Sections of liver and spleen from either normal or WHHL rabbits contained a few widely dispersed cells that were positively stained whereas sections of lung were devoid of immunostaining (data not shown). Clusters of cells within the rabbit bone marrow, however, were strongly immunoreactive for MCSF (Figure 2H).

To further study the capacity of arterial MFC to express and produce MCSF, we isolated large numbers of macrophage-derived foam cells from ballooned, cholesterol-fed rabbits and extracted the mRNA immediately after the isolation procedure. Figure 3 shows a Northern blot that demonstrates mRNA for MCSF in these arterial foam cells (lane 2). In contrast, alveolar macrophages isolated simultaneously from the same animals and exposed to the same isolation and purification procedure as the arterial foam cells, did not express the mRNA for MCSF (lane 1).

The freshly isolated macrophage-derived foam cells were also placed in culture and the conditioned media collected after 18–24 hours of incubation. As shown in Table 1, the conditioned media contained colony-stimulating activity assayed in a mouse bone marrow culture system. There was approximately a twofold increase in the number of total colonies formed in response to the conditioned media from four separate foam cell preparations as compared with nonconditioned media. This was approximately 40% of the activity observed with an equal volume of conditioned medium from mouse L-929 cells, which are known to actively secrete MCSF. To demonstrate what fraction of the biological activity in the MFC conditioned media was due to MCSF, the conditioned media were preincubated with an MCSF antibody before addition to the bone marrow cultures. The MCSF antibody neutralized most, but not all, of the colony-stimulating activity in the foam cell-conditioned media

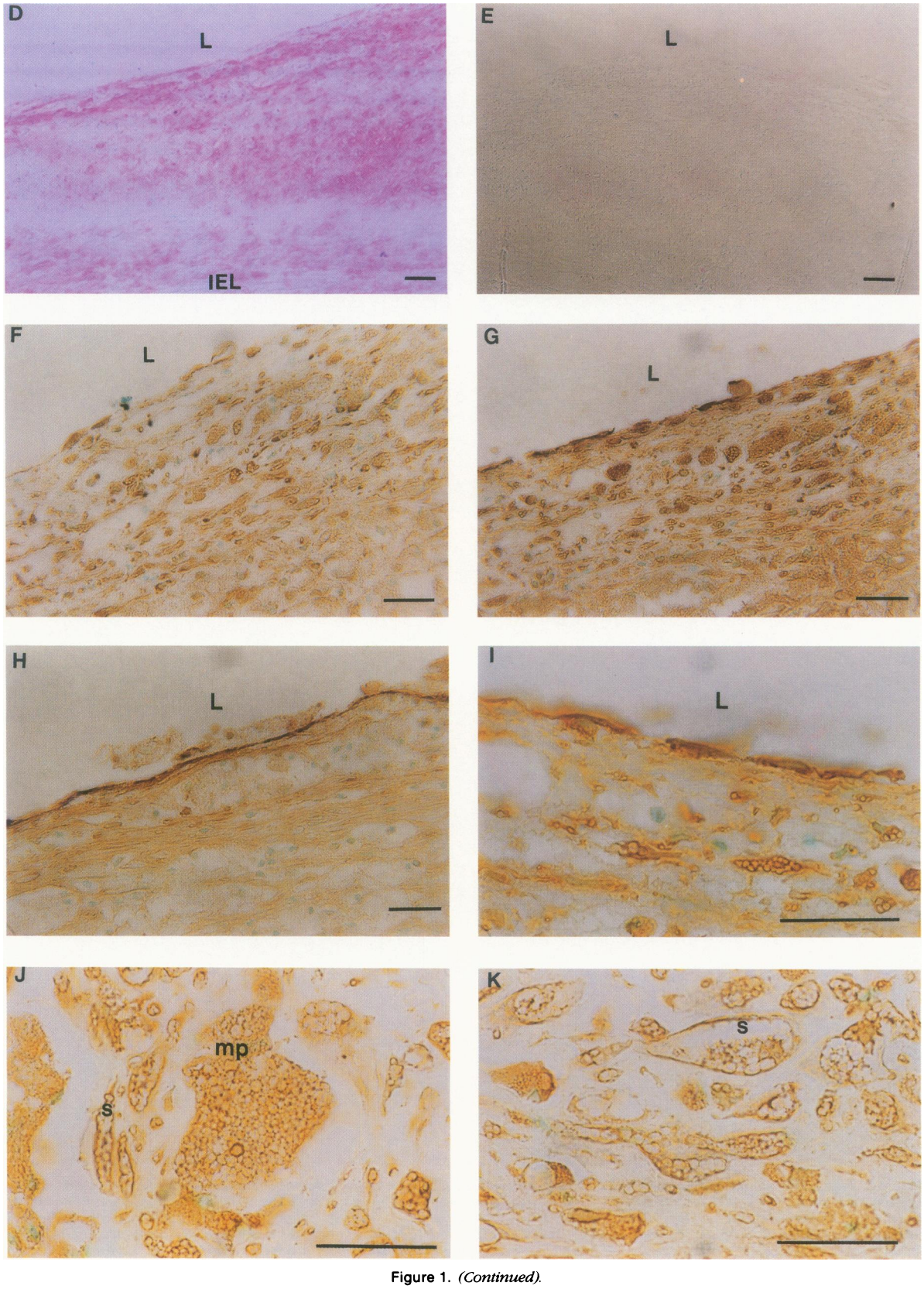

Figure 1. *(Continued).*

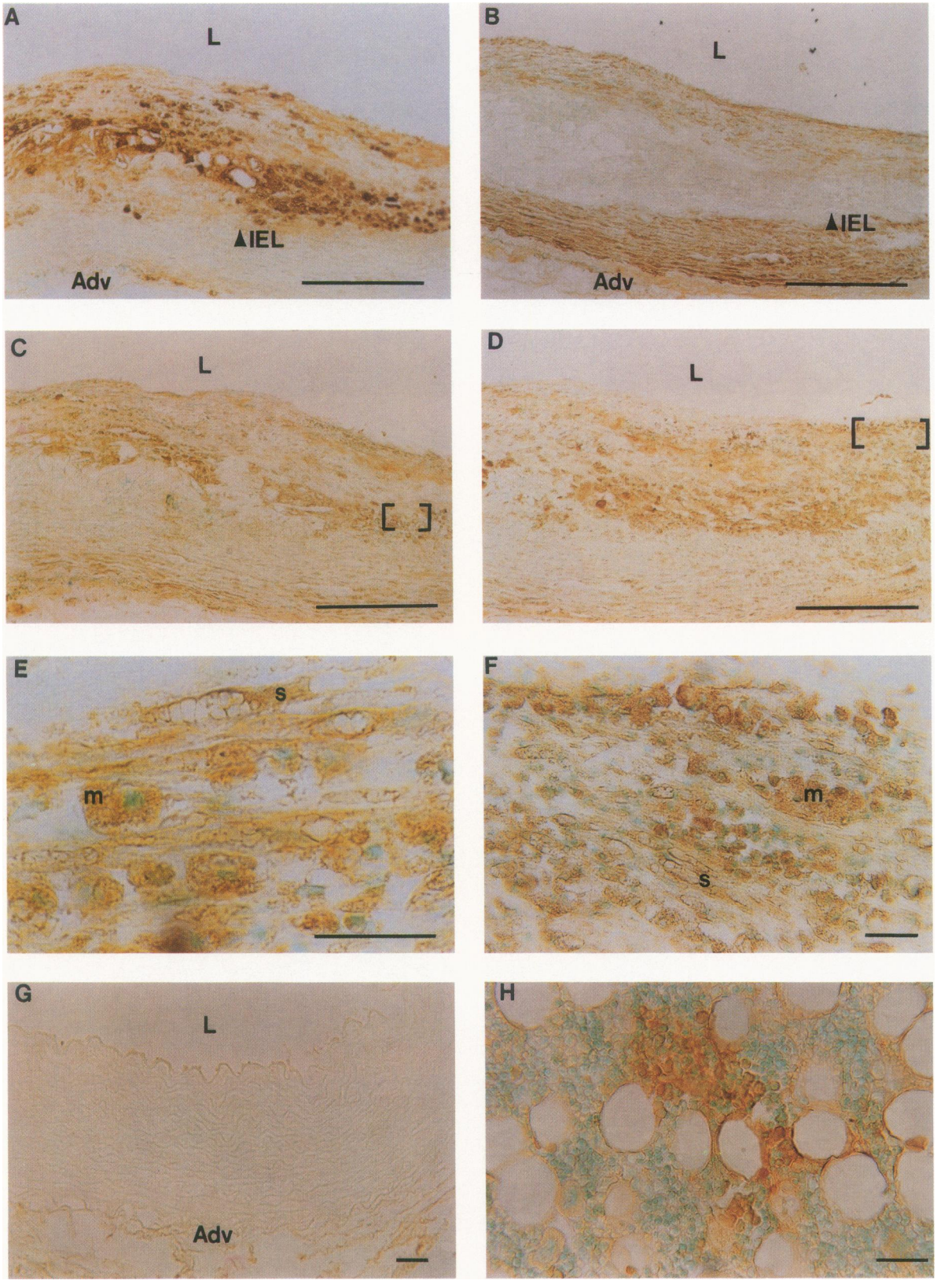

Figure 2. *Immunocytochemical analysis of MCSF in macrophage-rich WHHL rabbit atherosclerotic lesion and aorta and bone marrow from normal NZW rabbit. Serial sections of an advanced fatty streak from the thoracic aorta of a mature WHHl rabbit were immunostained as described in Methods.* **A**: *Macrophage specific antibody RAM-11 (dilution = 1:2,000).* **B**: *Smooth muscle actin specific antibody HHF-35 (dilution = 1:3,000).* **C**: *MCSF specific antibody HM7/41410 (dilution = 1:100), area in brackets is shown at high magnification in* **(E)**. **D**: *MCSF specific antibody HM7/7710 (dilution = 1:100), area in brackets is shown at high magnification in* **(F)**. **E**: *High magnification micrograph of macrophage and smooth muscle cells staining with MCSF specific antibody HM7/41410.* **F**: *High magnification micrograph of predominantly macrophages staining with MCSF specific antibody HM7/7710.* **G**: *Section of the thoracic aorta of a normal NZW rabbit stained with MCSF specific antibody HM7/7710.* **H**: *Bone marrow from normal NZW rabbit stained with MCSF specific antibody HM7/7710.* **A–D**: *Bar = 100 microns.* **E–G**: *Bar = 10 microns, L = lumen, Adv = adventitia, IEL = internal elastic lamina (arrowheads), m = macrophage, s = smooth muscle cell.*

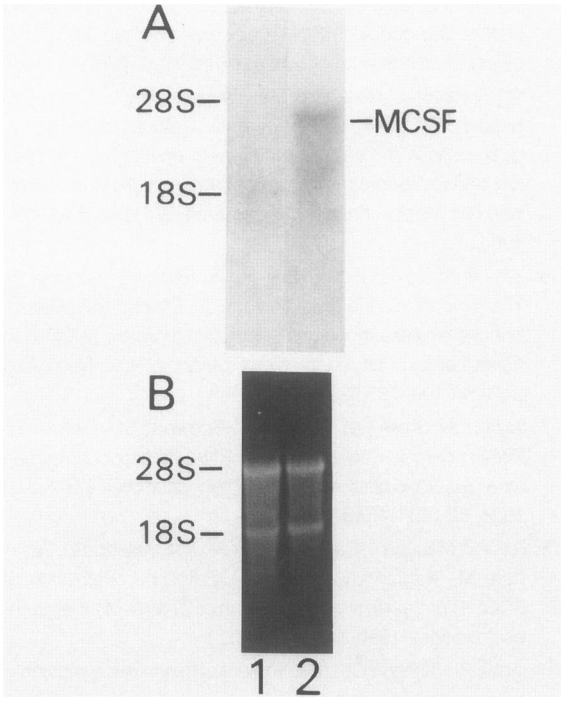

Figure 3. *Northern blot analysis of MCSF expression by MFC. Total RNA was isolated and subjected to northern blot analysis (25 μg total RNA/lane) as described in Methods using a human anti-sense MCSF cDNA probe.* **A**: *Lane 1, alveolar macrophages simultaneously isolated from the same rabbits used for isolating the MFC. Lane 2, MFC* **B**: *Ethidium-bromide staining of the RNA-gel. Lanes 1 and 2 are the same as in the upper panel. Positions of the 28S and 18S ribosomal RNA subunits are indicated.*

that were tested. LPS (10 ng/ml) or PMA (10 μg/ml) did not stimulate further secretion of MCSF by the isolated foam cells (data not shown).

Discussion

These studies show that the gene for MCSF is actively expressed in human and rabbit atherosclerotic lesions. Our results are in agreement with those of Rajavashisth et al.[14] who demonstrated MCSF message by Northen blot analysis of total RNA extracted from both normal arteries and atherosclerotic lesions of rabbit or swine (*in situ* hybridizations were not carried out). Our results show that all of the major cell types in the lesion express the gene. Our failure to find immunoreactive protein in sections of normal rabbit artery may reflect the limited sensitivity of the technique, since we were not using rabbit-specific antibodies, and/or a low level of expression of MCSF in normal arterial tissue.

The generalized nature of expression of MCSF by several cell types suggests a common mode of stimulation. Recent studies by Rajavashisth et al.[14] and Liao et al.[33] indicate that partially oxidized LDL induces the expression of MCSF in cultured endothelial cells and when injected intravenously caused the release of biologically active MCSF into the serum of mice *in vivo*. We, and others, have recently demonstrated the presence of oxidized LDL in human and rabbit atherosclerotic lesions.[26,34–36] Therefore, oxidized LDL could stimulate the expression of MCSF not only by endothelial cells but possibly also by macrophages and smooth muscle cells. Although endotoxin (LPS) did not stimulate the MFC to release additional colony-stimulating activity (data not shown), LPS has been shown to stimulate the expression of MCSF by monocytes *in vitro*.[37] As LPS associates with LDL particles,[38] it may be that LPS carried into the artery by LDL also stimulates the observed MCSF expression.

Other cytokines may also play an important role in stimulating the expression of MCSF by arterial cells. Several recent *in vitro* studies suggest that gamma-interferon, TNF-alpha, IL-1-beta, IL-3, GM-CSF, and IL-6 induce the expression of MCSF by monocytes and fibroblasts.[15,39–42] Recent *in vivo* studies of atherosclerotic lesions in primates[8,43] indicate that there is expression of IL-1-beta and TNF-alpha in the lesions. Rajavashisth et al.[14] have further demonstrated that cultured endothelial cells express GM-CSF in response to minimally oxidized LDL, whereas Lopponow and Libby have demonstrated that vascular smooth muscle cells make IL-6 after treatment with IL-1 or LPS.[44]

MCSF expression may play an important role in the atherogenic process. Studies by Wang et al.[25] have demonstrated that MCSF stimulates monocyte migration. Thus, the widespread cellular expression of MCSF, in combination with the expression of factors such as MCP-1 and PDGF[6–8] and the formation of oxidized LDL[2,25] may attract additional monocytes into the developing lesion. The presence of MCSF may also stimulate the differentiation of the newly arrived monocytes, including in particular the induction of scavenger receptors.[4,24]

Studies of cellular proliferation in atherosclerotic lesions from humans and rabbits, suggest that a significant percentage of cells taking up thymidine, or of those immunoreactive for the pericyclic nuclear antigen (Cyclin), are macrophages.[11,12] As *in vitro* studies indicate that MCSF stimulates proliferation of macrophages,[17,18] the localized expression of MCSF may be responsible for stimulating the observed arterial macrophage proliferation. However, even though MFC in culture secrete biologically active MCSF, it is still unclear whether the MCSF present in the lesions is biologically active or whether proliferating macrophages within the lesions express the MCSF receptor (c-*fms*) and thus, are capable of responding to the locally produced MCSF.

There is recent evidence that macrophages within atherosclerotic lesions express MHC and other activation specific cell surface antigens[9,10] and may be activated as part of an inflammatory or immune response.[9] Again,

in vitro studies have demonstrated that MCSF stimulates the expression of HLA-DR and other cell surface antigens such as the F_c gamma RIII receptor on monocytes[20,39,40] and enhances the capacity of monocytes and macrophages for antibody dependent and independent killing of bacteria.[19,20,40,43] Thus, expression of MCSF in atherosclerotic lesions may enhance the capacity of arterial macrophages to respond to inflammatory events or perhaps bind and present antigens to lymphocytes, which are now known to also reside in human atherosclerotic lesions.[9]

An additional role for MCSF may be as a maintenance factor for resident arterial macrophages. MCSF has been shown to be essential for the long-term survival of monocytes in both suspension and adherent cultures.[46,37] MCSF expression may have a similar effect *in vivo,* especially with macrophage-derived foam cells that may be compromised by their massive lipid content or as a result of the uptake of oxidized LDL.[47] In fact, it may be that a reduction in the expression of MCSF in more advanced lesions could contribute to the necrosis of foam cells and the formation of the acellular, lipid-rich necrotic core.

Acknowledgments

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