MCSF expression is induced in healing myocardial infarcts and may regulate monocyte and endothelial cell phenotype

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Submitted 2 December 2002; accepted in final form 7 April 2003

MCSF expression is induced in healing myocardial infarcts and may regulate monocyte and endothelial cell phenotype. Am J Physiol Heart Circ Physiol 285: H483–H492, 2003. First published April 10, 2003; 10.1152/ajpheart.01016.2002.—Myocardial infarction is associated with the rapid induction of mononuclear cell chemokat- tants that promote monocyte infiltration into the injured area. Monocyte-to-macrophage differentiation and macrophage proliferation allow a long survival of monocytes/cells, critical for effective healing of the infarct. In a canine infarct-reperfusion model, newly recruited myeloid leukocytes were markedly augmented during early reperfusion (5–72 h). By 7 days, the number of newly recruited myeloid cells was reduced, and the majority of the inflammatory cells remaining in the infarct were mature macrophages. Macrophage colony-stimulating factor (MCSF) is known to facilitate monocyte survival, monocyte-to-macrophage conversion, and macrophage proliferation. We demonstrated marked induction of MCSF mRNA in ischemic segments persisting for at least 5 days after reperfusion. MCSF expression was predominately localized to mature macrophages infiltrating the in- farcted myocardium; the expression of the MCSF receptor, c-Fms, a protein with tyrosine kinase activity, was found in these macrophages but was also observed in a subset of microvessels within the infarct. Many infarct macrophages expressed proliferating cell nuclear antigen, a marker of proliferative activity. In vitro MCSF induced monocyte chemotactant protein-1 synthesis in canine venous endothelial cells. MCSF-induced endothelial monocyte chemotactant protein-1 upregulation was inhibited by herbimycin A, a tyrosine kinase inhibitor, and by LY-294002, a phosphatidylinositol 3’-kinase inhibitor. We suggest that upregulation of MCSF in the infarcted myocardium may have an active role in healing not only through its effects on cells of monocyte/macrophage lineage, but also by regulating endothelial cell chemokine expression.

infection; reperfusion; chemokine; growth factors; mac- rophage colony-stimulating factor

METHODS

Ischemia-reperfusion protocols. All animal research proto- cols were approved by the Baylor College of Medicine Animal Research Committee. Healthy mongrel dogs (15–25 kg) of either sex were surgically instrumented as previously de- scribed (14, 28). Anesthesia was induced intravenously with 10 mg/kg methohexital sodium (Brevital, Lilly; Indianapolis, IN) and maintained with the inhalational anesthetic isoflu- rane (Anaquest; Madison, WI). A midline thoracotomy provided access to the heart and mediastinum. Subsequently, a hydraulically activated occluding device and a Doppler flow probe were secured around the circumflex coronary artery just proximal or just distal to the first branch. Indwelling catheters placed in the right atrium, left atrium, and femoral artery allowed blood sampling and pressure monitoring as needed. After surgery, the animals were allowed to recover for 72 h before coronary occlusion. Ischemia-reperfusion pro-
tocols were performed as previously described (10, 11). Coronary artery occlusion was achieved by inflating the coronary cuff occluder until mean flow in the coronary vessel was zero as determined by the Doppler flow probe. After 1 h, the cuff was deflated and the myocardium reperfused. Reperfusion intervals ranged from 1 h to 7 days. Circumflex blood flow arterial blood pressure, heart rate, and ECG (standard limb II) were recorded continuously. Analgesia was accomplished with intravenously administered 0.1–0.2 mg/kg pentazocine (Talwin; Winthrop Pharmaceuticals; New York, NY). After the reperfusion periods, the hearts were stopped by the rapid intravenous infusion of 30 meq KCl. The hearts were then removed from the chest for sectioning from apex to base into four transverse rings ~1 cm in thickness. The posterior papillary muscle and the posterior free wall were identified. Tissue samples were isolated from infarcted or normally perfused myocardium based on visual inspection. Myocardial segments were fixed for histological analysis or immediately frozen, homogenized, and processed for RNA extraction. Duplicate adjacent samples were also processed for blood flow determinations using radiolabeled microspheres as previously described. The monoclonal antibody to was based on light-microscopic examination of hematoxylin and eosin-stained tissue sections by the findings of contraction bands, “wavy fibers,” interstitial edema, and neutrophil infiltration. For experiments lasting 24 h or more after the injection bands, “wavy fibers,” interstitial edema, and neutrophil infiltration. For experiments lasting 24 h or more after the injection bands, “wavy fibers,” interstitial edema, and neutrophil infiltration. For experiments lasting 24 h or more after the injection bands, “wavy fibers,” interstitial edema, and neutrophil infiltration. For experiments lasting 24 h or more after the injection bands, “wavy fibers,” interstitial edema, and neutrophil infiltration. For experiments lasting 24 h or more after the injection bands, “wavy fibers,” interstitial edema, and neutrophil infiltration. For experiments lasting 24 h or more after the injection bands, “wavy fibers,” interstitial edema, and neutrophil infiltration. For experiments lasting 24 h or more after the injection bands, “wavy fibers,” interstitial edema, and neutrophil infiltration. For experiments lasting 24 h or more after the injection bands, “wavy fibers,” interstitial edema, and neutrophil infiltration. For experiments lasting 24 h or more after the injection bands, “wavy fibers,” interstitial edema, and neutrophil infiltration.
each reperfusion interval were used for quantitative analysis. Northern hybridization analysis and quantitation was performed by an investigator blinded to the tissue conditions and the blood flow determinations.

**Endothelial cell isolation and stimulation.** Endothelial cells were obtained as previously described (12). Jugular veins were everted on glass rods and incubated in collagenase solution (Boehringer Mannheim; Indianapolis, IN) for 20 min. Cells were collected by centrifugation and suspended in DMEM containing 5% FCS, 5% bovine calf serum, 50% mg/ml endothelial cell growth factor, 50 U/ml heparin, 1 mmol/l sodium pyruvate, and antibiotics. Cells were seeded in Primaria flasks (Becton Dickinson; San Jose, CA). After 2–4 days of incubation at 37°C in a CO2 incubator, areas of cells with cobblestone morphology were collected by scraping, transferred to gelatin-coated flasks (0.1% Difco), and grown to confluence. Endothelial cells were incubated with recombinant human MCSF (R&D Systems; Minneapolis, MN) for 2–24 h. To investigate the possible involvement of signaling kinases in MCSF-induced endothelial MCP-1 mRNA expression, we incubated canine endothelial cells with the tyrosine kinase inhibitor herbimycin A, the p38 mitogen-activated protein kinase inhibitor SB-203580, or with LY-294002 (all from Sigma), a specific phosphatidylinositol 3-kinase (PI3-kinase) inhibitor, 30 min before stimulation with MCSF. At the end of the experiment, endothelial cells were used for mRNA extraction as previously described. Quantitation of the Northern hybridization results was performed using densitometry. Relative density was normalized to the intensity of the 28S ribosomal RNA.

**Statistical analysis.** Statistical analysis was performed using ANOVA, followed by Student-Newman-Keuls t-test corrected for multiple comparisons. Data were expressed as means ± SE. Statistical significance level was set at 0.05.

**RESULTS**

**Monocyte-macrophages accumulate in ischemic and reperfused myocardium.** We used a dual immunohistochemical technique recently validated in our laboratory (15) to identify newly infiltrated myeloid cells and mature resident macrophages in canine infarcts. Newly recruited leukocytes (neutrophils and monocytes) were stained with the monoclonal antibody Mac387, which detects MRP14, a protein expressed in myeloid cells, but rapidly downregulated during monocyte to macrophage maturation (19). Mature resident macrophages were identified with the monoclonal antibody PM-2K (Fig. 1). Abundant Mac387-positive, newly infiltrated leukocytes accumulated in the infarct after 5–72 h of reperfusion (1-h ischemia/5-h reperfusion interval).

**Fig. 1.** Dual immunohistochemical staining identifies newly recruited macrophage Mac387-positive leukocytes (red) and monoclonal antibody PM-2K-positive mature macrophages (black) in the canine myocardium. Mac387 recognizes an epitope to the calcium binding protein MRP14, which is highly expressed in myeloid cells but is rapidly downregulated during monocyte to macrophage maturation. A: control myocardial areas show a resident macrophage population (black), whereas Mac387-positive myeloid cells (monocytes and neutrophils) are intravascular (red). B: canine infarct after 1 h of coronary occlusion and 24 h of reperfusion demonstrates abundant newly recruited leukocytes (red), and PM-2K-positive macrophages (black). C: after 7 days of reperfusion active recruitment of inflammatory cells is decreased and the healing infarct is now filled with mature macrophages. D: after 28 days of reperfusion the infarct is less cellular, containing a significant number of macrophages, and rare newly recruited cells. E: density of extravascular Mac387-positive leukocytes in the infarcted canine myocardium. Recruitment of myeloid cells was high after 5–72 h of reperfusion, but decreased significantly during the healing phase (120–168 h of reperfusion). F: in contrast, the number of PM-2K-positive mature macrophages in infarcted areas increased significantly after 72 h of reperfusion and remained elevated for at least 168 h of reperfusion. *P < 0.05 compared with control areas; **P < 0.01 vs. noninfarcted areas from the same experiment (n = 4 animals per reperfusion interval).
Fig. 2. Monocyte-to-macrophage differentiation is associated with collagen deposition in the healing infarct. Serial sections from canine infarcts after 24 h (A–C), 72 h (D–F), and 7 days (G–I) of reperfusion were stained for Mac387 (A, D, and G) a marker for newly recruited leukocytes, for PM-2K, a monoclonal antibody that identifies mature macrophages (B, E, and H) and collagen type III (C, F, and I). After 24 h of reperfusion (top) active leukocyte recruitment is noted (A, arrows), without significant new collagen deposition (C). After 72 h of reperfusion, many newly recruited Mac387-positive leukocytes are found in the infarct (D). Note that many PM-2K-positive mature macrophages (E) are found in an area of collagen deposition (F, arrow), whereas PM-2K-expressing cells are fewer in infarcted areas without replacement fibrosis. After 7 days of reperfusion, newly recruited Mac387-positive cells are rare (G) and the collagen-rich infarct (I) is filled with mature macrophages (H).
tion ischemic: 737.3 ± 151.4 cells/mm² vs. control: 16.2 ± 4.2; P < 0.01, n = 4) (Figs. 1, B and E, 2A, and 2D) however, leukocyte recruitment decreased significantly after 5 days (P < 0.05) and 7 days (P < 0.01) of reperfusion (Fig. 1C). In contrast, macrophage density steadily increased, peaking after 5–7 days of reperfusion (Fig. 1F and 2H). At this stage, the inflammatory cellular infiltrate in the infarcted myocardium predominantly contained differentiated mature macrophages and only rare newly recruited Mac387-expressing cells were noted (Fig. 1, C and D, and 2G). The macrophage differentiation process was associated with collagen deposition in the healing infarct and mature macrophages were found in areas exhibiting replacement fibrosis (Fig. 2).

Cloning of canine MCSF. The partial clone for canine MCSF (243 bp) demonstrated 89% homology with its human and 85% with its murine homologue.

MCSF mRNA induction after experimental canine myocardial infarction. Northern hybridization using tissues from endotoxin-stimulated dogs (12) demonstrated two distinct MCSF transcripts: a 4.5-kb transcript was common to all tissues examined and a smaller 1.4-kb transcript was expressed predominantly in the lymph node (Fig. 3A). Subsequently, we examined MCSF mRNA synthesis in the ischemic and reperfused canine myocardium. We found significant upregulation of MCSF mRNA in ischemic segments from experiments of coronary occlusion and reperfusion (Fig. 3B). Low levels of constitutive MCSF mRNA expression were found in control segments. Infarcted segments exhibited increased MCSF mRNA levels after 5 h of reperfusion (P < 0.01 vs. control segments from the same experiment) (Fig. 3D). MCSF expression remained elevated after 24–120 h of reperfusion (Fig. 3, C and D). Only the 4.5-kb MCSF transcript was found in the canine heart.

MCSF immunoreactivity after myocardial infarction. Immunohistochemical experiments localized MCSF immunoreactivity in mononuclear-like cells infiltrating the ischemic myocardium (Fig. 4). These cells first appeared after 1 h of ischemia and 3 h of reperfusion and became more numerous after 24–72 h of reperfusion. Minimal MCSF protein expression was noted in control myocardial segments. Dual immunohistochemical staining indicated that MCSF immunoreactivity was predominantly localized in PM-2K-positive macrophages infiltrating the healing infarct (Fig. 4).

Proliferating macrophages in infarcted myocardium. Dual immunohistochemistry combining peroxidase-based staining for PM-2K and alkaline-phosphatase immunohistochemistry for PCNA demonstrated a significant number of proliferating macrophages in the healing heart (Fig. 5). Few proliferating macrophages

Fig. 3. A: macrophage colony-stimulating factor (MCSF) mRNA expression in tissues from an endotoxin-stimulated dog. Northern hybridization experiments revealed two distinct MCSF transcripts: the 1.4-kb transcript was found predominantly in the lymph node (LN), whereas the 4.5-kb transcript was predominantly expressed in the spleen (S), lung (Lu), heart (H), and kidney (K). Li, liver. B: MCSF mRNA upregulation in canine infarcts. The 4.5-kb MCSF transcript was significantly upregulated in ischemic segments (I) after 1 h of ischemia and 5 h of reperfusion. Low levels of constitutive MCSF mRNA expression were found in control (C) segments. C: time course demonstrating MCSF mRNA synthesis in healing canine infarcts. MCSF mRNA was induced in myocardial infarcts after 1 h of reperfusion, and its expression remained elevated for at least 5 days of reperfusion. D: quantitative analysis of MCSF mRNA expression in canine infarcts. Sustained upregulation of MCSF mRNA synthesis was noted in infarcted segments after 5–120 h of reperfusion when compared with control segments from the same experiments. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control segments. Ischemic segments had blood flow <25% of control as determined by the method of radiolabeled microspheres and were obtained from four different experiments of coronary ischemia-reperfusion for each reperfusion interval (number of ischemic segments used for quantitative analysis: 3 h, n = 5; 5 h, n = 8; 24 h, n = 5; 120 h, n = 6).
DISCUSSION

Substantial evidence suggests that reperfusion of the ischemic myocardium exerts a positive effect on healing in both animal models (6, 30) and clinical investigations (22). The beneficial effect of reperfusion in cardiac repair may be related at least in part to the enhanced infiltration of the reperfused myocardium with mononuclear cells, capable of producing cytokines and growth factors, promoting healing. Mononuclear cell chemoattractants, such as C5a, transforming growth factor (TGF)-β1, and MCP-1 are released in the infarcted myocardium (23), mediating mononuclear cell infiltration in the healing heart (11). We suggest that effective cardiac repair may depend on the creation of a microenvironment capable of supporting survival, maturation, and differentiation of monocytes into macrophages in the infarcted heart. We hypothesized that colony-stimulating factors such as MCSF (39, 40), important in monocyte to macrophage maturation, may be induced after myocardial ischemia, mediating survival, proliferation, phenotypic changes, and differentiation of infiltrating mononuclear cells.

Reperfused myocardial infarction is associated with a rapid accumulation of inflammatory leukocytes in the injured area. Intense active infiltration with myeloid Mac387-positive cells is noted for 3 days after reperfusion, but decreases significantly after 5–7 days. At this
stage, the healing infarct exhibits a high number of mature macrophages, many of which demonstrate evidence of proliferative activity (Fig. 5). MCSF expression in the infarct may promote maturation and proliferation of monocytic cells (2, 40) creating an environment that favors macrophage growth. MCSF is a potent chemoattractant for mononuclear phagocytes (41). However, its primary role is to regulate the survival (2, 40), proliferation (1), and differentiation (1) of mononuclear phagocytes. Among its earliest effects on binding to mononuclear phagocyte cells are changes to the cell membrane itself, including ruffling and the formation of filopodia, vesicles, and vacuoles (5). Soon after, metabolic changes are observed, including increases in glucose uptake (18) and protein synthesis with a concomitant decrease in protein degradation. Furthermore, in vivo experiments demonstrated that intravenous infusion of MCSF in rodents (20) and primates (31) induces a marked increase in peripheral blood monocytes and tissue macrophages supporting its role as a critical regulator of the mononuclear phagocyte system.

Macrophages are capable of producing a wide variety of inflammatory mediators and growth factors and are important in regulating wound healing (25, 43). However, the potential role of MCSF in healing wounds has not been adequately investigated. MCSF expression was detected in wound fluid with the use of a sponge matrix model (9). In addition, MCSF-treated dermal ulcers showed accelerated healing and elevated TGF-β1 mRNA expression, suggesting local macrophage activation and growth (24). We present the first demonstration of MCSF mRNA and protein induction in healing myocardial infarcts accompanied by evidence of macrophage proliferation. MCSF mRNA up-regulation was first noted in the ischemic and reperfused myocardium after 1 h of reperfusion and its expression remained elevated for at least 5 days of reperfusion (Fig. 2C). Monocyte macrophages were the...
main source of MCSF after myocardial ischemia raising the possibility for an autocrine mechanism responsible for macrophage growth and activation. Macrophage-derived MCSF may allow for autonomous survival and growth of macrophages without endangering systemic homeostasis. The early stages of infarction are associated with upregulation of monocyte chemoattractants and marked leukocyte recruitment (Fig. 1). At this stage, local MCSF expression may be crucial for monocyte survival and differentiation creating a microenvironment capable of sustaining macrophage growth. Macrophages may be important in regulating fibroblast proliferation and extracellular matrix deposition in the healing infarct through expression of fibrogenic growth factors and production of metalloproteinases and their inhibitors. The localization of mature macrophages in areas of collagen deposition (Fig. 2) suggests that monocyte to macrophage differentiation may play an important role in healing and scar formation.

The exact role of MCSF in regulating monocyte to macrophage differentiation remains unclear. Osteopetrotic mice lack functional MCSF and have few tissue macrophages (42). However, transgenic osteopetrotic mice that overexpress human bel-2 in monocytes to inhibit their apoptotic death, show significant tissue macrophage replenishment (24) suggesting that monocytes may be capable of differentiating into macrophages in the absence of functional MCSF, and MCSF may simply serve as a factor that augments monocyte survival.

Although the role of MCSF in regulating monocyte/macrophage phenotype and activity has been extensively investigated, little information is available on its potential effects on other cell types involved in wound healing and cardiac repair. Shyy and co-workers (37) demonstrated that recombinant human MCSF induces MCP-1 mRNA synthesis in human umbilical vein endothelial cells and increases monocyte adhesion to endothelial monolayers. In addition, MCSF induces expression of the C-X-C chemokine interferon-γ-inducible protein 10, a mononuclear cell chemoattractant, in canine jugular vein endothelial cells (12). Our experiments (Fig. 6) demonstrated that MCSF induced a transient MCP-1 upregulation in canine jugular vein endothelial cells, which peaked after 4–6 h of stimulation and decreased to baseline levels after 24 h. The effects of MCSF on cells of the monocyte/macrophage lineage are mediated by the MCSF receptor, encoded by the c-Fms protooncogene, a protein with tyrosine kinase activity (34, 36). However, the mechanisms of MCSF signaling on endothelial cells have not been investigated. We found that endothelial MCSF-induced MCP-1 mRNA synthesis is markedly inhibited by herbimycin A, a specific inhibitor of tyrosine kinase and LY-294002, a PI3-kinase inhibitor. The involvement of a tyrosine kinase pathway is consistent with an MCSF/c-Fms interaction in the venular endothelium and may suggest the expression of c-Fms in vascular endothelial cells. Recently, Minehata et al. (29) demonstrated that endothelial cell precursors express the MCSF receptor and suggested that MCSF may

Fig. 8. Expression of the MCSF receptor c-Fms in healing myocardial infarcts. Serial section staining for c-Fms (A) and the macrophage marker PM-2K (B) in an experiment of myocardial ischemia-reperfusion (1 h of ischemia/72 h of reperfusion) demonstrating, as expected, that macrophages in the healing infarct (arrows) express c-Fms. Serial section staining for c-Fms (C) and the endothelial cell marker CD31 (D) showed that a subset of microvascular endothelial cells (arrows) exhibit c-Fms immunoreactivity.
stimulate differentiation of endothelial cell precursors. Primitive bone marrow cells infiltrate and transdifferentiate in the healing infarct forming vascular structures and myocytes (32). MCSF expression may provide an important differentiation signal for immature hematopoietic cells accumulating in the ischemic area.

MCSF is a critical regulator of monocyte to macrophage differentiation and proliferation. Its local up-regulation in healing infarcts may create a microenvironment necessary for macrophage growth. Macrophages are major contributors to healing through their production of critical cytokines and growth factors. However, MCSF may also promote cardiac repair by modulating endothelial cell phenotype, enhancing production of mononuclear cell chemotacticants such as MCP-1. MCP-1 induction in the microvascular endothelium will not only amplify monocyte chemotaxis (26), but may also have direct angiogenic effects in the infarcted heart (35). The effects of MCSF stimulation on the vascular endothelium suggest that its activity is not limited to cells of the monocyte/macrophage lineage and support its role as an important mediator in wound healing.

The authors thank Lisa Thurmon, Alida Evans, and Stephanie Butler for outstanding technical assistance and Concepcion Mata and Sharon Malinowski for expert secretarial assistance in preparing the manuscript.

DISCLOSURES
This work was supported by National Heart, Lung, and Blood Institute Grant HL-42550, a grant from the American Heart Association, and support its role as an important mediator in wound healing.

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