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## Oncostatin M differentially regulates CXC chemokines in mouse cardiac fibroblasts

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Sandra B. Haudek,<sup>1</sup> C. Wayne Smith,<sup>2</sup> and Mark L. Entman<sup>1</sup>

<sup>1</sup>Department of Medicine, Cardiovascular Sciences, DeBakey Heart Center, The Methodist Hospital, and Baylor College of Medicine, Houston; and <sup>2</sup>Department of Pediatrics, Leukocyte Biology, Children's Nutrition Research Center, Baylor College of Medicine, Houston, Texas

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**Lafontant, Pascal J., Alan R. Burns, Elizabeth Donnachie, Sandra B. Haudek, C. Wayne Smith, and Mark L. Entman.** Oncostatin M differentially regulates CXC chemokines in mouse cardiac fibroblasts. *Am J Physiol Cell Physiol* 291: C18–C26, 2006. First published February 1, 2006; doi:10.1152/ajpcell.00322.2005.— Ischemia-reperfusion injury in the heart is characterized by marked infiltration of neutrophils in the myocardial interstitial space. Studies in human, canine, and murine models have revealed oncostatin M (OSM) expression in infiltrating leukocytes. In an effort to assess possible roles of OSM in the myocardium, we used cardiac fibroblasts (mCFs) isolated from adult mouse heart to determine whether recombinant murine OSM regulates the synthesis and release of MIP2/CXCL2, KC/CXCL1, and LIX/CXCL5, which are three potent neutrophil chemoattractants in the mouse. Our results demonstrate that mCFs express OSM receptors and that, within the IL-6 cytokine family, OSM uniquely induces significant release of KC and LIX in mCFs. In addition, although OSM activates the JAK-signal transducers and activators of transcription and MAPK pathways, we demonstrate that the OSM-mediated CXC chemokine release in mCFs is also dependent on the activation of the phosphatidylinositol 3-kinase pathway.

inflammation; cytokine; neutrophil

ONCOSTATIN M (OSM) belongs to the IL-6 family of cytokines, which includes IL-6, leukemia-inhibitory factor (LIF), IL-11, cardiotrophin-1 (CT-1), and ciliary neurotrophic factor (37). The IL-6 family of cytokine receptors requires dimerization with glycoprotein 130 (gp130), a glycoprotein cell surface receptor, for intracellular signaling. The members of the IL-6 family of cytokines frequently cause overlapping molecular responses [e.g., signal transducer and activator of transcription (STAT3) activation], although specific nonredundant functions for many members of the family have been established (20). OSM as well as several members of the IL-6 cytokine family are known to activate fibroblasts and to regulate the synthesis of matrix metalloproteinases and their inhibitors in these cells (24, 25, 42). However, the results of studies in the human, rat, and mouse have supported the notion that OSM is involved uniquely in the regulation of inflammation (15, 22, 23, 26, 27, 28, 31). OSM is primarily produced in and released by activated monocytes, T lymphocytes, and neutrophils (4, 16, 32), and it is found in a variety of inflammatory sites. In the human lung during acute lung injury, infiltrating neutrophils secrete OSM (15). OSM levels also are elevated in the sera of patients

with rheumatoid arthritis (28) as well as in patients with inflamed skin (36) and periodontitis (23). In addition, *in vitro* studies have demonstrated that OSM not only regulates the remodeling function of fibroblasts but also elicits inflammatory responses in these cells. OSM induces the CC chemokines eotaxin (26) (an eosinophil chemoattractant) and monocyte chemoattractant protein 1 (25) in mouse lung and in synovial fibroblasts, respectively. Moreover, overexpression of OSM in the mouse lung results in increased recruitment of eosinophils (26). Altogether, these studies suggest that OSM regulates inflammatory function in fibroblasts and that fibroblasts may be implicated in the recruitment of leukocytes upon activation by OSM.

Members of the IL-6 family of cytokines are also found in stressed or injured myocardium (3, 13, 17, 29, 40, 43). Previous studies conducted at our laboratory showed that IL-6 is induced and synthesized in the myocardial infarction border zone after reperfusion (17). More recently, Gwechenberger et al. (18) demonstrated that OSM was present in leukocytes during a 7-day observation period in a canine model of myocardial reperfusion. Ischemia-reperfusion injury in the heart is characterized by early extravasation of neutrophils and monocytes into the myocardial interstitial space (7, 9, 11), and myocardial damage after reperfusion of ischemic tissue is caused primarily by infiltrating neutrophils. The recruitment of neutrophils into the extravascular space of various organs is regulated in part by the  $\alpha$  (CXC) chemokine family of inflammatory and immunoregulatory cytokines, which include human IL-8/CXC ligand 8 (IL-8/CXCL8), the growth-related oncogene isoforms (GRO- $\alpha$ , - $\beta$ , and - $\gamma$ /CXCL1, CXCL2, and CXCL3), epithelial neutrophil-activating peptide (ENA-78/CXCL5), and mouse macrophage inflammatory protein 2/CXCL2 (MIP2/CXCL2), renal keratinocyte-derived chemokine (KC/CXCL1), and LPS-induced chemokine (LIX/CXCL5) (6, 30). Whether OSM regulates the recruitment of leukocytes in the ischemic reperfused heart is not known.

Among interstitial cells, mast cells and resident macrophages have been suggested to contribute to leukocyte recruitment in extravascular spaces (10, 14). Whether cardiac fibroblasts, the most abundant resident interstitial cells in the heart, contribute to leukocyte recruitment in the reperfused heart is not known. Although it was previously established that cardiac fibroblasts can synthesize and release a variety of cytokines *in vitro* (8, 44), whether they synthesize the chemokines involved

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in the recruitment of leukocytes at the site of inflammation is not known and whether OSM can regulate chemokine synthesis in cardiac fibroblasts has not been studied. Therefore, using mouse cardiac fibroblasts (mCFs) isolated from adult mouse heart, we sought to determine in the present study whether recombinant mouse OSM can regulate the synthesis and release of MIP2/CXCL2, KC/CXCL1, and LIX/CXCL5, which

are three potent neutrophil chemoattractants in the mouse. We have demonstrated selectivity among the members of the IL-6 family of cytokines to regulate CXC chemokines in mCFs, and that OSM triggers highly specific chemokine expression in mCFs. In addition, we have demonstrated that the OSM-mediated response is dependent on the phosphatidylinositol 3-kinase (PI3-kinase) pathway. We compared the response of

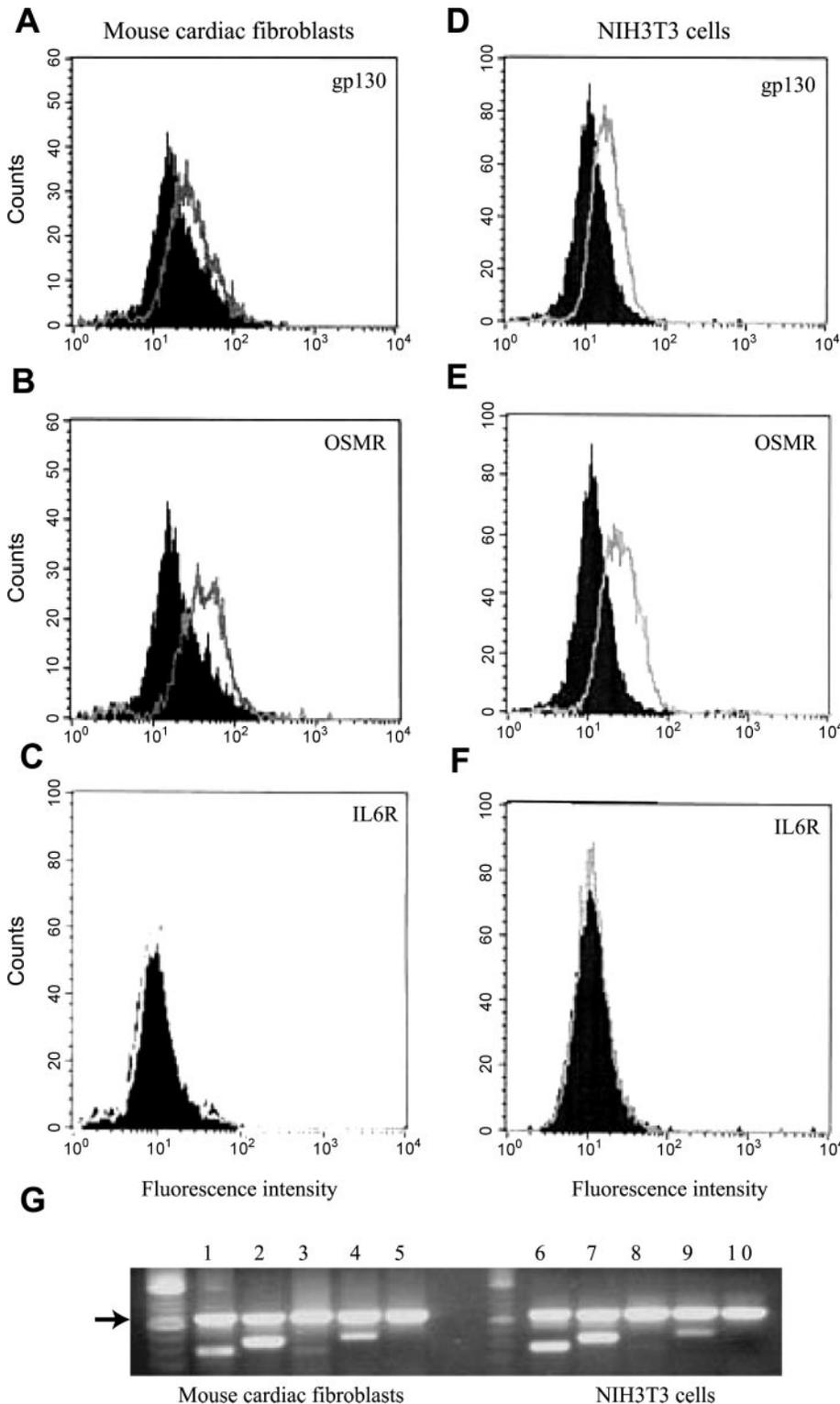


Fig. 1. Flow cytometric analysis of the surface expression of oncostatin M (OSM) receptor (OSMR), IL-6 receptor (IL6R), glycoprotein 130 (gp130), as well as RT-PCR analysis of mRNA expression of IL-6 cytokine family receptors in adult murine cardiac fibroblasts (mCFs) isolated from adult mouse heart and NIH 3T3 cells. mCFs (A–C) and NIH 3T3 cells (D–F) were incubated with MAbs to mouse OSMR and IL6R, with goat PAb against mouse gp130, and with the appropriate rat or goat IgG isotype controls. The cells were incubated with FITC- or phycoerythrin (PE)-labeled secondary antibody and were analyzed as described in MATERIALS AND METHODS. Histograms are representative of 3 experiments for mCFs and of 2 experiments for NIH 3T3 cells. Rightward shifts of the histograms (receptors) from filled histograms (IgG control) indicate the presence of the specific receptor on the cell surfaces. G: mRNA expression of cytokine receptors by RT-PCR in unstimulated mCFs (lanes 1–5), and NIH 3T3 cells (lanes 6–10). Top bands (arrow), GAPDH; bottom bands, gp130, lanes 1 and 6; OSMR, lanes 2 and 7; IL6R, lanes 3 and 8; leukemia-inhibitory factor (LIF) receptor, lanes 4 and 9; IL-11 receptor, lanes 5 and 10.

primary mCFs with that of embryonic NIH 3T3 cells to elucidate the pertinent signaling pathways involved in this regulation.

## MATERIALS AND METHODS

**Preparation of cardiac fibroblasts.** Mouse cardiac fibroblasts (mCFs) were isolated and cultured according to the method described previously by Eghbali (8) and Zeydel et al. (44) with little modification. Left and right ventricles and septa obtained from three to five 12-wk-old C57BL/6J mouse hearts were minced and digested in 100 U/ml type 2 collagenase (CLS2; Worthington Biochemical, Lakewood, NJ) at 37°C. Supernatants were filtered through a 70- $\mu$ m filter. Cells passed through the filter were pelleted, washed to remove the collagenase, and then resuspended in DMEM containing 10% FBS and antibiotics (100 U/ml penicillin G, 100 g/ml streptomycin sulfate, and 0.25  $\mu$ g/ml amphotericin B; GIBCO-BRL, Grand Island, NY), plated on 25- or 75-cm<sup>2</sup> flasks, and incubated at 37°C. Adherent cells were characterized at *passage 1* using immunofluorescence microscopy and found to be positive for vimentin and smooth muscle  $\alpha$ -actin but negative for desmin and CD31 (platelet endothelial cell adhesion molecule 1, a leukocyte and endothelial cell marker). Fibroblasts up to *passage 4* were used in these studies. NIH 3T3 cells (American Type Culture Collection, Manassas, VA) were cultured in the same medium as mCFs. Before (16 h) each experiment, cells were incubated in DMEM with 2% serum. Cells were washed three times in HBSS before being treated with cytokines. All experiments were performed in serum-free DMEM.

**Cytokines and inhibitors.** Mouse recombinant OSM, LIF, IL-6, IL-11, CT-1, and TNF- $\alpha$ , and neutralization antibody against mouse OSM were purchased from R&D Systems (San Diego, CA). Endotoxin levels were <0.1 ng/ $\mu$ g recombinant mouse cytokine as tested by the manufacturer using the limulus amoebocyte lysate method. The pharmacological inhibitors LY-294002 (a specific inhibitor of the PI3-kinase pathway), PD-98059 (a pharmacological inhibitor of the MAPK pathway), and AG 490 (a pharmacological inhibitor of JAK2) were purchased from Calbiochem (San Diego, CA). MAb against MIP2, KC, and LIX for capture, as well as biotinylated PAb MIP2, KC, LIX, and horseradish peroxidase (HRP) and substrate for detection were obtained from R&D Systems. Primary antibodies against mouse OSM receptor (OSMR) and IL-6 receptor for fluorescence-activated cell sorting were obtained from BD Pharmingen (San José, CA), and anti-mouse gp130 was purchased from R&D Systems. FITC- and phycoerythrin (PE)-conjugated secondary antibodies were obtained from Sigma (St. Louis, MO).

**ELISA.** Fibroblasts were seeded at an initial concentration of 5,000 cells/cm<sup>2</sup> in 12- or 24-well plates. After cytokine stimulation, supernatants were collected and frozen at -80°C when not used immediately. ELISA was performed in 96-well plates (Thermo Electron, Waltham, MA). Plates were coated with anti-mouse KC, LIX, or MIP2 MAb overnight and then blocked for 1 h with PBS containing 1% BSA. Samples were incubated for 2 h, washed, and biotin-conjugated secondary antibodies were added for 2 h. Streptavidin-HRP and substrates were used for color development. Plates were read and analyzed on a SpectraMax 96-well plate reader running SoftMAX Pro 3.1 software (Molecular Devices, Sunnyvale, CA). MIP2 and KC protein standards were obtained from R&D Systems, and LIX was from PeptoTech (Rocky Hill, NJ).

**Flow cytometry.** Mouse cardiac fibroblasts were detached from plates using trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA.4Na), washed in ice-cold PBS buffer, resuspended, incubated with primary antibody for 20 min, washed and incubated with FITC- or PE-labeled secondary antibody, and fixed in 1% paraformaldehyde. Analysis was performed on a FACScan device using CellQuest software (BD Biosciences, San Diego, CA).

**Western blot analysis.** Mouse cardiac fibroblasts and NIH 3T3 cells were lysed in ice-cold SDS lysis buffer containing 100  $\mu$ g/ml PMSF.

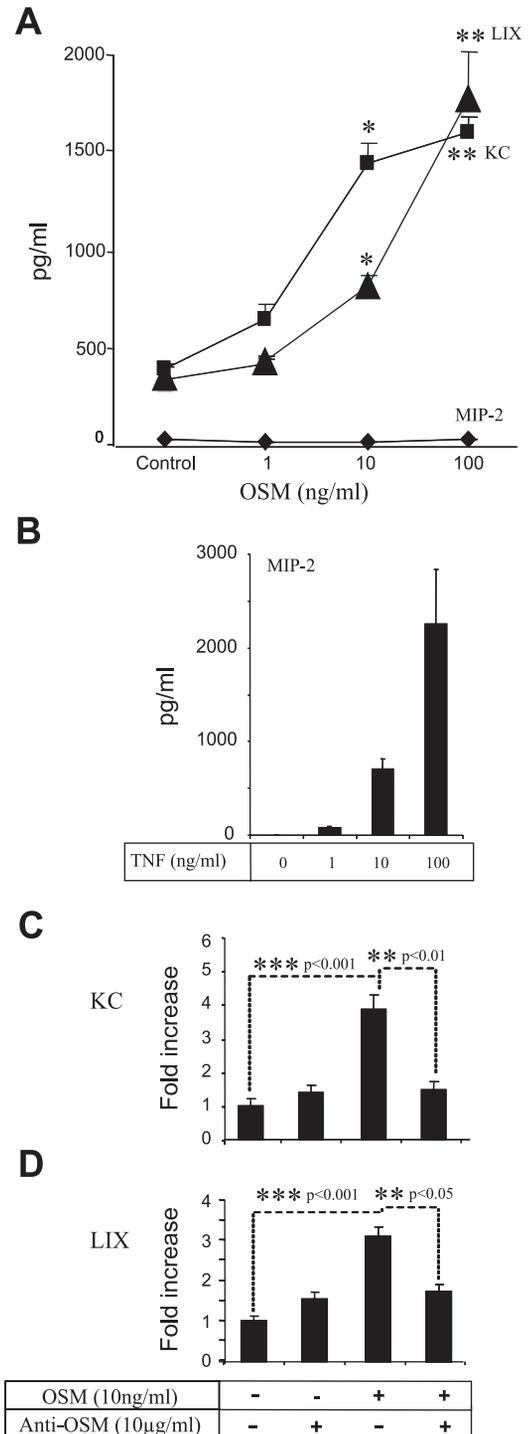


Fig. 2. Dose-response relationships of OSM-stimulated release of  $\alpha$  (CXC) chemokine family of inflammatory and immunoregulatory cytokines renal keratinocyte-derived chemokine (KC), LPS-induced CXC (LIX), and macrophage inflammatory protein 2 (MIP2). mCFs were plated in 24-well plates and treated in serum-free media with increasing doses of OSM for 24 h (A) or TNF- $\alpha$  (B). For blocking studies, mCFs were incubated with 10  $\mu$ g/ml OSM-neutralizing antibodies before addition of recombinant OSM for 24 h as described in MATERIALS AND METHODS. Protein amounts of KC, LIX, and MIP2 released in cell culture supernatants were measured by performing specific sandwich ELISA. Data represent means  $\pm$  SE of 4 independent experiments performed in triplicate (A, C, and D). Data in B represent 3 experiments. \* $P$  < 0.05, \*\* $P$  < 0.01, and \*\*\* $P$  < 0.001 vs. control.

Lysates were sonicated and then centrifuged at 14,000 *g* for 10 min. Lysate supernatant was collected and frozen at  $-80^{\circ}\text{C}$  if not used immediately. Protein concentration was determined using the bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL), and equal amounts of protein were loaded onto 10% SDS-PAGE gels. Proteins were then transferred onto PVDF membrane (MSI, Westborough, MA). Membranes were blocked in PBS containing 5% low-fat milk powder or horse serum, probed with antibodies to ERK1/2 (BD Pharmingen), to STAT1 and STAT3 (BD Pharmingen), to Akt1/2 (Santa Cruz Biotechnology, Santa Cruz, CA), and to their phosphorylated states. Blots were incubated with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology), and bands were visualized using an ECL Plus detection system (Amersham Pharmacia Biotech, Piscataway, NJ). Signals from phosphorylated proteins were assessed first, and then the blots were stripped and subsequently probed with antibodies against total protein.

**RNA isolation and analysis by RNase protection assay.** Total RNA was isolated from mCFs using acid guanidinium thiocyanate-phenol-chloroform. The quantitation and quality of RNA were assessed on the basis of  $A^{260}/A^{280}$  UV absorption. The mRNA expression levels of KC and MIP2 were determined using a RNase protection assay (RiboQuant; BD Pharmingen) according to the manufacturer's protocol. Phosphorimaging of the gels was performed (Storm 860; Molecular Dynamics, Sunnyvale, CA), and signals were quantified using ImageQuant software (Molecular Dynamics) and normalized to housekeeping gene *L32*.

**RT-PCR.** mRNA for mouse OSM, IL-6, LIF, and IL-11 receptors, as well as mRNA for gp130 and GAPDH, were assayed in unstimulated mCFs and NIH 3T3 cells by RT-PCR using specific primers. LIX mRNA in OSM-treated mCFs was assayed by RT-PCR using specific primers (see Supplemental Data; <http://ajp-cell.physiology.org/cgi/content/full/00322.2005/DC1/>).

**Statistical analysis.** Data are means  $\pm$  SE. For statistical analysis, we used one-way ANOVA, followed by Student-Newman-Keuls posttest. Differences in which  $P < 0.05$  were considered significant.

## RESULTS

**Expression of OSMR on mCFs and CXC chemokine secretion after OSM treatment.** Figure 1 shows that mCFs bind antibodies to the OSMR and its associated gp130 coreceptor (Fig. 1, *A* and *B*). The flow cytometric pattern for mCFs was similar to that observed in NIH 3T3 fibroblasts (Fig. 1, *D* and *E*). In addition, RT-PCR analysis revealed expression of OSMR and gp130 mRNA in these cells (Fig. 1*G*). In both the mCFs and NIH 3T3 cells, IL-6 receptor expression was not detected using flow cytometry or by RT-PCR (Fig. 1, *C*, *F*, and *G*). RT-PCR analysis also revealed the presence of LIF receptor mRNA, but not IL-11 receptor mRNA, in mCFs and NIH 3T3 cells. The CXC chemokines MIP2, KC, and LIX were examined in supernatants collected from cultured mCFs treated for 24 h with recombinant mouse OSM. Figure 2 shows that mCFs treated with OSM exhibited dose-dependent increases in the amount of KC and LIX released into the culture supernatant (Fig. 2*A*). OSM did not increase MIP2 secretion. However, TNF- $\alpha$  induced a dose-dependent release of MIP2 (Fig. 2*B*), confirming that the mCFs were capable of MIP2 secretion. KC and LIX secretion was significantly inhibited in the presence of an anti-mouse OSM neutralizing MAb (Fig. 2, *C* and *D*).

**Secretion of CXC chemokines by mCFs in response to other members of the IL-6 family of cytokines.** To determine whether the ability of OSM to regulate CXC chemokine induction in mCFs is unique among members of the IL-6 family of cyto-

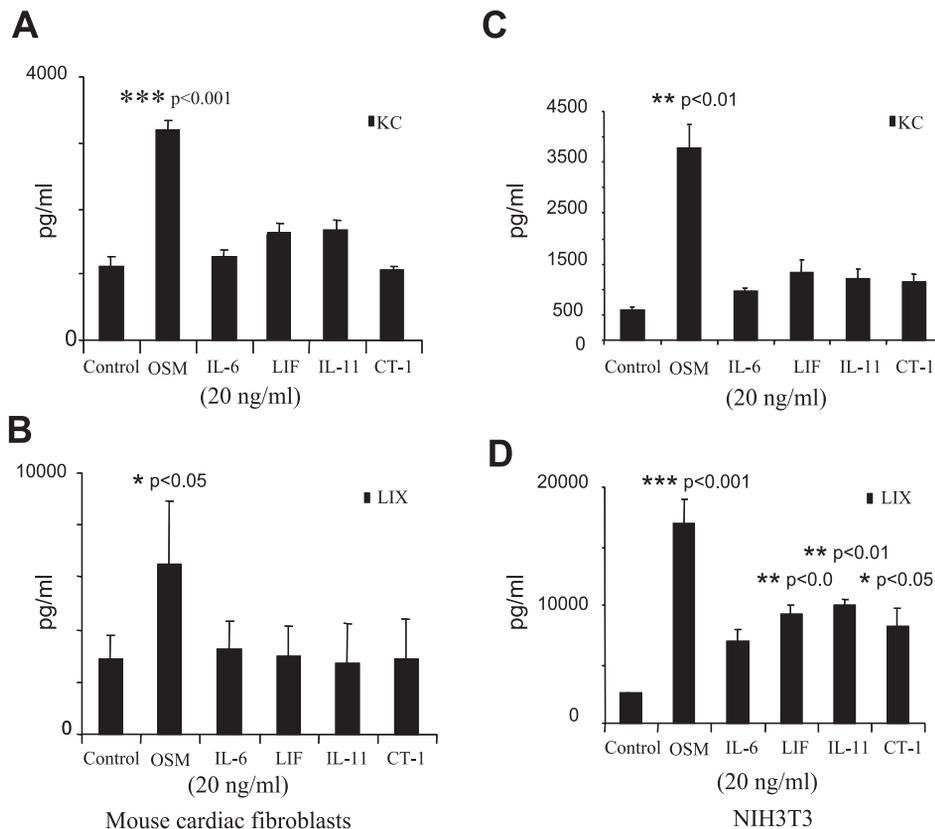


Fig. 3. Regulation of KC and LIX release by the IL-6 family of cytokines in adult mCFs and NIH 3T3 cells. mCFs (*A* and *B*) and NIH 3T3 cells (*C* and *D*) were plated in 24-well plates and treated in serum-free media with 20 ng/ml of each member of the IL-6 family of cytokine OSM for 24 h in quadruplicate. Protein amounts of KC, LIX, and MIP2 released in supernatants were measured by ELISA. Data represent means  $\pm$  SE of 2 independent experiments conducted in quadruplicate. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  vs. control.

kines, we stimulated mCFs and NIH 3T3 cells with a 20 ng/ml concentration of each of the following: OSM, IL-6, LIF, IL-11, and CT-1. In mCFs, only OSM significantly increased the release of KC and LIX (Fig. 3, A and B). Even with an elevated dose (100 ng/ml), IL-6, LIF, IL-11, and CT-1 failed to induce CXC chemokine secretion (data not shown). Neither OSM (Fig. 2A) nor other members of the IL-6 family of cytokines at 10, 20, or 100 ng/ml increased the release of MIP2 (data not shown). In NIH 3T3 cells, OSM significantly increased the release of KC (Fig. 3C) and LIX (Fig. 3D). However, in contrast to mCFs, LIX release was observed in response to LIF, IL-11, and CT-1, but to a lesser extent than in response to OSM, confirming the biological activity of these cytokines.

**Activation of JAK-STAT, MAPK, and PI3-kinase pathways in mCFs.** To determine whether OSM activates the JAK-STAT, MAPK, and PI3-kinase pathways in mCFs, the lysates of mCFs treated with OSM were probed after Western blot analysis using antibodies against phosphorylated and total STAT1, STAT3, ERK1/2, and Akt. We found that at 10 ng/ml, OSM activated STAT1, STAT3, ERK1/2, and Akt (Fig. 4A) in a time-dependent manner. Neither IL-6, LIF, IL-11, or CT-1 activated STAT1 or STAT3 in mCFs (Fig. 4B). Phosphorylation of the STATs was not detected at 10 min, even when IL-6, LIF, IL-11, and CT-1 concentrations were increased to 100 ng/ml (data not shown). However, in addition to OSM, LIF and IL-11 activated STAT3 and Akt in NIH 3T3 cells, although to a lesser degree (Fig. 4C).

**Regulation of OSM-induced CXC chemokine expression in mCFs.** OSM induction of the CC chemokine eotaxin in mouse NIH 3T3 cells is partially dependent on the activation of the MAPK pathway (26). To determine whether activation of the MAPK and PI3-kinase pathways is involved in OSM-induced CXC chemokine release, we treated mCFs with increasing doses of PD-98059 (a pharmacological inhibitor of MEK1 of the MAPK pathway) or LY-294002 (a specific inhibitor of PI3-kinase) before treatment with OSM. Also, using the pharmacological inhibitor AG 490, we tested whether JAK2 of the JAK-STAT pathway is involved in OSM-induced chemokine release. At 24 h, LY-294002 produced a dose-dependent decrease in the OSM-induced release of KC and LIX (Fig. 5, A and B). By contrast, PD-98059 (Fig. 5, C and D) and the JAK2 inhibitor AG 490 (Fig. 5, E and F), under the same conditions, did not decrease LIX or KC secretion significantly.

To determine whether OSM regulates the induction of the CXC chemokines at the level of transcription, we performed a RNase protection assay on total RNA isolated from the lysates of mCFs treated with 10 ng/ml OSM at time intervals up to 24 h. We found that KC mRNA was not detected at *time 0* but was elevated at 30 min and 1 h after OSM stimulation (Fig. 6A). KC mRNA was not detectable beyond 2 h. Consistent with the observed lack of MIP2 protein induction after OSM treatment, OSM failed to increase MIP2 mRNA expression. KC mRNA induction demonstrated dose dependence on OSM concentration, but MIP2 mRNA expression did not (Fig. 6B). mCFs were capable of MIP2 mRNA expression, as evidenced by an inductive response to both TNF and LPS (data not shown). Although LIX mRNA was present in mCFs, we were unable to determine changes in mRNA expression (see Supplemental Data).

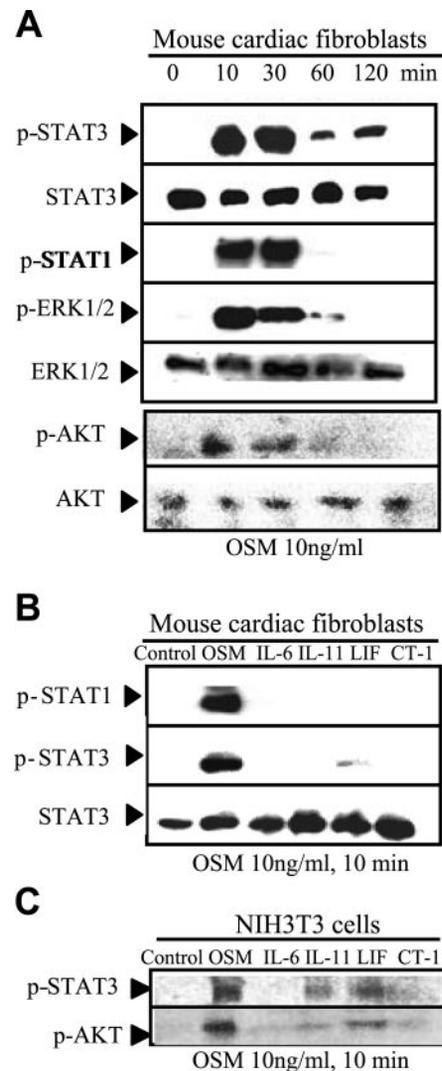


Fig. 4. JAK-signal transducer and activator of transcription (JAK-STAT), MAPK, and phosphatidylinositol 3-kinase (PI3-kinase)-Akt pathway activation by the IL-6 family of cytokines in mCFs and NIH 3T3 cells. mCFs and NIH 3T3 cells were allowed to rest in serum-free media for 2 h before being stimulated with 10 ng/ml OSM, IL-6, LIF, IL-11, cardiotrophin-1 (CT-1), or increasing doses of OSM. For time-dependent response to OSM, total cell lysates were collected at 10, 30, 60, and 120 min. Western blot analysis of lysates was performed using antibodies against total and phosphorylated forms of STAT1, STAT3, ERK1/2, and Akt. Western blot analysis shown in A represents 2 experiments conducted to assess time-dependent activation of STAT1, STAT3, ERK1/2, and Akt by OSM, and Western blot analyses shown in B and C represent responses of mCFs and NIH 3T3 cells to stimulation by 10 ng/ml concentrations of IL-6 family of cytokines for 10 min.

## DISCUSSION

In this study, we have demonstrated for the first time that mCFs in culture express OSMR and that OSM, a member of the IL-6 family of cytokines, induces CXC chemokine expression in mCFs. Previous reports documented OSMR mRNA expression in the developing mouse heart (35) and suggested the expression of OSMR protein in human cardiac myocytes (41). However, no reports have been published to date regarding the surface expression of OSMR on mCFs. Our results provide direct evidence for the expression of the OSMR on the

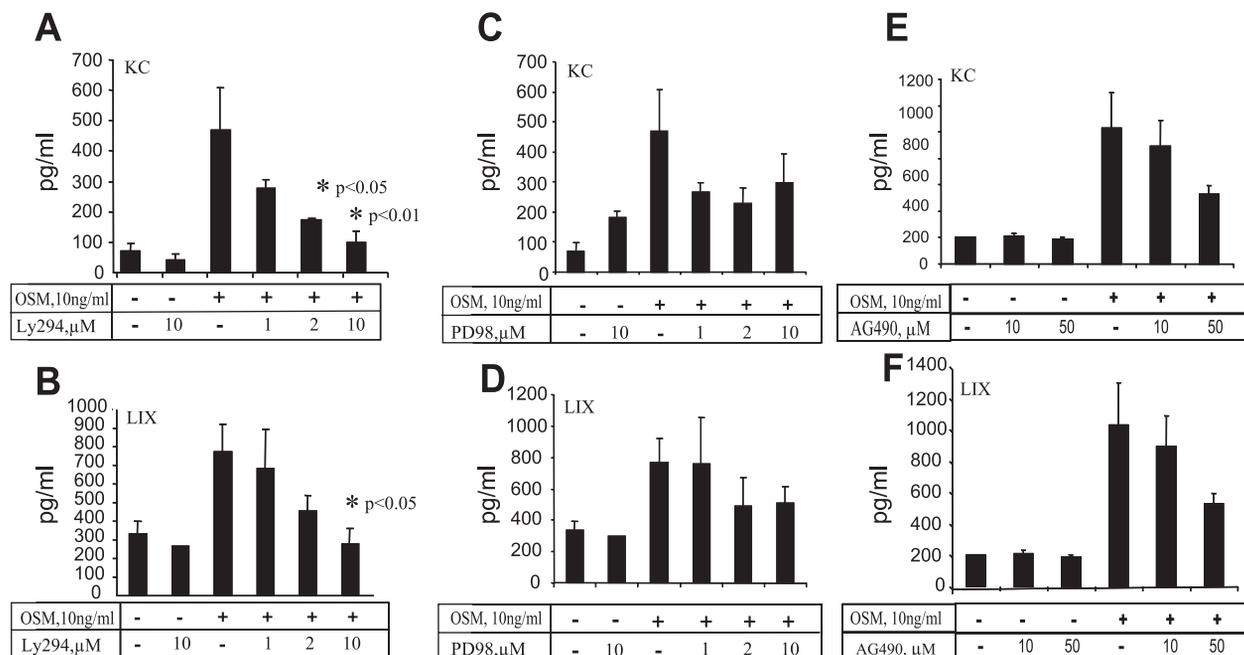


Fig. 5. Role of PI3-kinase, MAPK, and JAK-STAT pathways in OSM-mediated regulation of KC and LIX release in mCFs. mCFs were plated in 24-well plates and treated in triplicate with increasing doses of PI3-kinase inhibitor LY-294002 (LY294), MAPK pathway inhibitor PD-98059 (PD98), and JAK2 inhibitor AG 490 in serum-free media for 1 h. Next, mCFs were treated with 10 ng/ml OSM for 24 h. Protein amounts of KC and LIX released in cell culture supernatants were measured by ELISA. Values shown for LY-294002 (A and B)-, PD-98059 (C and D)-, and AG 490 (E and F)-treated cells are means  $\pm$  SE of 3 independent experiments conducted in triplicate. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  vs. control.

surface of adult mCFs (Fig. 1B) and confirm the surface expression of gp130, the common cell membrane receptor for the IL-6 family of cytokines (Fig. 1A). In response to OSM, mCFs secrete the CXC chemokines KC and LIX, both of which are potent neutrophil chemoattractants. On the basis of

inhibitor studies of the JAK-STAT, MAPK, and PI3-kinase pathways, we have shown that OSM induction of KC and LIX release involves PI3-kinase activation. Altogether, these data suggest an important role for OSM as an inducer of specific CXC chemokine release from resident cardiac fibroblasts.

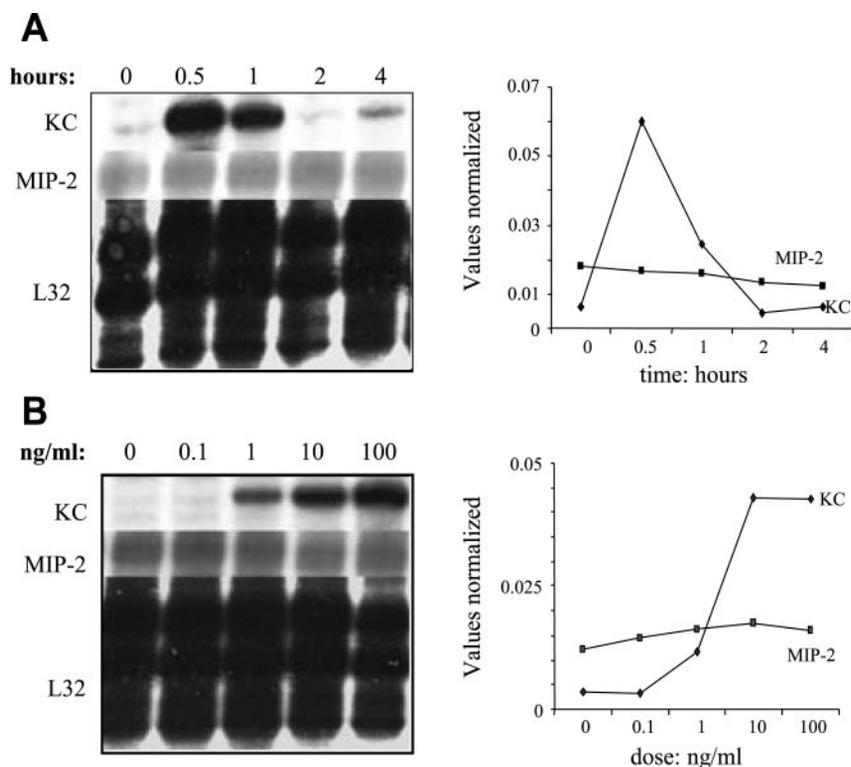


Fig. 6. OSM regulation of KC and MIP2 mRNA levels in mCFs. mCFs were stimulated with OSM in serum-free media. Total RNA was isolated as described in MATERIALS AND METHODS and analyzed for KC and MIP2 expression by performing a RNase protection assay. For induction kinetics studies, 10 ng/ml OSM was added for different time periods (A). For dose-dependent studies, increasing doses of OSM were used and cells were harvested after 30 min of stimulation (B). Data in graphs represent relative band intensities of mRNA obtained using a PhosphorImager and normalized to the housekeeping gene L32.

The presence of the OSMR, along with gp130, supports the notion that OSM may regulate cardiac fibroblast function directly. In response to OSM treatment, mCFs secrete the CXC chemokines KC and LIX, two of the three known potent neutrophil chemoattractants in the mouse (Fig. 2A). These results are consistent with previously published findings documenting that OSM induces endothelial synthesis of GROs and ENA-78, functional orthologs of KC and LIX, respectively (27). Consistent with KC release, OSM induced a time (Fig. 6A)- and dose (Fig. 6B)-dependent increase in KC mRNA transcription levels. It is well established that chemokine up-regulation, including the CXC chemokine IL-8, can be the result of increased transcriptional activity as well as of increased posttranscriptional mRNA stability (21, 34, 39). Whether the increased level of OSM-induced KC mRNA transcripts is due to an increase in mRNA stability or the induction of mRNA transcription, or both, is not known. However, it is worth noting that basal levels of KC mRNA were undetectable before stimulation. OSM treatment has no effect on the MIP2 mRNA transcription level, which is consistent with the lack of MIP2 secretion. Our RT-PCR experiments have shown that LIX mRNA transcripts were detectable in mCFs; however, OSM treatment did not appear to alter its levels (Supplemental data for this article may be found at <http://ajpcell.physiology.org/cgi/content/full/00322.2005/DC1>). Selectivity in CXC chemokine induction by OSM has been observed in other species. For example, in humans (lung fibroblasts, synovial fibroblasts, and brain endothelial cells), OSM does not induce the synthesis or release of IL-8 (31) or the release of MIP2 in rat mesangial cells (19). The inability of OSM to induce the synthesis of MIP2 in mCFs cannot be explained by the cells' inability to synthesize MIP2, because mCFs synthesize and release MIP2 upon stimulation with TNF- $\alpha$  (Fig. 2B).

Our study has demonstrated the apparent uniqueness of OSM among the members of the IL-6 family of cytokines in its ability to induce CXC chemokines in mCFs (Fig. 3, A and B). Even at high cytokine concentrations, OSM but not IL-6, LIF, IL-11, or CT-1 increased the release of KC and LIX (data not shown). This distinctive ability of OSM to regulate CXC chemokine expression is similar to observations in mouse lung fibroblasts, in which OSM but not other IL-6 family members significantly induced the release of the CC chemokine eotaxin (26). The inability of IL-6 to induce CXC chemokine secretion in mCFs is consistent with the apparent lack of IL-6 receptors in mCFs as measured using flow cytometry and RT-PCR. Whether the inability of LIF, IL-11, or CT-1 to regulate CXC chemokine secretion in mCFs is due to the lack of, or to inadequate levels of, surface expression of their respective receptors or is due to their inability to activate the necessary pathways for CXC chemokine synthesis is not clear. Our studies with NIH 3T3 cells also have shown that OSM alone was able to induce secretion of KC. However, in contrast to mCFs, LIF, IL-11, and CT-1 induced the release of LIX in NIH 3T3 cells (Fig. 3C), suggesting a differential regulation of KC and LIX in these cells. Still, the release of LIX by NIH 3T3 cells treated with LIF, IL-11, and CT-1 was much less than that released by NIH 3T3 cells treated with OSM.

To understand OSM regulation of CXC chemokines in mCFs, we studied the main signaling pathways activated by OSM (20). We have demonstrated that OSM induces rapid (10

min), marked activation of the JAK-STAT, MAPK, and PI3-kinase-Akt pathways in mCFs (Fig. 4A). Activation of ERK by OSM in mCFs is consistent with published data showing ERK activation by OSM in NIH 3T3 cells (26). Activation of the PI3-kinase pathway by OSM is also consistent with previous data regarding the activation of PI3-kinase by OSM in Kaposi sarcoma (33). LIF, IL-11, and CT-1 did not activate STAT1 and STAT3 in mCFs. These results support the notion that, on the surface of primary cultured mCFs, the receptors for IL-6, LIF, IL-11, and CT-1 are not expressed or that their expression levels are not sufficient to activate the JAK-STAT pathway. However, it should be noted that LIF regulates collagen synthesis in mCFs (40), suggesting that the LIF receptor is expressed in mCFs. It also should be noted that CT-1 regulates remodeling in rat and dog cardiac fibroblasts (3, 12, 13). This finding suggests that the expression of these receptors is species specific and/or is inducible under different culture conditions. In contrast to the mCFs, LIF and IL-11, in addition to OSM, activated the JAK-STAT pathway in NIH 3T3 cells, although we could not detect IL-11 receptor mRNA in the NIH 3T3 cells. Although they were less potent than OSM, these responses suggest that, in contrast to mCFs, the LIF and IL-11 receptors are sufficiently expressed in NIH 3T3 cells.

Although the PI3-kinase pathway has been recognized as an important mediator of IL-6 cytokine family activity (1, 2, 5, 33, 38), its role in inflammation is not clear. Our study has demonstrated that the PI3-kinase pathway contributes to OSM-induced CXC chemokine release by mCFs because PI3-kinase inhibitor LY-294002 significantly decreased the release of KC and LIX in OSM-treated mCFs in a dose-dependent manner (Fig. 5, A and B). The MEK1 inhibitor PD-98059 (Fig. 5, C and D) and JAK2 blocker AG 490 (Fig. 5, E and F) did not significantly decrease the release of KC and LIX from mCFs. These results suggest that MEK1 and JAK2 are not involved in OSM-mediated CXC chemokine release. However, a role for other branches of the MAPK pathway, for JAK1, or for other upstream signaling proteins cannot be ruled out. Our results clearly demonstrate that the PI3-kinase pathway plays an important role in OSM-induced chemokine release in mCFs; however, the mechanisms involved remain to be determined.

In conclusion, we report herein for the first time that the OSMR is expressed in adult mCFs *in vitro* and that OSM is unique among the IL-6 family of cytokines in its ability to induce CXC chemokine expression in cardiac fibroblasts. This regulation is at least partially dependent on PI3-kinase activation. A growing body of literature supports a role for fibroblasts in a variety of acute and chronic inflammatory settings. Our results suggest that cardiac fibroblasts may also contribute to the evolution of inflammation in the heart. Specifically, in the setting of acute myocardial infarction, OSM released in the reperfused myocardium may trigger cardiac fibroblasts to synthesize and release CXC chemokines, thereby providing a mechanism for enhanced neutrophil recruitment and amplification of the acute inflammatory response.

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