Oncostatin M Production and Regulation by Human Polymorphonuclear Neutrophils

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Oncostatin M (OSM) is an interleukin-6 (IL-6) family cytokine known in particular to induce the synthesis of acute-phase proteins by hepatocytes. Because human polymorphonuclear neutrophils (PMN) can secrete numerous cytokines, the potential production of OSM by PMN was investigated. Highly purified PMN were found to contain an intracellular stock of preformed OSM that was rapidly mobilized by degranulating agents such as phorbol myristate acetate and granulocyte-macrophage colony-stimulating factor (GM-CSF). Moreover, PMN produced OSM after a few hours of stimulation by various agonists. The most potent effect was observed with the combination of lipopolysaccharide and GM-CSF, which had a concentration- and time-dependent effect at both the protein and mRNA levels. Actinomycin D strongly reduced OSM mRNA induction, suggesting the involvement of gene transcription. Cycloheximide inhibited OSM protein synthesis but did not affect the release of preformed stores. In addition, OSM production was downregulated by dexamethasone, whereas IL-10 had no effect. The OSM produced by PMN was biologically active, as demonstrated by its ability to induce α1- acid glycoprotein synthesis by HepG2 cells. OSM secretion thus occurs through a two-step mechanism in PMN, consisting of early release of a preformed stock, followed by de novo protein synthesis. This would allow rapid and sustained OSM release to occur at inflammatory sites, and may contribute to the modulation of local inflammation.

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MATERIALS AND METHODS

Purification of human PMN. Human PMN were purified as previously described. Pure PMN were cultured for up to 48 hours at 37°C with 5% CO2 in 24-well tissue culture plates (Costar, Cambridge, MA). The cell culture medium was RPMI 1640 (Sigma, St Louis, MO) supplemented with 2 mmol/L glutamine, antibiotics, and 10% fetal bovine serum. PMN were washed twice with cold medium and counted by light microscopy. Gating on a Coulter EPICS profile 50 flow cytometer showed the absence of CD14+ monocytes, confirming the recovery of highly purified PMN.

Cell culture. Pure PMN (10^7/mL) were cultured for up to 48 hours

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10% heat-inactivated fetal calf serum (BioWhittaker, Gagny, France). Stimulating agents were added to the culture medium at the following optimal concentrations determined in preliminary concentration-response experiments: lipopolysaccharide at 100 ng/mL (LPS; from Escherichia coli 055:B5), phorbol myristate acetate at 100 ng/mL (PMA), N-formylmethionyl-leucyl-phenylalanine at 10⁻⁵ mol/L (fMLP), TNF-α at 100 U/mL (TNF), IFN-γ at 500 U/mL (IFN), and GM-CSF at 100 U/mL; all reagents were from Sigma, except for recombinant cytokines, which were from Genzyme (Cambridge, MA). The inhibitory effects of IL-10 (kindly provided by Schering-Plough Research Institute, Kenilworth, NJ) and dexamethasone (DEX; Sigma) were studied by adding them for 30 minutes at 37°C before stimulation with LPS plus GM-CSF. In selected experiments, pure resting T lymphocytes (5 × 10⁶/mL) were cultured alone or cocultured with 10⁷ PMN/mL after stimulation with LPS plus GM-CSF to test the role of contaminating resting T cells in OSM production. Cell-free PMN culture supernatants were collected at various times and stored at −20°C until OSM assay. To determine cell-associated OSM, 10⁷ PMN/mL were incubated for 15 minutes at 37°C with or without 100 ng/mL PMA or 100 U/mL GM-CSF; cell-free supernatants were collected and the cell pellets were sonicated for 30 seconds to measure cell-associated OSM. Both supernatants and cell pellets were stored at −20°C until OSM assay. In some experiments, neutrophils (10⁷/mL) were preincubated with or without 10 μg/mL of cycloheximide (CHX) for 30 minutes at 37°C and then further incubated with 100 U/mL of GM-CSF for 15 minutes or 3 or 8 hours.

**OSM assay.** OSM was quantified by using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Quantikine; R&D Systems, Abingdon, UK) following the manufacturer’s instructions; the detection limit was 2.1 pg/mL.

**Immunocytochemical staining of intracellular OSM.** Unstimulated blood smears from healthy controls were air-dried for 24 hours and fixed in cold methanol/acetic acid. Smears were then incubated with a polyclonal rabbit antihuman-OSM antibody (50 μg/mL; Genzyme), followed by incubation with a biotinylated antibody and then alkaline phosphatase-labeled streptavidin, as recommended by the manufacturer (LSAB kit-AP; Dako, Carpenteria, CA). Staining was completed by incubation with a chromogenic substrate solution; smears were counterstained with hematoxylin and ammonia water. Positive staining developed as a fuschia-colored reaction product. Smears incubated with a control IgG (Sigma) served as negative controls and smears incubated with an anti-CD11b monoclonal antibody (Immunotech, Marseille, France) served as positive controls.

**Northern blot analysis.** For RNA analysis, 5 × 10⁷ PMN were incubated for up to 18 hours in 2 mL of standard culture medium containing the appropriate stimuli. In selected experiments, actinomycin D (5 μg/mL; Sigma) was added to the medium to block transcription. Total cellular RNA was isolated from PMN with RNA-B (Bioprobe, Cergy-Pontoise, France) according to the manufacturer’s instructions, and the RNA concentration was determined at 260 nm. Twenty micrograms of total RNA was analyzed by electrophoresis on 1% agarose-formaldehyde gel and transferred to nylon filters (Amersham, Les Ulis, France). The filters were prehybridized in 5× SSC, 25 mmol/L sodium phosphate (pH 6.4), 5× Denhardt’s reagent, 0.1% sodium dodecyl sulfate (SDS), and 0.2 mg/mL denatured salmon sperm DNA for 12 hours at 42°C. Human OSM mRNA was detected by hybridization with ³²P-labeled oligonucleotide probes (R&D Systems) for 16 hours at 42°C in 5× SSC, 25 mmol/L sodium phosphate (pH 6.4), 5× Denhardt’s reagent, 0.1% SDS, 50% formamide, and 0.2 mg/mL denatured salmon sperm DNA. The membranes were then washed in 2× SSC, 0.1% SDS for 5 minutes at room temperature and then for 30 minutes at 42°C. The oligonucleotide probes were labeled with [γ³²P]ATP (specific activity, 3,000 Ci/μmol; Amersham) by using the Ready To Go T4-Poly nucleotide Kinase (Pharmacia) and further purified on Micro Bio-Spin columns (Biorad, Ivy, France) following the manufacturers’ instructions. The glyceraldehyde phosphate dehydrogenase (GAPDH) and actin cDNA probes (Clontech, Palo Alto, CA) were labeled with [α³²P]dCTP by random priming using the RediPrime DNA labeling system (Amersham). The blots were then exposed for autoradiography.

**Biological activity of PMN OSM.** The HepG2 hepatoma cell line was cultured in minimum essential medium Eagle (MEM) containing Glutamax, 25 mmol/L HEPES, and Earle’s salts (Life Technologies, Cergy Pontoise, France) plus 10% heat-inactivated fetal calf serum. Cells were plated in 24-well culture plates (Costar) and allowed to grow to confluence at 37°C in humidified air containing 5% CO₂. Monolayers were then incubated for 24 hours with 1 mL of RPMI 1640 medium supplemented with 10% fetal calf serum, antibiotics, 2 mmol/L glutamine, pyruvate (Life Technologies), and 10⁻⁶ mol/L dexamethasone and were stimulated with either 10 ng/mL recombinant human OSM (rhOSM; Genzyme) or the culture supernatant of PMN stimulated with LPS + GM-CSF (PMN conditioned medium [CMF]). In some cases, before HepG2 stimulation, rhOSM and CMF were preincubated at 37°C for 30 minutes with polyclonal rabbit antibodies against human OSM (20 μg/mL; Genzyme).

The cell-free supernatants of HepG2 cultures were then harvested and stored at −20°C until ELISA measurement of α1-acid glycoprotein.¹⁰ Cell total DNA content was quantified¹¹ to express the amount of secreted α1-acid glycoprotein in nanograms per microgram of DNA. All samples were tested in duplicate.

**Statistical analysis.** Data are expressed as the means ± SEM; differences were considered statistically significant if P < .05 in Wilcoxon’s paired test.

**RESULTS**

**OSM synthesis can be induced by various stimuli.** Because PMN have been described to synthesize several cytokines de novo, the ability of PMN to synthesize OSM in various conditions of stimulation for 20 hours of culture was tested. As shown in Fig 1, fMLP, IFN-γ, and LPS alone moderately induced OSM release as compared with unstimulated PMN (respectively, 218 ± 50, 251 ± 88, and 455 ± 93 pg/10⁷ PMN; n = 6). TNF-α and GM-CSF were potent inducers (respectively, 508 ± 163 and 828 ± 173 pg/10⁷ PMN; P < .05). The strongest OSM production was obtained using PMN alone or LPS plus GM-CSF, giving values of, respectively, 1,300 ± 400 and 1,294 ± 133 pg/10⁷ PMN (P < .05). OSM upregulation by LPS, GM-CSF, LPS plus GM-CSF, and PMN was observed at both the protein and mRNA levels. As shown in Fig 2, OSM mRNA was undetectable after 1 hour in control PMN, whereas stimulated PMN accumulated OSM mRNA. As shown in Fig 3, PMN incubation at the outset of culture with the transcriptional inhibitor actinomycin D strongly reduced the OSM mRNA accumulation induced by LPS plus GM-CSF. After stimulation with these agonists for 1 hour (steady-state mRNA peak in Fig 2), actinomycin D was added to assess OSM transcript stability. In these conditions, OSM mRNA levels decreased rapidly (Fig 4), with a calculated half-life of 45 ± 8 minutes (n = 3, regression analysis). Taken together, our data suggest that the induction of this gene by LPS plus GM-CSF might take place at the transcriptional level.

**OSM production increased in a concentration-dependent manner and reached a steady level with 100 U/mL GM-CSF and 100 ng/mL LPS (Fig 5). A fixed concentration of GM-CSF (100 U/mL) combined with increasing concentrations of LPS (1 to
1,000 ng/mL) had an additive effect (not shown). Other combinations, including LPS plus IFN-γ or TNF-α, had the same additive effects (not shown).

Because resting T cells are the only cells potentially not removed by HLA class II-coated magnetic beads, their participation in OSM production was evaluated. T lymphocytes alone (5 x 10⁵) did not produce detectable OSM (<2.1 pg/mL) when stimulated by LPS plus GM-CSF for 20 hours; moreover, PMN alone (10⁷) and combined with 5 x 10⁵ autologous T lymphocytes produced similar levels of OSM (data not shown).

**Time course study of OSM release by PMN.** The small amounts of OSM released by control cells were detectable after 4 hours of culture. By contrast, in optimal conditions of stimulation (LPS + GM-CSF), OSM was detectable after 1 hour.

**Fig 1.** Stimulation of PMN OSM production. PMN (10⁷/mL) were incubated for 20 hours with various stimulating agents at the concentrations indicated in Materials and Methods. OSM was assayed in the cell-free supernatants. Control cells (CTRL) were incubated with medium alone. Results are expressed as the means ± SEM of six independent experiments. *P < .05 compared with control cells.

**Fig 2.** OSM mRNA expression in human PMN. PMN (5 x 10⁷) were cultured for up to 18 hours in the presence of LPS (100 ng/mL) plus GM-CSF (100 U/mL) (right panel). In other experiments, PMN were cultured for 1 hour in the presence of LPS and/or GM-CSF, PMA (100 ng/mL), or in medium alone (control) (left panel). Total RNA was extracted and Northern blots of OSM and GAPDH were run as specified in Materials and Methods.

**Fig 3.** Effect of actinomycin D on expression of OSM transcripts induced by LPS plus GM-CSF. PMN (5 x 10⁷) were incubated for 15 minutes in the presence or absence of 5 μg/mL actinomycin D (ACT D) and then stimulated for 1 hour with LPS (100 ng/mL) plus GM-CSF (100 U/mL). Controls cells (CTRL) were incubated in the medium alone. Total RNA was extracted and processed for Northern blot analysis of OSM and actin mRNAs as described in Materials and Methods. Results are from one experiment representative of two.
hour, reached a plateau by 24 hours, and gradually accumulated for up to 48 hours of culture (n = 3; Fig 6). It is noteworthy that 70% of the total amount of OSM was synthesized within 8 hours. Maximal expression of OSM mRNA by PMN stimulated by LPS plus GM-CSF was observed as early as 1 hour and disappeared after 6 hours (Fig 2).

**Human PMN contain an intracellular pool of OSM.** Because time-course studies showed that OSM was released as early as 1 hour after PMN stimulation, we investigated whether PMN contained a preformed stock of OSM in two different ways. First, immunocytochemistry showed the presence of intracellular OSM in unstimulated PMN in whole-blood smears (Fig 7). Second, degranulating experiments were conducted with pure isolated PMN incubated with or without PMA or GM-CSF (both potent inducers of neutrophil degranulation) for 15 minutes at 37°C. Released and cell-associated OSM were then measured separately. As shown in Fig 8, the amount of cell-associated OSM was 70 ± 6 pg per 10^7 PMN, whereas the OSM concentration was below the detection limit in the supernatant of resting PMN maintained for 15 minutes at 37°C in the absence of degranulating agents. PMA or GM-CSF stimulation led to a reduction in cell-associated OSM, matched by a parallel increase in the extracellular OSM level (Fig 8); the total amount of extracellular plus cell-associated OSM was similar to the total amount of cell-associated OSM in resting PMN. These results suggested that a preexisting pool of OSM was released. Similar experiments with incubation times of 5 and 30 minutes gave similar results, in keeping with a maximal degranulating effect of PMA after as little as 5 minutes (data not shown).

Because GM-CSF is a physiologic inducer of both neutrophil degranulation and OSM synthesis, a kinetic study was performed in the presence and absence of cycloheximide, an inhibitor of protein synthesis. The amounts of OSM detected after 15 minutes of stimulation with GM-CSF were not affected by cycloheximide (Fig 9). In contrast, after 3 and 8 hours of GM-CSF stimulation, a significant reduction in OSM synthesis was observed after CHX treatment (62% and 68% inhibition, respectively) as compared with untreated cells (P < .05, n = 4).

Taken together, these data strengthen the hypothesis that the observed OSM secretion by PMN is first due to the release of a preexisting intracellular pool, followed by de novo synthesis.

**DEX but not IL-10 inhibits OSM production induced by LPS plus GM-CSF.** Because IL-10 and DEX are potent modulators of cytokine production by PMN, we tested their influence on inducible OSM production. As shown in Fig 10, pretreatment of PMN with DEX resulted in significant concentration-dependent inhibition of OSM production induced by LPS plus GM-CSF (inhibition of 0%, 42%, 61%, and 65% with DEX concentrations of 10^-10, 10^-9, 10^-8, and 10^-7 mol/L, respectively; n = 3). By contrast, IL-10 (1 to 1,000 ng/mL) failed to modulate OSM production by PMN in optimal conditions of stimulation (not shown). The same pattern was observed at the OSM mRNA level, as DEX reduced the level of OSM transcripts in stimulated PMN, whereas IL-10 failed to affect it (not shown).

**OSM bioactivity.** OSM is an IL-6–related cytokine that stimulates hepatocytes and induces the expression of acute-phase proteins such as α-1 acid glycoprotein (AGP). We investigated the ability of CMP (culture supernatant of PMN stimulated with LPS plus GM-CSF) to stimulate α-1-acid glycoprotein synthesis by HepG2 cells. In a preliminary experiment, we found that AGP synthesis was unaffected by direct
Fig 6. Time course of OSM release by PMN. PMN (10⁷/mL) were stimulated with GM-CSF (100 U/mL) plus LPS (100 ng/mL). Control cells were incubated with the medium alone. Supernatants were collected at the times indicated and OSM was assayed in an ELISA method. Results are expressed as the mean ± SEM of three independent experiments.

Fig 7. Immunocytochemical staining of PMN in whole-blood smears. (A) Negative control; no staining was seen with a control Ig. (B) Intracellular fuschia staining was observed in PMN with specific polyclonal anti-OSM antibodies; smears were examined by light microscopy at ×1,200.
stimulation with LPS plus GM-CSF. Conditioned medium (500 µL) containing 4 ng of OSM (measured by ELISA) was added to HepG2 cell culture medium for 24 hours. As shown in Fig 11, CMP and rhOSM (10 ng/mL) stimulated α1-acid glycoprotein synthesis as compared with unstimulated HepG2 cells (n = 5; P < .05), and antibodies raised against OSM led to a 35% inhibition of CMP-induced AGP secretion (from 29.3 to 18.9 ng AGP/µg DNA; P < .05 as compared with CMP alone), whereas it completely abolished rhOSM-induced AGP secretion (control).

**DISCUSSION**

This study suggests that human PMN contain an intracellular pool of OSM that is rapidly released in degranulating conditions. This release is followed by OSM mRNA accumulation and protein synthesis in PMN stimulated by various agonists. Given the role of OSM in the acute-phase response, these findings support the pivotal role of PMN in the inflammatory process via a newly described two-step mechanism. Indeed, some cytokines are stored in PMN, such as vascular endothelial growth factor (VEGF), whereas others can be synthesized de novo, such as TNF-α, but we concomitantly explored for the first time both secretion processes for the same cytokine.

The PMN isolation method used here yielded highly purified preparations (>99% PMN). Immunomagnetic depletion of HLA class II-positive cells (B cells, activated T cells, and monocytes) may have left a very small proportion of HLA class II-negative cells (resting T lymphocytes), but these cells were ruled out as a source of the observed OSM release, by means of PMN and T-lymphocyte coculture assays. Furthermore, we failed to detect IL-6 (<10 pg/mL/10⁷ cells, ELISA) in our PMN preparations even after LPS stimulation (data not shown), confirming the absence of contaminating monocytes.

Although the intracellular pool of OSM rapidly released during degranulation induced by PMA or GM-CSF seemed small (48 ± 6 pg and 37 ± 9 pg/10⁷ PMN, respectively) as compared with the amount synthesized after 20 hours of culture, this content of pre-existing OSM is of importance, because it allows a rapid and significant secretion of OSM at the inflammatory site and could be an early event in the multistep process of PMN activation. The existence of this intracellular pool was confirmed by using CHX, which failed to affect early OSM release. Other cytokines stocked in PMN granules and rapidly released after exposure to degranulating agents include VEGF in specific granules and TGF-α in an undefined granule type. PMA and GM-CSF failed to release the entire OSM pool, as shown by a measurable residual level of cell-associated OSM. GM-CSF induces degranulation of specific and azurophilic granules, whereas the effect of GM-CSF is limited to specific granules. OSM may thus also be located in another type of granules or in membrane-bound state.

PMN are relatively short-lived end-stage cells that can synthesize mRNA encoding various cytokines. We found that OSM production by PMN was upregulated by a wide variety of stimuli, including chemoattractants and cytokines, corresponding to different mechanisms of cell activation. GM-CSF,
TNF-α, and IFN-γ, all of which upregulate the expression of several cytokine genes in PMN, also induced OSM synthesis; among them, the most potent effect was observed with GM-CSF. Formyl peptide (fMLP), a chemotactic agent for PMN, showed weak activity. The protein kinase C activator PMA yielded the most potent induction of OSM synthesis. LPS alone was a relatively moderate inducer of OSM, and its combination with GM-CSF (or IFN-γ or TNF-α) was additive, pointing to the use of different signaling pathways. The low level of OSM produced by unstimulated cells cultured for 20 hours was usually found with other cytokines and could be due to stimulation by cell adherence. Interindividual variations were also observed, as commonly described with other cytokines.

Kinetic studies showed that the amount of OSM (70 pg/10⁷ PMN) measured after 1 hour of stimulation by LPS plus GM-CSF (the optimal combination of physiological stimuli) was similar to the intracellular stock of OSM, suggesting that the OSM induced by LPS plus GM-CSF is initially cell-associated. OSM production then increased and was strong within 4 hours of stimulation (40% of the total OSM amount), suggesting direct stimulation rather than induction of a second messenger. Similar results were obtained with GM-CSF alone. Moreover, CHX significantly reduced OSM production at 8 hours (by up to 68%), confirming de novo protein synthesis. Northern blotting showed that OSM mRNA expression by stimulated PMN was rapid (as early as 1 hour). In the same way, Cassatella et al. and Fujishima and Aikawa demonstrated that IL-8 mRNA expression was maximal at 1 hour. This maximal expression of OSM mRNA in PMN was transient, with a short half-life. The combination of LPS plus GM-CSF acts at least partly at the transcriptional level, as suggested by Northern blot analysis in the presence of actinomycin D. Taken together, these findings confirm that PMN make an early and sustained contribution to the inflammatory process through two different mechanisms of OSM release: liberation of a preformed stock followed by de novo protein synthesis. It is noteworthy that neither IL-6 (data not shown) nor LIF (data not shown) was found in PMN culture supernatants. Therefore, in contrast to monocytes, among the three major cytokines belonging to the IL-6 family, only OSM was released by PMN whatever the conditions of stimulation used.

Some immunoregulatory mediators downregulate cytokine production by PMN. In our study, DEX strongly inhibited OSM production at both the mRNA and protein levels, in keeping with the inhibitory effects of DEX on other cytokines produced by PMN, such as IL-8. Interestingly, IL-10 preincubation did not inhibit OSM production induced by LPS plus GM-CSF, whereas IL-8 and TNF-α release was downregulated in the same experiments (data not shown). The modulation of cytokine production by IL-10 is complex and dependent on the nature of the stimulus and cell type. Richards et al. have shown that OSM has strong hepatocyte-stimulating activity and that α₁-acid glycoprotein, among other acute-phase proteins, is potently induced by OSM in the hepatocyte cell line HepG2. In this work, we found that the
supernatant of stimulated PMN upregulated α1-acid glycoprotein production by these cells; moreover, α1-acid glycoprotein secretion was, at least in part, inhibited by anti-OSM antibodies, suggesting that OSM present in the conditioned medium was biologically active.

OSM produced by PMN may serve numerous functions, including the stimulation of acute-phase reactant synthesis and the modulation of other regulatory cytokines. OSM produced locally by PMN in inflamed tissues may act as an anti-inflammatory mediator. First, OSM inhibits IL-1-induced expression of IL-8 by lung fibroblasts, suggesting that PMN may limit their own recruitment to sites of local inflammation. Second, OSM is a potent inducer of several antiproteases in cells of extrahepatic origin. For example, OSM upregulates the synthesis of α1-antitrypsin and α1-antichymotrypsin by epithelial cells originating from the jejunum, lung or skin. Therefore, OSM released by activated PMN could participate in this local induction of antiproteases, which could be a mechanism against the detrimental effects of PMN proteases (ie, elastase). Most acute-phase proteins induced by OSM possess anti-inflammatory properties in vivo and in vitro. For example, α1-antichymotrypsin and α1-antitrypsin inhibit PMN superoxide anion production and may have a role in the modulation of reactive oxygen species-induced tissue damage. Besides this anti-inflammatory effect, recent data suggest that OSM could also be directly proinflammatory. Indeed, Modur et al recently showed that OSM induces PMN adhesion and transmigration, as well as chemokine production by endothelial cells. OSM produced by PMN may not only participate in the repair discussed above, but also play a role in initiating the inflammatory response.

In conclusion, our data indicate that PMN at inflammatory sites can rapidly release an intracellular stock of OSM, followed by de novo OSM synthesis. This two-step mechanism of cytokine secretion by PMN would allow rapid and sustained OSM release to occur at inflammatory sites and may contribute to the modulation of local inflammation.

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