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Oncostatin M is a member of a cytokine family that includes leukemia-inhibitory factor, granulocyte colony-stimulating factor, and interleukin 6

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ABSTRACT **Oncostatin M (OSM), a glycoprotein of $M_r \approx$ 28,000 produced by activated monocyte and T-lymphocyte cell lines, was previously identified by its ability to inhibit the growth of cells from melanoma and other solid tumors. We have detected significant similarities in the primary amino acid sequences and predicted secondary structures of OSM, leukemia-inhibitory factor (LIF), granulocyte colony-stimulating factor (G-CSF), and interleukin 6 (IL-6). Analysis of the genes encoding these proteins revealed a shared exon organization, suggesting evolutionary descent from a common ancestral gene. Using a panel of DNAs from somatic cell hybrids, we have shown that OSM, like LIF, is located on human chromosome 22. We have also demonstrated that OSM has the ability to inhibit the proliferation of murine M1 myeloid leukemic cells and can induce their differentiation into macrophage-like cells, a function shared by LIF, G-CSF, and IL-6. We propose that OSM, LIF, G-CSF, and IL-6 are structurally related members of a cytokine family that have in common the ability to modulate differentiation of a variety of cell types.**

Oncostatin M (OSM), a glycoprotein of $M_r \approx 28,000$, was originally isolated from the conditioned medium of U937 human histiocytic leukemia cells that had been induced to differentiate into macrophage-like cells by treatment with phorbol 12-myristate 13-acetate (1). OSM was identified by its ability to inhibit the growth of human A375 melanoma cells but not normal human fibroblasts. Treatment with recombinant OSM leads to the inhibition of proliferation and changes in cellular morphology of a number of tumor cell lines derived from a wide variety of tissue types (2). High-affinity binding sites for OSM have been detected on normal and tumor cell lines, and chemical cross-linking studies have demonstrated the presence of a M_r 150,000–160,000 receptor (3). While OSM is expressed in activated monocytic and lymphocytic cell lines and in normal adherent macrophages (4, 5), no functional role has been reported for OSM in the growth or development of hematopoietic cells.

Leukemia-inhibitory factor (LIF), granulocyte colony-stimulating factor (G-CSF), and interleukin 6 (IL-6) are cytokines that affect the growth and differentiation of multiple cell types, including those of hematopoietic origin. Although unique growth-regulatory activities have been determined for these factors, they also exhibit several common functions. All three factors have been shown to induce the differentiation of the murine M1 myeloid leukemia cell line (6–10) and they all enhance interleukin-3-dependent colony formation of very primitive blast colony-forming cells (11). LIF and IL-6 have in common the ability to induce neuronal differentiation (12, 13) and stimulate the production of acute-phase proteins in hepatocytes (14, 15). The expression of these factors is detected not only in adult tissue (16–18), but also during early embryonic development (19, 20). These results suggest that LIF, IL-6, and G-CSF play important roles in the regulation of early embryonic and hematopoietic stem cells. While these three factors demonstrate some functional similarities, no relationship between the protein and gene sequences of LIF, G-CSF, and IL-6 has been reported.

In this study, a computer-aided homology search has identified a similarity between the amino acid sequences of OSM, LIF, G-CSF, and IL-6. Protein sequence alignments coupled with protein and gene structure comparisons suggest that these four cytokines have evolved from a common ancestor and are members of a single cytokine family. Functional studies presented here demonstrate that OSM, like LIF, IL-6, and G-CSF, can induce phenotypic differentiation of the M1 myeloid leukemic cell line.

MATERIALS AND METHODS

Computer Analysis. PatMat software (21), obtained from J. Wallace (Seattle), was used to perform amino acid homology searches; GenePro software (Riverside Scientific, Seattle), and P/C Gene software (IntelliGenetics) were used for protein sequence alignments and predictions of secondary structure. ScorEdit software, obtained from J. Durand (Seattle), was used for alignment and scoring of multiple sequences. The "Motif" program of Smith *et al.* (22) as implemented in the Protomat\MotifJ software obtained from S. Henikoff (Seattle) was used for detecting sequence motifs.

Chromosome Localization. Samples of DNA from a panel of somatic hybrids of hamster cells that contain different human chromosomes were obtained from Bios (New Haven, CT). An oligonucleotide primer pair (5′-CAGACTGGCCGACTTAGA-3′ and 5′-CAAGGGGTGCTCTCGAGGCTA-3′) defining a 454-base-pair (bp) domain in the 3′ coding and noncoding region of OSM and a primer pair (5′-GCTAAGGCTGGCCCTCCAGC-3′ and 5′-CACTGAGTGCATGAA-3′) defining an 815-bp domain in the second intron and adjacent coding region were synthesized. Using the two sets of oligonucleotides, we performed individual polymerase chain reactions on the panel of DNA samples in a Perkin–Elmer Thermocycler (Norwalk, CA), and we analyzed amplified DNA fragments by electrophoresis in an agarose gel.

Polypeptide Growth Factors. Recombinant human OSM was isolated from Chinese hamster ovary cells transfected with the human OSM cDNA. The factor was purified to homogeneity essentially as described for the native molecule (1) and the concentration and identity were verified by reverse-phase HPLC and amino acid analysis. Recombinant

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Abbreviations: OSM, oncostatin M; LIF, leukemia-inhibitory factor; G-CSF, granulocyte colony-stimulating factor; IL-6, interleukin 6; GH, growth hormone.

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human LIF and human IL-6 were purchased from Amgen Biologicals. Recombinant human G-CSF was purchased from R & D Systems (Minneapolis).

***In Vitro* Assays.** The murine myeloid leukemic M1 cells were obtained from the American Type Culture Collection. Cultures were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum. For growth inhibitory assays, the cells were diluted to 5×10^5 cells per ml and 50-μl aliquots were added per well to a 96-well plate. Samples (50 μl) of media or factor diluted in media were added to the wells in triplicate and the cells were incubated at 37°C under 5% CO_2 for 72 hr. After pulsing with [^{3}H]thymidine for 4 hr, the specific incorporation was determined. To determine cellular differentiation, treated cells were cytocentrifuged onto glass slides, stained with SureStain Wright-Giemsa stain (Fisher), and observed under a light microscope, as described (9).

RESULTS

A computer-aided homology search of the Protein Identification Resource data base, Release 25, identified an amino acid sequence similarity between human OSM and murine LIF. Alignment of the two sequences revealed a striking similarity, with 47 identical residues in 173 positions (27%) between the 196-residue mature OSM and the 180-residue mature LIF, including 4 cysteine residues. A comparison of the sequence of OSM with other known cytokines revealed additional similarities with the sequences of G-CSF and IL-6.

Gene Structure Analysis. Analysis of the genes for OSM and LIF revealed identical exon organizations. Both genes contain three exons of similar size with two intron–exon junctions. The first junction occurs within the residues encoding the N-terminal signal peptide and the second is positioned approximately 34–44 residues from the N terminus of the mature peptide. The majority of the coding sequence and the entire 3′ noncoding region of both genes resides within the single final exon. The phasing of each exon junction is identical for the two genes (Fig. 1).

The exon organizations of G-CSF and IL-6 are identical. Like OSM and LIF, these genes contain an exon boundary within their signal sequences and a second boundary, which is positioned 35–44 residues downstream of the N terminus of the mature protein. The phasing of these two boundaries is identical to that seen in both OSM and LIF. Whereas the majority of the coding region of OSM and LIF is contained within a single final exon, the analogous region within G-CSF and IL-6 is interrupted by two additional exon–intron junctions. The phasing of both of these junctions is identical and is maintained within both genes.

Analysis of the gene structures of granulocyte/macrophage (GM)-CSF, macrophage (M)-CSF, IL-1, -2, -3, -4, and -5, tumor necrosis factor, steel factor, and the interferons demonstrates that, in contrast to the group described above, these cytokine genes do not contain the characteristic splice junction within their signal sequences and the structure and number of their exons are different. Although similarities were apparent in the presence and phasing of splice junctions within the C-terminal coding regions of some of the genes, including GM-CSF and IL-2, -3, -4, and -5, the exon positions and sizes were not analogous and no obvious sequence similarity was detected.

Amino Acid Sequence Comparison. An alignment of the amino acid sequences of both the human and mouse homologues of LIF, IL-6, and G-CSF and the human and simian homologues of OSM is presented in Fig. 2. Analogous exon boundaries within the mature proteins were aligned and the sequence alignment was optimized. Significant similarity is detected throughout the entire coding regions of OSM, LIF, G-CSF, and IL-6 and obvious conserved sequence patterns are present. The percentage of matching residues in the alignments was determined (Fig. 3*A*); it showed that OSM is more closely related to LIF (22–29%) and to G-CSF (23–25%) than LIF and G-CSF are to each other (12–16%). IL-6 is more closely related to G-CSF (16–20%) and OSM (11–19%) than it is to LIF (10–12%). The similarities between the sequences were also quantified allowing for conservative substitutions of amino acid residues, and alignment scores ranging from 17% to 35% were obtained (Fig. 3*B*).

A comparison of the sequences revealed the presence of several extended motifs within blocks of aligned residues. The most important motif surrounded the position of cysteine 5 (OSM) near the C-terminal region and contained the sequence pattern $\begin{matrix}A\\V\end{matrix}FQ\begin{matrix}KK\\RR\end{matrix}XXG\begin{matrix}C\\V\end{matrix}$, which is found in the sequences of human and simian OSM and in the human and murine homologues of LIF and G-CSF. In addition, this motif contained the sequence pattern $\begin{matrix}FL\\YM\end{matrix}$, which occurs in all the sequences except LIF, which contains the hydrophobic residues $\begin{matrix}I\\V\end{matrix}I$. Many motifs contained conserved leucine and isoleucine residues, and a highly repeated pattern of apolar residues occupying the i and $i + 3$ positions of repeated heptads was detected. Such a periodicity is predictive of coiled-coil α-helices and has been implicated in the interactions between two or more α-helices (30). Four regions, each containing three to four consecutive heptad repeats, were found in all four proteins, and greater than 80% of the i and $i + 3$ positions contained apolar residues.

Four cysteine residues were present in analogous positions in the sequences of human and simian OSM and human and murine LIF. In human OSM, these residues have been shown to be involved in intrachain disulfide linkages (ref. 31; as denoted in Fig. 1). Although disulfide linkages have not yet been established for LIF, the similarities in cysteine residues suggest that analogous disulfide linkages exist in LIF. LIF lacks a cysteine residue corresponding to the OSM cysteine 3

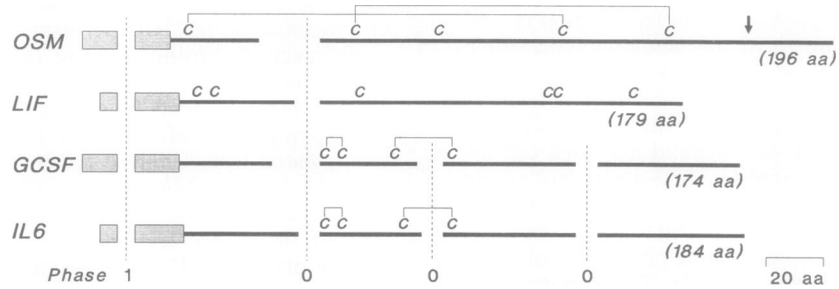

FIG. 1. Schematic representation of exon boundaries within the protein precursors for human OSM (4), LIF (23), G-CSF (24), and IL-6 (25). Solid lines represent mature polypeptides and boxes represent signal sequences. An arrow marks a maturation site within the C-terminal region of OSM (4). Sizes of the mature polypeptides are indicated as number of amino acids (aa). Conserved cysteine residues (C) and known disulfide linkages are shown. Exon boundaries are denoted with dashed vertical lines and the phasing of the junction is given.

FIG. 2. Amino acid sequence alignment of the precursor polypeptides of human (h-) and simian (s-) OSM (ref. 4 and unpublished results) and human and murine (m-) LIF (26, 27), G-CSF (24, 28), and IL-6 (25, 29). Exon boundaries are denoted with an * above a vertical line. Residues occurring in two or more separate genes are boxed and cysteines in OSM are numbered. Unknown residues within the simian OSM sequence are denoted (.). Protease maturation sites are indicated (/) and a potential N-linked glycosylation site occurring in both OSM and LIF is indicated (#).

which, in OSM, is apparently not involved in a disulfide linkage. On the other hand, LIF has two additional cysteine residues, which are located adjacent to the cysteines in positions 1 and 4. There are four cysteine residues in analogous positions in the human and murine sequences of G-CSF and IL-6. Although two of these residues can be aligned with those found in positions 2 and 3 of OSM (Fig. 2), disulfide linkage patterns different from that seen with OSM have been demonstrated for G-CSF (32) and IL-6 (33), as shown in Fig. 1.

Secondary structure analysis of primary amino acid sequences predicted extensive α-helical configurations for OSM, LIF, G-CSF, and IL-6 (data not shown). Based on a combination of predictive algorithms, molecular models have previously been proposed for G-CSF and IL-6 (34, 35). A model consisting of a four α-helical bundle structure patterned after the structure determined for growth hormone (GH) by x-ray crystallography has been proposed (35). Structural models for OSM and LIF suggest that these factors could also adopt a similar α-helical bundle structure (see Fig. 4). The proposed helical domains of OSM and LIF each contain heptad periodicities of apolar residues. The length and occupancy of these repeats are virtually identical to those seen in GH. In addition, a disulfide bond analogous to that found in GH connecting helix IV with the loop region between helices I and II (36) is also found in OSM. The presence of this disulfide bond has been shown to be essential for activity of OSM (31). Although OSM contains an additional disulfide bond between the N-terminal sequence and loop III, this second bond is not essential for activity (31).

OSM Is Localized to Human Chromosome 22. A panel of somatic cell hybrids containing different human chromosomes was screened for the presence of the OSM gene by polymerase chain reaction using two pairs of specific oligonucleotide primers which define 454- and 815-bp segments of the OSM gene. Specific DNA fragments of the correct size were obtained in DNA samples from hybrids 683, 803, and 1099 (Fig. 5). Only human chromosomes 5 and 22 were common to these hybrids. No specific fragment was detected in hybrids 937 and 1006, which contained chromosome 5 but not chromosome 22. No fragments were amplified from DNA samples containing only hamster chromosomes or samples from the remaining hybrids representing all the human chromosomes except chromosome 22.

OSM Inhibits the Proliferation of Mouse M1 Myeloid Leukemic Cells. The effect of OSM on the proliferation of M1 cells was compared to that seen with LIF, IL-6, and G-CSF by assaying the incorporation of tritiated thymidine after treatment with the various factors. The dose–response curves for OSM and LIF are essentially identical, with a half-maximal dose of 0.59 ng/ml (27 pM) and 0.43 ng/ml (22 pM), respectively, and a 65% maximal reduction in thymidine incorporation (Fig. 6). The half-maximal dose with IL-6 was 10-fold higher, at 5.7 ng/ml (273 pM), and the maximal reduction in thymidine incorporation was 80%. G-CSF did not cause inhibition of proliferation in our assay, which is consistent with previous results, where little effect was detected at 60 ng/ml even though the half-maximal dose for induction of differentiation was 10 ng/ml (530 pM) (10).

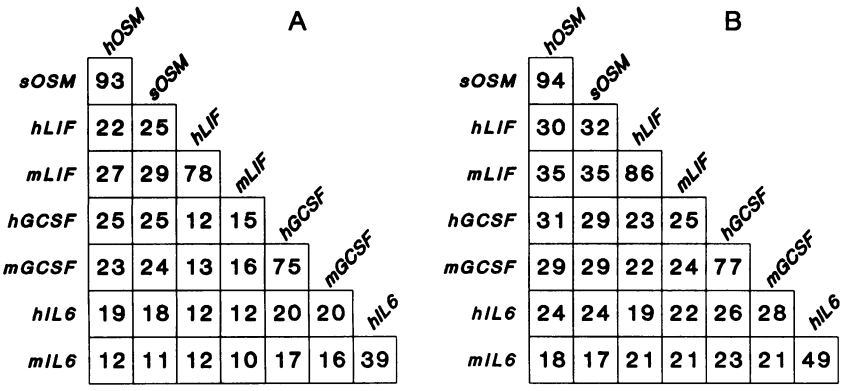

FIG. 3. Amino acid similarity scores based on the alignment in Fig. 2. (*A*) Percentage of identical residues. (*B*) Percentage including conservative amino acid changes: F/Y, A/G, K/R, L/I/V, N/Q, D/E, and S/T. Percentages were obtained from the ratio of the number of identical residues to the number of aligned residues for each pair of sequences, with any gap counting as one mismatch.

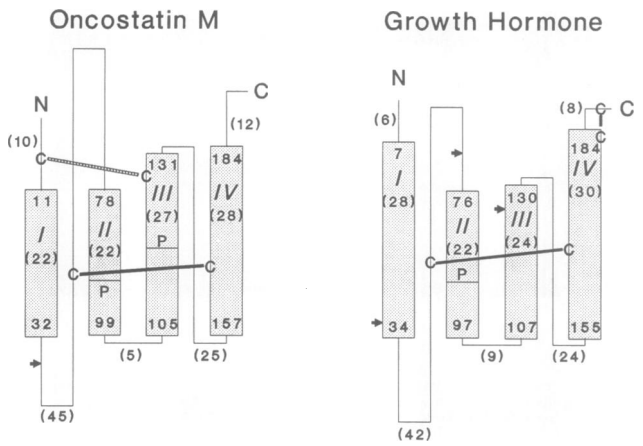

FIG. 4. Schematic representations of the four-α-helical bundle structure predicted for the 196-residue human OSM and that determined for the 188-residue porcine GH by x-ray crystallography (36). Numbers in parentheses give the lengths of predicted helical domains and connecting chains, and the approximate amino acid residue positions are indicated. The disulfide bond in OSM that can be deleted without loss of activity is given as a broken line. The positions of exon boundaries in OSM (4) and GH (37) are denoted by arrows and the positions of putative kinks in the helical domains induced by proline residues (P) are shown.

These results show that OSM is as potent in M1 cells as it is in A375 human melanoma cells, the most sensitive cell line reported to date.

M1 cells were treated with doses of the different factors that gave half the maximal activity in the inhibition assay (200 ng/ml for G-CSF). With each of the four factors, by 72 hr, greater than 50% of cells had a macrophage-like appearance with a decreased nucleus-to-cell ratio and the obvious presence of vacuoles (results not shown). This morphology was not observed in untreated M1 cells.

DISCUSSION

We have detected significant similarities between the primary amino acid sequences and predicted secondary structures of OSM, LIF, G-CSF, and IL-6, and analysis of the gene structures revealed a shared exon organization. We have shown that OSM, like LIF, G-CSF, and IL-6, has the ability to induce the phenotypic differentiation of murine myeloid leukemia M1 cells into macrophage-like cells. We propose that OSM, LIF, G-CSF, and IL-6 are structurally related members of a cytokine family that have in common the ability to modulate the differentiation of a variety of cell types.

OSM is most closely related to LIF on the basis of their sequence similarity and identical gene structure. At the level of amino acid sequence, human OSM is as similar to G-CSF (25%) and IL-6 (19%) as it is to LIF (22%). However, the presence of two additional exon boundaries in the gene structures of G-CSF and IL-6 suggests that they are most closely related to each other. Evolution from a common ancestral gene through gene duplication, coupled with either loss or capture of introns, could explain the difference in exon organization of these genes. The relationship of G-CSF and IL-6 to LIF (12% amino acid sequence similarity) is less obvious and becomes meaningful only through their common homology to OSM. Although there is a comparatively low sequence similarity between the members of this gene family, helical proteins are known for tolerating significant changes in their amino acid sequence. This is demonstrated by the globin family, where the sequence identity between family members is as low as 16%, yet their tertiary structures are very similar (38).

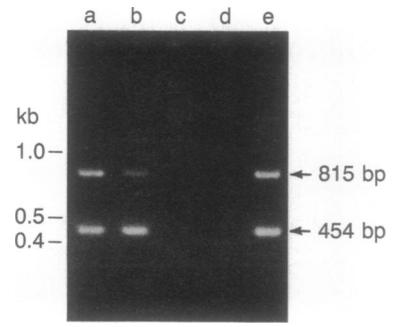

FIG. 5. Chromosomal localization of the human OSM gene. Polymerase chain reactions were performed on DNAs obtained from a panel of 25 hamster–human somatic cell hybrids, using two oligonucleotide primer pairs specifying 815- and 454-bp gene fragments specific to OSM. The reactions were combined for analysis and the results from hybrids 683 (containing human chromosomes 5, 11, 12, 14, 19, 21, and 22; lane a), 803 (4, 5, 8, 11, 22, and X; lane b), 937 (1, 5, 14, 15, 17, and 21; lane c), 1006 (4, 5, 7, 8, 13, 15, 19, and 21; lane d), and 1099 (1, 5 [deleted at 5p15.1–5p15.2], 13, 19, 21, and 22; lane e) are shown. kb, Kilobases.

Secondary structure predictions suggest that OSM, LIF, G-CSF, and IL-6 could adopt similar four-α-helical bundle conformations. However, the differences in the disulfide linkages suggest that these structures would not be identical. We have proposed that OSM could have an α-helical bundle conformation similar to that determined for GH (36). Mutational analyses of both OSM and GH have implicated similar discontinuous domains, including a C-terminal amphiphilic α-helix, as being involved in receptor binding (31, 39). In both molecules this helix is linked by a disulfide bond to an analogous N-terminal domain that links helices I and II. Alignment of amino acid sequences in the C-terminal domain of both OSM and human GH (data not shown) reveals the presence of matching phenylalanines, residue 176 in OSM and 177 in GH, which are essential for the activity of OSM and GH (31, 39). This residue is part of the sequence motif conserved within the C-terminal helical domains of OSM, G-CSF, and IL-6. Although the overall sequence similarity of GH to OSM, LIF, G-CSF, and IL-6 is low (approximately 10%), conserved amino acid patterns and structural motifs, such as helical regions and periodicity of apolar residues, are present. In addition, the exon organization and phasing within the GH gene is identical to that found in G-CSF and IL-6, as has been noted earlier (35). This suggests the possibility that even more distantly related members of the OSM, LIF, G-CSF, and IL-6 cytokine family exist and could include GH and possibly prolactin, erythropoietin, and in-

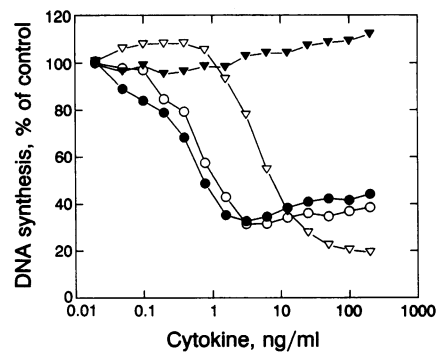

FIG. 6. Inhibition of proliferation of mouse M1 myeloid leukemia cells treated with different concentrations of the human cytokines OSM (○), LIF (●), IL-6 (▽), and G-CSF (▼). The level of DNA synthesis was determined from the incorporation of [^{3}H]thymidine and is expressed as percent of the untreated control.

terleukin 7, which also have the same gene structure and amino acid similarities. It is interesting to note that the receptors for G-CSF, IL-6, GH, prolactin, and erythropoietin have been cloned and, on the basis of sequence homology, have been included within a larger family of related cytokine receptors (40). The receptors for OSM and LIF have not yet been fully characterized, but it seems likely that they would also belong to this receptor family.

We have determined that OSM is localized to human chromosome 22. The human gene for LIF has also been localized to chromosome 22, 22q12 (41, 42). This colocalization is another indication of common evolutionary origin and raises the possibility that these genes could be adjacent on chromosome 22. In the mouse genome, G-CSF (43) and LIF (44) have both been localized to chromosome 11, which is syntenic with human chromosome 22.

In addition to the structural similarities detected between OSM, LIF, G-CSF, and IL-6, we have shown that these factors share an ability to induce the differentiation of M1 myeloid leukemic cells. Additional functional similarities have been determined for these factors, including the ability to (*i*) enhance interleukin-3-dependent colony formation of primitive blast colony-forming cells (LIF, G-CSF, and IL-6) (11), (*ii*) induce differentiation of neuronal cells (LIF and IL-6) (12, 13), and (*iii*) induce production of acute-phase proteins in hepatocytes (LIF and IL-6) (14, 15). Other common functions may exist; however, the divergence in amino acid sequences suggests that these four factors have evolved to perform different functions. In fact, unique functions have been attributed to each individual factor, including inhibition of stem cell differentiation (LIF) (45, 46), induction of B-cell differentiation (IL-6) (47), and stimulation of neutrophil proliferation (G-CSF) (48). The extent of the functional similarities and differences between members of this cytokine family needs to be examined further. Our results suggest that OSM, as a member of this family, may play a role in the regulation and growth of hematopoietic cells.

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