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Regulation of Bone Marrow Stromal Cell Differentiation by Cytokines Whose Receptors Share the gp130 Protein

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Abstract The bone marrow stroma consists of a heterogeneous population of cells which participate in osteogenic, adipogenic, and hematopoietic events. The murine stromal cell line, BMS2, exhibits the adipocytic and osteoblastic phenotypes in vitro. BMS2 differentiation was examined in response to cytokines which share the gp130 signal transducing protein within their receptor complex. Four of the cytokines (interleukin 6, interleukin 11, leukemia inhibitory factor, and oncostatin M) inhibited hydrocortisone-induced adipocyte differentiation in a dose dependent manner based on lipid accumulation and lipoprotein lipase enzyme activity. Inhibition occurred only when the cytokines were present during the initial 24 h of the induction period; after 48 h, their effects were diminished. Likewise, these cytokines increased alkaline phosphatase enzyme activity twofold in preadipocyte BMS2 cells. Both leukemia inhibitory factor and oncostatin M induced early active gene expression in resting preadipocyte BMS2 cells and decreased the steady state mRNA level of a unique osteoblastic gene marker, osteocalcin. A fifth cytokine whose receptor complex shares the gp130 protein, ciliary neurotrophic factor, did not significantly regulate stromal cell differentiation when added by itself. However, with the addition of a missing component of its receptor complex, ciliary neurotrophic factor receptor α protein, this cytokine also inhibited BMS2 adipogenesis. Together, these data indicate that the cytokines whose receptors share the gp130 protein can modulate stromal cell commitment to the adipocyte and osteoblast differentiation pathways.

Key words: adipocytes, ciliary neurotrophic factor, interleukin 6, interleukin 11, leukemia inhibitory factor, oncostatin M, osteoblasts

The bone marrow stroma consists of a heterogeneous cell population of macrophages, fibroblast/endothelial cells, adipocytes, and osteoblasts [Beresford, 1989; Gimble, 1990; Kincade et al., 1989; Owen, 1988; Weiss and Saki, 1984]. These cells contribute to the bone marrow microenvironment and are directly involved in a number of metabolic functions. Through the localized production of cytokines and direct cell-cell interactions, stromal cells nurture the maturation and proliferation of lymphohemopoietic progenitor cells, thereby regulating the composition of the circulating blood cell pool and effecting immune function. Stromal osteoblasts lay down new bone matrix and influence the maturation of osteoclasts, thereby contributing to bone growth and remodeling. Stromal adipocytes serve as a lipid storage site which may be utilized during unique periods of stress, such as hemolytic anemia or prolonged starvation [Gimble, 1990]. These processes are coordinated through a complex communication network mediated by locally acting cytokines and hormones [Kincade et al., 1989]. Dysregulation in stromal cell function and differentiation may contribute to the pathophysiology of osteoporosis, myelofibrosis, and aplastic anemia [Beresford, 1989; Gimble, 1990; Kincade et al., 1989; Owen, 1988; Weiss and Saki, 1984].

It has been hypothesized that a single multipotent progenitor cell gives rise to each of the stromal cell lineages (lymphohemopoietic support cell, adipocyte, and osteoblast) [Beresford, 1989; Gimble, 1990; Owen, 1988]. With advances in long-term bone marrow culture tech-

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niques, stromal cell lines have been cloned and characterized in vitro [Kincade et al., 1989]. One of these cell lines, the murine bone marrow-derived BMS2 cell clone, exhibits the ability to support lymphohemopoiesis in vitro, to undergo adipocyte differentiation, and to express genes unique to the osteoblastic lineage [Dorheim et al., 1993; Gimble et al., 1989; Gimble et al., 1990; Pietrangeli et al., 1988]. Each of these functions is subject to regulation by cytokine and hormonal agonists and antagonists. Thus, the BMS2 cell line provides an in vitro model for a multipotent stromal cell progenitor. With this, it is now possible to directly examine the effect of specific cytokines on stromal cell differentiation and function.

The cytokines interleukin 6 (IL-6), interleukin 11 (IL-11), leukemia inhibitory factor (LIF), oncostatin M (OSM), and ciliary neurotropic factor (CNTF) were originally identified as unique factors with distinct properties: IL-6 and IL-11 as B cell growth factors [Paul et al., 1990; Taga and Kishimoto, 1993], LIF as myeloid leukemia inhibitor [Hilton and Gough, 1991], OSM as a human melanoma inhibitor [Zarling et al., 1986], and CNTF as a survival promoting factor for ciliary neurons [Adler et al., 1979; Lin et al., 1989; Stockli et al., 1989]. Based on structural analysis, it has been determined that four of these cytokines (CNTF, IL-6, LIF, and OSM) descend from a common ancestral gene and represent a distinct family [Bazan, 1991; Rose and Bruce, 1991]. Each of these cytokines interacts with a heterodimeric or heterotrimeric receptor complex consisting of a shared signal transducing protein, gp130, associated with an α and/or β chain [Baumann et al., 1993; Davis et al., 1991, 1993a,b; Gearing et al., 1992; Hibi et al., 1990; Ip et al., 1993; Liu et al., 1992; Murakami et al., 1993; Stahl et al., 1993; Taga et al., 1992; Yin et al., 1992, 1993]. Unique α chains are contained in the receptor complexes for IL-6 and CNTF, respectively (i.e., IL-6R α and CNTFR α) [Davis et al., 1991, 1993b; Taga and Kishimoto, 1993]. While the IL-6R α protein is widely distributed, the CNTFR α protein has been observed only in the nervous tissue and skeletal muscle [Davis et al., 1991, 1993a]. A common β chain, known as LIFR β, is shared by the receptor complexes for LIF, CNTF, and OSM [Baumann et al., 1993; Davis et al., 1993b; Gearing et al., 1992; Ip et al., 1992; Liu et al., 1992; Murakami et al., 1993; Stahl et al., 1993; Taga and Kishimoto, 1993].

The existence of a shared signal transduction pathway, mediated through gp130, may explain the functional redundancy and pleiotropic effects evident within this cytokine family [Taga and Kishimoto, 1993]. Three of these cytokines (IL-6, IL-11, and LIF) have been demonstrated as stromal cell products, suggesting that signals mediated through gp130 may play a regulatory role within the bone marrow microenvironment [Gimble et al., 1991; Paul et al., 1990; Wetzler et al., 1991]. The current work determines the effects of CNTF, IL-6, IL-11, LIF, and OSM on the differentiation and gene expression of the BMS2 stromal cell line. These data indicate that the BMS2 cells display a common response pattern to at least four of these cytokines.

MATERIALS AND METHODS

Materials

The cytokines rat ciliary neurotropic factor (CNTF), murine interleukin 6 (IL-6), human leukemia inhibitory factor (LIF), and oncostatin M (OSM) were purchased from PeproTech, Inc (Rocky Hill, NJ). Recombinant human interleukin 11 (IL-11) was purchased from R & D Systems (Minneapolis, MN). The ciliary neurotrophic factor receptor α protein was obtained through Regeneron Pharmaceuticals (Tarrytown, NY). The cDNA probes utilized were obtained from the following sources: actin and adipsin (courtesy L. Choy, W. Wilkison, and B. Spiegelman, Dana Farber Cancer Institute) [Wilkison et al., 1990]; rat angiotensinogen (courtesy K. Lynch, University of Virginia) [Lynch et al., 1986]; aP2 (courtesy H. Green, Harvard University) [Spiegelman et al., 1983]; CHO-B and TIS11 (courtesy R. Wall, UCLA) [Harpold et al., 1979; Lim et al., 1987; Nakajima and Wall, 1991]; complement C3 (ATCC 63097/63098) [Domdey et al., 1982]; v-*fos* (ATCC 41040) [Curran et al., 1982]; human gp130 (courtesy of T. Taga, T. Kishimoto, Osaka University) [Hibi et al., 1990]; IL-6 (courtesy S. Clark, Genetics Institute, Inc); JE (courtesy C. Stiles, Dana Farber Cancer Institute) [Rollins et al., 1989]; c-*jun* (courtesy R. Bravo, Bristol-Myers Squibb) [Ryseck et al., 1988]; *jun* B (ATCC 63025) [Ryder et al., 1988]; *jun* D (ATCC 63024) [Ryder et al., 1989]; serum amyloid A (SAA; courtesy of C. Webb, OMRF) [Webb et al., 1989]; murine lipoprotein lipase (ATCC 63117) [Gimble et al., 1992]; murine osteocalcin (courtesy A. Celeste, Genetics Institute, Inc.) [Celeste et al., 1986]; human osteopontin (ATCC 61052) [Kiefer

et al., 1989]. All chemicals were obtained from Sigma Chemical Co., St. Louis, MO, unless otherwise indicated.

Cell Culture

The BMS2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco/BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 1 mM Na pyruvate, 50 μM 2-mercaptoethanol, 100 mg/ml streptomycin, and 100 units/ml penicillin [Dorheim et al., 1993]. Cells were grown to confluence prior to induction of adipogenesis by the addition of 0.5 mM methylisobutylxanthine, 0.5 μM hydrocortisone (Elkin Sinn, Cherry Hill, NJ), and 60 μM indomethacin (MHI) for a period of 48 or 72 h [Gimble et al., 1992]. Inductions were conducted in the presence of cytokines at concentrations of 0 to 1,000 ng/ml. After the induction period, the medium was replaced with fresh medium without MHI; the cytokines were present throughout the length of the study at the indicated concentrations. In studies with β-glycerophosphate, freshly plated cells were grown in medium supplemented with 10 mM β-glycerophosphate and 50 μM ascorbate in addition to cytokines [Dorheim et al., 1993]. All cultures were fed every 3 days.

Fluorescence Activated Cell Sorting

Cells grown in 24-well plates (Corning, Corning, NY) were stained with Nile red and harvested as previously described [Dorheim et al., 1993; Smyth and Wharton, 1992]. Gold fluorescence emission was detected between 564 nm and 604 nm with a bandpass filter 582/42, using a FACscan (Becton-Dickinson, San Jose, CA) multiparameter flow cytometer. The median channel number (fluorescence intensity) was held constant for the uninduced cell population between samples. Samples of 7.5×10^3 to 10^4 cells were analyzed for each data point.

Northern Blot Analysis

Total RNA was isolated from cells according to published methods [Chomczynski and Sacchi, 1987]. Poly A^+ mRNA was prepared using oligo-dT spin columns according to the manufacturer's instructions (Five Prime-Three Prime, Boulder, CO). Northern blots were prepared with approximately 10 μg of total RNA or 2 μg poly A^+ mRNA per lane, transferred to MSI-NT membranes (MSI, Westboro, MA), and hybridized with the indicated probes and washed at 60°C [Thomas, 1980; Church and Gilbert, 1984]. Probes were radio-labeled with random hexamer primers [Feinberg and Vogelstein, 1983].

Lipoprotein Lipase Assay

Culture supernatants in the presence of heparin (10 u/ml) were harvested and assayed for lipoprotein lipase activity as previously described [Gimble et al., 1989; Nilsson-Ehle and Schotz, 1976]. Activities are expressed as nmol of free fatty acid released/h/ml of supernatant or normalized relative to control values, defined as 100%.

Alkaline Phosphatase Assay

Cells were plated at 3×10^4 cells per 35 mm dish, incubated overnight in DMEM, 2% fetal bovine serum, penicillin 100 units/ml, streptomycin 100 μg/ml, with or without 10 mM β-glycerophosphate and 50 μg/ml ascorbate, and then treated with CNTF, IL-6, LIF, or OSM at 20 ng/ml or IL-11 at 30 ng/ml for 3 days. All conditions were performed in duplicate. Cells were harvested and enzyme activity measured as previously described [Dorheim et al., 1993]. Activity was normalized relative to the protein concentration (reported as nmol hydrolyzed/min/μg cell protein) as determined by the BCA Protein Assay Reagent according to the manufacturer's instructions (Pierce, Rockford, IL).

Statistics

Data were analyzed statistically by the student *t* test according to published methods using the Sigmastat software package (Jandel, San Rafeal, CA) [Winer, 1971].

RESULTS

Adipogenic Response

To examine the adipogenic response to cytokines, confluent pre-adipocyte BMS2 cultures were induced with a combination of adipogenic agonists (MHI, methylisobutylxanthine/hydrocortisone/indomethacin) for 2 days in the absence or presence of cytokines (CNTF, LIF, and OSM). As a control for the inhibition of adipocyte differentiation, BMS2 cells were induced with MHI in the presence of conditioned media from lipopolysaccharide (LPS)-treated macrophages, a potent source of monokines (10% conditioned medium, CM). Cytokines or conditioned medium were present throughout the

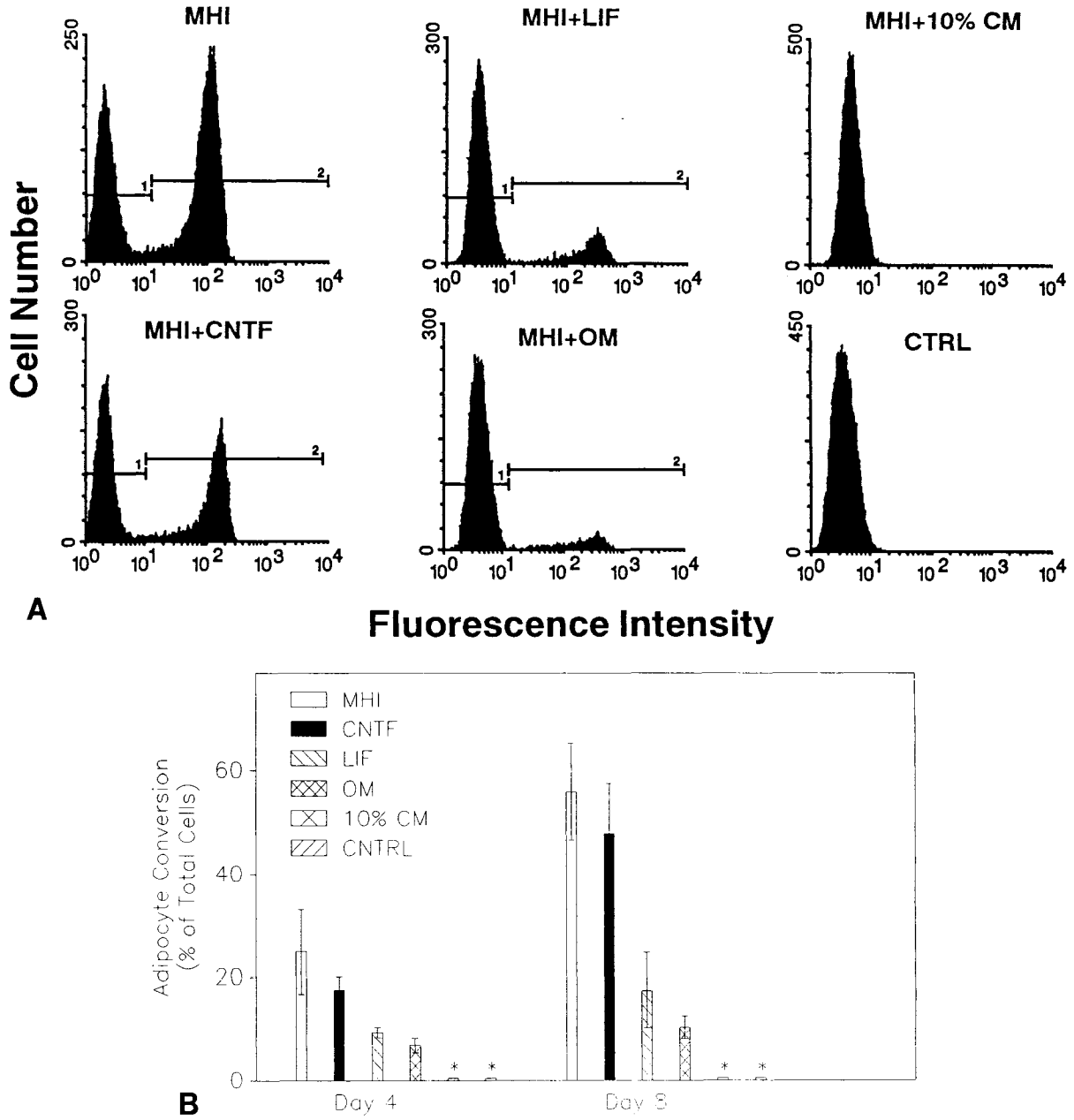

Fig. 1. Adipogenesis in the presence of cytokines. Confluent, quiescent BMS2 preadipocytes were induced to undergo adipocyte differentiation with the combination of *m*ethylisobutylxanthine, *h*ydrocortisone, *i*ndomethacin (*MHI*) for 48 h. Control (*CTRL*) cells were incubated in the absence of MHI. Inductions were carried out with the additional presence of *CNTF* (20 ng/ml), *LIF* (20 ng/ml), *OSM* (20 ng/ml), or 10% conditioned medium from LPS stimulated J774 macrophages (*10% CM*). Cells were harvested for fluorescence activated analysis on days 4 and 8. **A:** Fluorescence activated cell sorting (FACS) analysis of day 8 induced adipocytes. FACS analysis was conducted on 7.5×10^3 to 10^4 cells (Y axis) stained with nile red and the fluorescence emission intensity between 564 nm and 604 nm (X axis) monitored. **B:** The percent of total cells under each culture condition which differentiated into adipocytes on days 4 and 8. Each data point represents the mean ± S.D. of four separate wells.

course of each study (4 or 8 days of culture). Cells grown in the absence of both MHI and cytokines served as an additional control (CTRL). The extent of adipocyte differentiation was monitored by quantitative flow cytometry of cells stained with the dye Nile red, which increases its fluorescence emission in a lipid environment. A representative flow cytometric analysis of cells on day 8 following adipocyte induction is shown in Figure 1A. The percentage of total cells con-

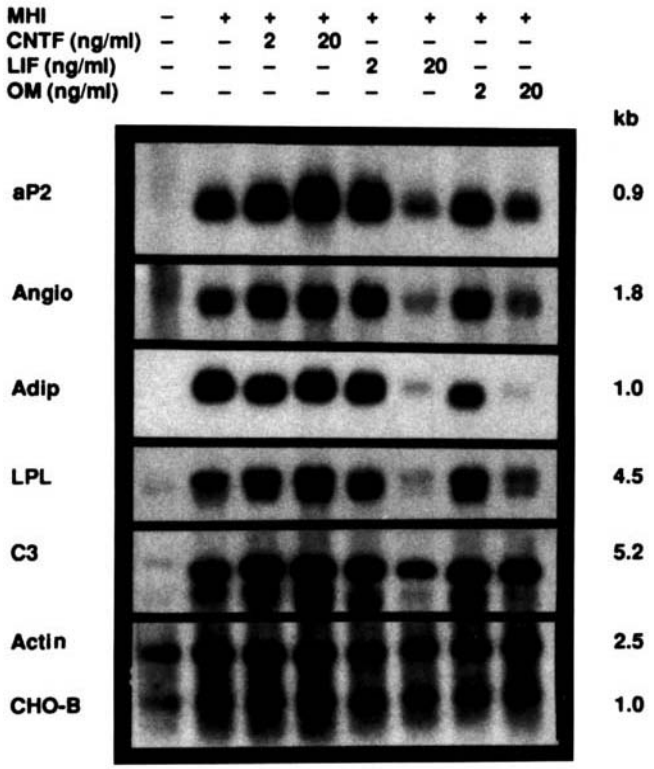

Fig. 2. Analysis of adipocyte gene markers at day 4 of induction in the presence and absence of cytokines. Confluent, quiescent BMS2 pre-adipocytes were induced to undergo adipogenesis with MHI in the presence or absence of CNTF, LIF, or OSM at 2 or 20 ng/ml. After 48 h, the media was replaced with fresh medium containing the cytokines but without MHI. Heparin treated supernatants and total RNA were harvested on day 4 of the study. Northern blots prepared with total RNA (10 μg/lane) were hybridized with the following cDNA probes: aP2, angiotensinogen (Angio), adipsin (Adip), lipoprotein lipase (LPL), complement C3 (C3), actin, and CHO-B. The sizes of the mRNA transcripts shown in kilobases (kb) were calculated relative to the electrophoretic migration of the 18 S and 28 S ribosomal RNAs.

verted to adipocytes (mean values, n = 4) on days 4 and 8 is shown in Figure 1B. Maximum adipocyte differentiation was induced by MHI alone. Although CNTF lowered these values, the differences were not significant. However, both LIF and OSM reduced adipocyte differentiation to 20–30% of maximum MHI levels. No adipocyte differentiation was detected in cells induced in the presence of macrophage conditioned medium or in control cells incubated in the absence of MHI.

The response to the cytokines was dose dependent. When BMS2 cells were induced with MHI in the presence of LIF or OSM at 2 ng/ml, the development of the adipocytic phenotype was not markedly changed; this required cytokine levels of 20 ng/ml (data not shown). Inhibition was paralleled by decreased steady-state mRNA levels for a number of adipocyte-specific gene markers. These included aP2, a fatty acid binding protein; adipsin/complement factor D and angiotensinogen, members of the serine protease protein family; and lipoprotein lipase (LPL), a key enzyme in lipid metabolism (Fig. 2). One exception to this pattern was the complement C3 gene; its expression level was not inhibited at the LIF and OSM concentrations examined. The heparin releasable LPL enzyme activity levels correlated directly with the steady-state mRNA levels; reduced LPL activity occurred at 20 ng/ml concentrations of LIF and OSM (Table I).

The temporal relationship between adipocyte induction and sensitivity to inhibitory cytokines was examined. The ability of IL-6, IL-11, LIF, or OSM to block adipocyte differentiation was limited to the initial induction period (Table IIA). If the addition of these cytokines was delayed by 48 h after induction with MHI, their inhibitory action was abolished. Treatment with IL-11, LIF, or OSM for only the initial 6 h of MHI induction was sufficient to inhibit adipogenesis (Table IIB). However, both IL-6 and LIF required a 24 h period of exposure for maximum effectiveness. A comparable pattern has been reported in the 3T3 3 cell line, where the inhibitory effects of TGFβ on adipogenesis occur only if the cytokine is present during the initial induction period [Sparks et al., 1992]. Although the

degree of adipocyte differentiation was reduced in some studies with CNTF, these values were not statistically significant. Hybridization of northern blots prepared with BMS2 poly A^+ RNA with probes for the gp130 and CNTFR α cDNAs indicated that the gp130 mRNA was present and its level did not change during the course of adipocyte differentiation; however, no evidence of the 2 kb CNTFR α mRNA was detected (data not shown).

Inhibition of Lipoprotein Lipase Activity

The action of cytokines on mature BMS2 adipocytes was measured based on inhibition of LPL activity. The cytokines IL-11, LIF, and OSM reduced the heparin-releasable LPL enzyme activity level of differentiated BMS2 adipocytes in a dose dependent manner, achieving significant effects at 2–3 ng/ml (Table III). For a similar level of inhibition, IL-6 required a concentration of 20 ng/ml. The cytokines reduced LPL activity by up to 45% of control values. Northern blot analysis determined that LPL mRNA levels did not change in response to any of these cytokines (data not shown), suggesting translational as opposed to transcriptional regulation of LPL activity.

TABLE I. Effect of Cytokines on the MHI Induction of Lipoprotein Lipase†

Inducing agent	Heparin-releasable LPL activity (nmol/h/ml)
Control	178 ± 5*
MHI	1,761 ± 170
MHI + CNTF (2 ng/ml)	1,471 ± 90
MHI + CNTF (20 ng/ml)	1,511 ± 21
MHI + LIF (2 ng/ml)	1,687 ± 57
MHI + LIF (20 ng/ml)	110 ± 17*
MHI + OSM (2 ng/ml)	1,401 ± 50
MHI + OSM (20 ng/ml)	95 ± 7*

†Values are the mean ± S.D. of duplicate plates each assayed in triplicate. Confluent cells were cultured in the presence of cytokines for a total of 4 days; MHI was present only during the initial 48 h of treatment. The cells were incubated in fresh medium containing heparin (10 units/ml) for 1 h and the harvested supernatants assayed for LPL as described in Materials and Methods.
*Significance values compared with MHI, $P < 0.01$.

Immediate/Early Gene Response

Confluent, quiescent cultures of murine preadipocyte BMS2 stromal cells were treated with LIF or OSM at concentrations of 20 ng/ml for increasing periods of time. Analysis of steady state mRNA levels by northern blot (Fig. 3) demonstrated a time dependent activation of immediate/early gene response. Within 30 min of exposure to cytokines, the signal for the immediate/early genes *c-fos,* jun B, and TIS 11 genes peaked. After 1 h, the maximum signal intensity was observed for the steady-state mRNA levels of *c-jun* and the cytokine genes IL-6 and JE/IL-8. The acute phase response gene serum amyloid A (SAA) reached maximum intensity after 24 h of cytokine exposure.

Osteoblastic Gene Expression

The BMS2 stromal cells express genes consistent with an osteoblastic phenotype [Dorheim et al., 1993]. These include osteocalcin, a protein related to bone mineralization and unique to osteoblasts; osteopontin, an extracellular matrix protein expressed in osteoblasts, kidney, and other tissues; and alkaline phosphatase

TABLE II. Adipogenesis in the Presence of Cytokines†

Delay after MHI	MHI + CNTF	MHI + IL-6	MHI + IL-11	MHI + LIF	MHI + OSM
A. Delayed addition of cytokine following MHI induction					
0 h	104 ± 29	20** ± 12	15** ± 10	7** ± 4	3** ± 1
6 h	62 ± 24	41** ± 14	14** ± 7	19** ± 8	5** ± 2
24 h	70 ± 12	42** ± 26	24** ± 16	48** ± 20	12** ± 4
48 h	139 ± 34	94 ± 34	43** ± 12	124 ± 41	90 ± 17
B. Early removal of cytokine after MHI induction					
6 h	105 ± 44	78 ± 33	23** ± 9	44** ± 28	14** ± 14
24 h	64 ± 38	30** ± 14	18** ± 17	25** ± 13	12** ± 11
48 h	98 ± 56	29** ± 12	15** ± 10	15** ± 8	8** ± 4
Continuous	84 ± 49	28** ± 14	15** ± 10	12** ± 5	7** ± 3

†Values are normalized relative to the adipogenic agonist (MHI) positive control, defined as 100% of maximum adipocyte induction, and expressed as mean ± S.D. All cytokines were present at a concentration of 20 ng/ml with the exception of IL-11, which was present at a concentration of 30 ng/ml. Data in each individual study was determined by FACS analysis on 7.5–10 × 10^3 cells from each of four separate cultures, 6 days after the induction of adipogenesis. Significant values based on comparison with that of the MHI control (100%): $^{**}P = / < 0.001$.

TABLE III. Inhibition of Heparin-Releasable Lipoprotein Lipase Activity in Mature Adipocytes†

Agent (ng/ml)	CNTF	IL-6	IL-11	LIF	OSM
0.2–0.3	129 ± 44	103 ± 19	93 ± 23	80 ± 15	84 ± 35
2–3	87 ± 7	106 ± 22	58 ± 9*	61 ± 18*	59 ± 17*
20–30	80 ± 17	60 ± 20	54 ± 13*	54 ± 11*	55 ± 16*

†Mature cultures of BMS2 adipocytes were incubated in the presence of cytokines for a period of 20 h, after which the cells were harvested with medium containing heparin (10 units/ml) as described in Table I. All cytokines were present at concentrations of 0.2, 2, and 20 ng/ml with the exception of IL-11, which was added at concentrations of 0.3, 3, and 30 ng/ml. Values are normalized relative to heparin-releasable LPL activity from untreated adipocytes (0 ng/ml), defined as 100%, expressed as the mean ± S.D. from at least six data points collected in two or three individual experiments. Significance values compared to controls (0 ng/ml): $^*P < 0.001$.

(ALP), an enzyme associated with bone growth. The osteoblastic phenotype is believed to be enhanced when cells are cultured in the presence of β-glycerophosphate and ascorbate, agents which promote mineralization. BMS2 cells were incubated in the absence and presence of CNTF, LIF, and OSM (20 ng/ml) for a period of 6 days and the steady state level of osteoblastic gene markers examined by Northern blot analysis (Fig. 4). While control cells or cells grown in the presence of CNTF demonstrated detectable steady state levels of osteocalcin mRNA, the signal for this gene was not detected when cells were treated with LIF or OSM. In contrast, the osteopontin mRNA steady-state level was increased only by LIF, consistent with earlier osteoblast experiments [Noda et al., 1990]. In parallel studies, IL-6, IL-11, LIF, and OSM all increased alkaline phosphatase enzyme activity while CNTF had no effect (Table IV). In control medium, ALP activity was increased 50% by IL-11, LIF, and OSM and 100% by IL-6. In the presence of β-glycerophosphate/ascorbate, all three cytokines increased ALP activity by 100%.

Adipogenic Inhibition by CNTF in the Presence of Soluble CNTFR α

The lack of a CNTF response in previous experiments was believed to be due to the lack of stromal cell expression of the CNTFR α protein, a necessary component of the CNTF receptor complex. Therefore, the effect of soluble CNTFR α protein on the ability of CNTF to inhibit adipogenesis was examined (Table V). Adipogenesis was induced with MHI in the presence of CNTF and/or CNTFR α at concentrations of 0, 100, or 1,000 ng/ml. By itself, the CNTFR α protein did not significantly inhibit adipogenesis. However, the combination of CNTF and CNTFR α at 1,000 ng/ml reduced adipocyte differentiation to less than 10% of control levels. Lesser effects were observed with CNTF alone or both proteins together at lower concentrations. Thus, in the presence of its soluble receptor protein, CNTF can modulate some aspects of BMS2 gene expression.

DISCUSSION

These results indicate that members of the cytokine family whose receptors share the gp130 signal transducing protein can regulate the adipocytic and osteoblastic differentiation potential of bone marrow stromal cells. For each cytokine, the receptor complex consists of a heterodimer or heterotrimer involving gp130, a β chain (LIF receptor β or a second gp130 molecule), and in some cases an α chain (CNTFR α or IL-6 receptor α) [Taga and Kishimoto, 1993]. These receptor complex differences may account for the varying degree of stromal cell response to members of this cytokine family. For example, the mRNA for the CNTFR α protein was not detected in BMS2 stromal cells. This protein is an essential component of the CNTF receptor complex and its distribution appears to be limited to the nervous system and skeletal muscle [Davis et al., 1991, 1993b]. Consequently, adipogenesis in BMS2 cells was inhibited by CNTF only when the exogenous soluble CNTFR α protein was also present during the induction period.

The cytokines inhibited stromal cell adipocyte differentiation and the heparin-releasable activity of the lipid metabolic enzyme, lipoprotein lipase in the following order: IL-11, OSM, LIF > IL-6 > CNTF. This is consistent with reported in vivo and in vitro findings. Long-term treatment of mice with LIF or IL-6 has been associated with loss of adipose tissue stores [Greenberg et al., 1992; Hilton and Gough, 1991]. Treatment of 3T3-L1 adipocytes with IL-6 inhibited the heparin-releasable LPL enzyme activity in a dose-dependent manner; half maximal inhi-

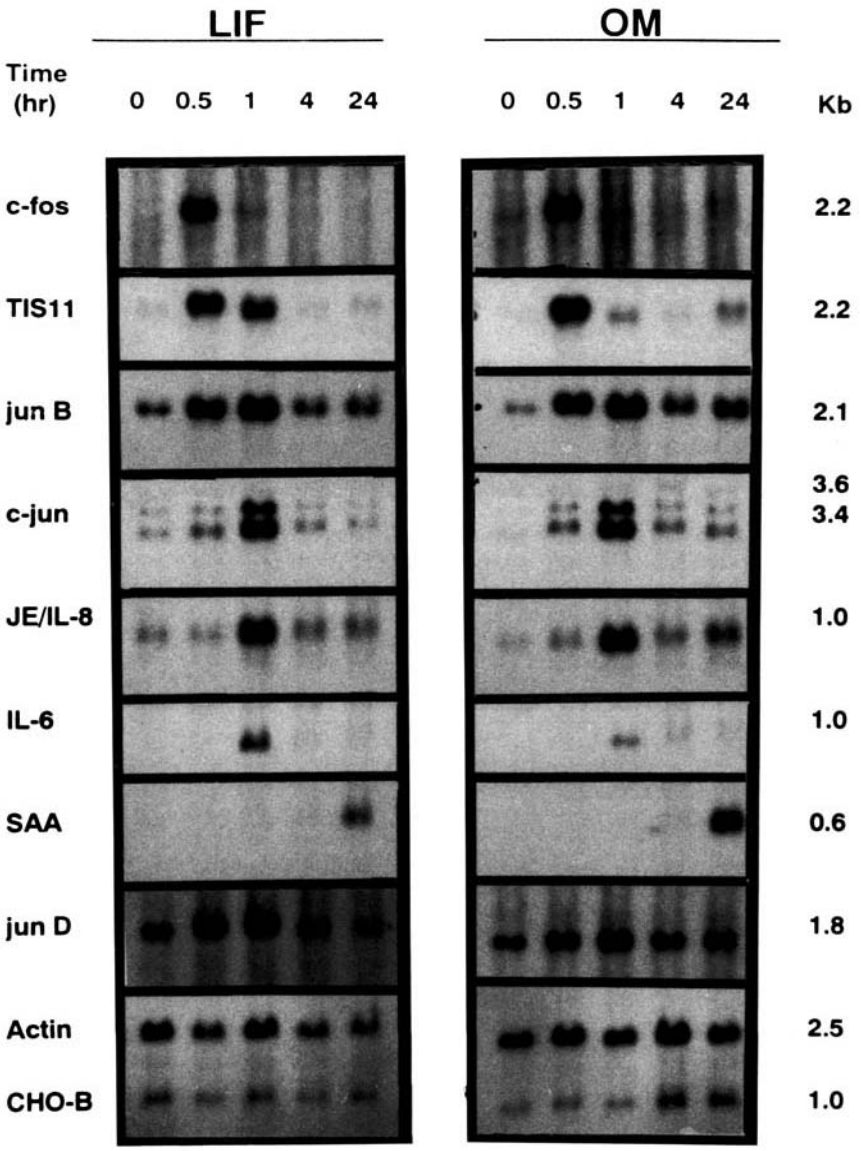

Fig. 3. Immediate/early gene response. Confluent, quiescent BMS2 pre-adipocytes were induced for periods of 0 to 24 h with either Leukemia Inhibitory Factor (LIF) or Oncostatin M (OM) at 20 ng/ml. Total RNA harvested from the cells was electrophoresed (10 μg/lane) and examined on Northern blots hybridized with probes for the indicated genes. The size of the detected mRNAs are shown in kilobases (Kb). The constantly expressed Actin and CHO-B genes demonstrate relatively equal loading between lanes.

bition required IL-6 concentrations of 5,000 units/ml [Greenberg et al., 1992]. Using a similar 3T3-L1 assay, a melanoma-derived LPL inhibitor (MLPLI) has recently been purified and putatively identified as LIF, based on N-terminal amino acid sequence analysis [Mori et al., 1989]. The stromal cell derived adipogenic inhibitory factor (ADIF) was found to be identical to IL-11 by the same process [Kawashima et al., 1991]. Previous investigators have suggested that expression of LIF and IL-6 may be partially responsible for cancer cachexia [Hilten and Gough, 1991; Greenberg et al., 1992]. The current findings indicate that IL-11 and OSM expression may also contribute to this symptom.

In vitro, LIF and OSM elicited an immediate/early gene response from preadipocyte stromal cells similar to that reported in other cell lineages induced with IL-6, IL-11, LIF, or OSM [Brown et al., 1991; Lord et al., 1991; Nakajami and Wall, 1991; Baumann and Schendel, 1991]. In lymphoid cells exposed to IL-6, expression of

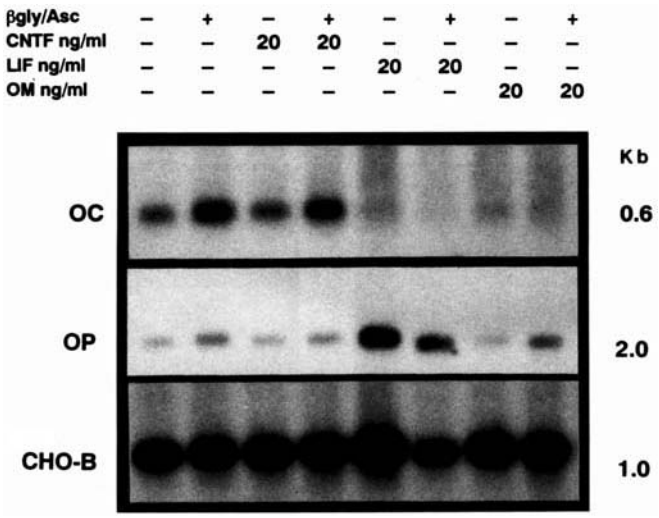

Fig. 4. Northern blot analysis of osteoblastic gene markers. Confluent, quiescent cultures of preadipocyte BMS2 cells were incubated in the absence (CNTRL) or presence of CNTF, LIF, or OSM (20 ng/ml) for 6 days in either standard medium or in medium supplemented with 10 mM β-glycerophosphate and 50 μg/ml ascorbate. Poly A^+ mRNA prepared from the cells was analyzed on northern blots (approximately 2 μg/lane) hybridized successively with probes for osteocalcin (OC), osteopontin (OP), and the housekeeping gene CHO-B.

TABLE IV. Alkaline Phosphatase Activity†

Agent	Control	CNTF	IL-6	IL-11	LIF	OSM
Media	1.22 ± 0.34	1.29 ± 0.22	2.47** ± 0.45	1.99** ± 0.76	1.76* ± 0.29	1.80* ± 0.36
β Gly-Asc	0.87 ± 0.11	1.13** ± 0.19	2.48** ± 0.27	2.08** ± 0.73	2.28** ± 0.13	2.32** ± 0.38

†Cells were incubated in the presence of cytokines as described in Materials and Methods. Cytokines were present at concentrations of 20 ng/ml with the exception of IL-11, added at 30 ng/ml. Activity is reported as nmol substrate hydrolyzed/min/μg cell protein. Values are the mean ± S.D. of two or three separate experiments assayed in duplicate. Significance values relative to CONTROL. *$P < 0.01$; **$P < 0.001$.

TABLE V. Effect of Soluble CNTF Receptor on Adipogenesis†

MHI	+	+	+	+
CNTF	1,000	0	100	1,000
CNTFR	0	1,000	100	1,000
% Adip.	62 ± 23	95 ± 28	66 ± 26	9** ± 8

†Values are normalized relative to the adipogenic agonist (MHI) positive control, defined as 100% of maximum adipocyte induction, expressed as the mean percent adipogenesis ± S.D. of two separate experiments. The concentrations of CNTF and CNTFR are indicated in ng/ml; these agents were present throughout the course of the study. Data in each individual study was determined by FACS analysis on 10^4 cells from each of four separate cultures, 6 days after the induction of adipogenesis. Significance values are based on comparison with that of the MHI control (100%): **P=/< 0.001.

TIS11 and *junB* was increased [Nakajami and Wall, 1991]. Following exposure to either IL-6 or LIF, myeloid cells increased their expression of *c-jun, junB,* and *junD* [Lord et al., 1991]. Likewise, human endothelial cells exposed to OSM responded with increased production of IL-6 [Brown et al., 1991]. In hepatic cells, CNTF, IL-6, IL-11, LIF, and OSM all induced acute phase response genes [Baumann and Schendel, 1991; Richards et al., 1992; Schooltink et al., 1992; Taga et al., 1992]. The acute phase response gene, serum amyloid A (SAA), was among the stromal cell genes induced by LIF and OSM.

In vivo treatment of mice with LIF has been associated with increased bone turnover and resorption [Cornish et al., 1993; Lowe et al., 1991; Metcalfe and Gearing, 1989]. Both IL-6 and LIF regulate the production of osteoclast-like cells which are responsible for the process of bone remodeling/resorption [Kurihara et al., 1990; Ishimi et al., 1990; Reid et al., 1990]. Osteoclasts are derived from the macrophage lineage and are recruited within the bone marrow by osteoblast-derived chemotactic cytokines such as JE/IL-8 [Takeshita et al., 1993]. The observed induction of stromal cell IL-6 and JE/IL-8 expression by LIF and OSM may partially

explain the mechanism underlying osteoclast recruitment and activation in vivo.

Literature reports of the alkaline phosphatase response of osteoblasts treated in vitro with LIF have been variable [Hakeda et al., 1991; Rodan et al., 1990]. In the murine calvaria-derived MC3T3E1 cell line, LIF treatment reduced alkaline phosphatase enzyme activity [Hakeda et al., 1991]. In the rat RCT-1 cell line, LIF treatment had no effect by itself but, in combination with retinoic acid, it enhanced alkaline phosphatase enzyme activity and mRNA steady state levels [Rodan et al., 1990]. In the BMS2 cells, IL-6, LIF, and OSM enhanced alkaline phosphatase enzyme activity. Inter-specie variations and heterogeneity among murine bone marrow stromal cell lines may account for these differences.

The cellular organization and communication networks within the bone marrow microenvironment remain complex. The present studies demonstrate that pleiotropic cytokines which share a common receptor regulate BMS2 stromal cell differentiation in a similar manner. This suggests that stromal cells may exhibit a common response to other cytokine families whose members act through the same or similar receptor proteins. If true, this will simplify the conceptual model depicting cytokine mediated regulation of stromal cell function. Further studies will be necessary to test this hypothesis.

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