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ARTICLES

Human BMP-7/OP-1 Induces the Growth and Differentiation of Adipocytes and Osteoblasts in Bone Marrow Stromal Cell Cultures

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Abstract We studied the effects of BMP-7/OP-1 on growth and differentiation of bone marrow stromal cells. BMS2, a mouse bone marrow stromal cell line capable of differentiating into adipocytes and osteoblasts, were treated in a serum-free medium containing differentiation agents that favor the expression of both lineages. BMP-7/ OP-1 stimulated cell proliferation and differentiation concomitantly. These effects were dose- and growth phase-dependent. Cells were more sensitive to the treatment early in the culture (30–40% confluence) with a significant increase in cell proliferation and markers of differentiation at low concentrations. When treated later in the growth phase (90–100% confluence), no significant increase in cell proliferation was seen. The concentration requirement for cells later in the culture to reach an equivalent degree of differentiation was 3–10- fold higher than for cells treated early. In both cases, the effects on adipocyte differentiation were biphasic; low concentrations stimulated adipocyte differentiation which was inhibited at higher concentrations where stimulation of osteoblast markers were observed. We conclude that cell proliferation and cell differentiation into adipocyte/osteoblast can occur simultaneously under BMP-7/OP-1 treatment. J. Cell. Biochem. 82: 187–199, 2001. †Published 2001 Wiley-Liss, Inc.

Key words: bone marrow stromal cells; BMP-7/OP-1; cell differentiation; adipocyte; osteoblast

Bone marrow stromal cells contain stem cells as a source for multiple cell types including myocytes, chondrocytes, adipocytes and osteoblasts [Owen, 1988; Prockop, 1997; Aubin, 1998]. The differentiation of stromal stem cells into adipocytes or osteoblasts is of particular interest because of clinical evidence which suggests an inverse relationship existing between bone mass and adipose tissue in osteopenia and age-related osteoporosis [Meunier et al., 1971; Demmler and Burkhardt, 1978; Burkhardt et al., 1987]. With increasing age, the number and size of marrow adipocytes increase in a linear manner [Martin and Zissimos, 1991]. Studies in an age-related osteopenia animal model, SAMP6 mice, have shown increased adipogenesis and myelopoiesis in the bone marrow to be associated with reduced number of osteoblast progenitors and decreased bone formation [Kajkenova et al., 1997].

Numerous studies using a variety of stromal cell lines have demonstrated that the differentiation of adipocytes, chondrocytes, and osteoblasts can be influenced by the presence of hormones [Cheng et al., 1994; Locklin et al., 1995; Kelly and Gimble, 1998], cytokines [Keller et al., 1993; Gimble et al., 1994], and local growth factors [Benayahu et al., 1993; Wang et al., 1993; Gimble et al., 1995]. In rat bone marrow stromal cell cultures, Beresford et al. [1992] have shown an inverse relationship between formation of adipocytes and osteo-

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blasts. Factors that promote osteoblast differentiation are generally inhibitory to adipocyte differentiation and vice versa [Gori et al., 1999; Johnson et al., 1999].

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor superfamily and play a significant role in the formation of skeletal tissues [Sakou, 1998]. The original molecules were cloned based on peptides isolated from bone extract for their ability to induce ectopic bone formation in vivo [Wozney et al., 1988]. The BMP subfamily has since expanded to comprise more than 40 related molecules [Reddi, 1997]. Among them, the recombinant molecules of BMP-2, BMP-4, and osteogenic protein-1 (OP-1 or BMP-7) are well studied for their ability to stimulate proliferation and differentiation of osteoblasts in cell cultures, to induce ectopic bone formation and to promote bone healing in animal models [Wang et al., 1990; Chen et al., 1991; Sampath et al., 1992]. BMPs act on multipotential marrow stromal cells to induce cell differentiation along the osteoblast pathway [Thies et al., 1992; Yamaguchi et al., 1996; Fromigue et al., 1998; Gori et al., 1999] while inhibiting the formation of myoblasts [Yamaguchi et al., 1991].

The effects of BMPs on adipocyte differentiation are less defined. As osteogenic and chondrogenic inductive factors, BMPs are expected to act as a negative regulator for adipogenesis. Data from a multipotent murine bone marrow stromal cell line, BMS2 cells, support this hypothesis. Gimble et al. [1995] have shown in this cell line, BMP-2 induced alkaline phosphatase, an osteogenic-related enzyme, but suppressed the expression of adipocyte phenotypes. Different results have been obtained from C3H10T1/2 cells, a murine multipotential fibroblastic cell line where BMP-2 induced adipocyte differentiation at a low concentration but chondrocyte phenotype expression at high concentrations [Wang et al., 1993]. Similar findings were reported by Asahina et al using BMP-7/OP-1 [Asahina et al., 1996].

In addition to their differentiation effects, BMPs can affect cell proliferation. This effect depends on the cell type and culture conditions. In rat osteoblast-like cells, BMP-4 stimulates cell proliferation as well as osteoblast phenotype expression [Chen et al., 1991]. While in a murine osteoblast cell line, MC3T3-E1 cells, cell proliferation was not affected by BMP-2 or osteogenin (BMP-3) [Takuwa et al., 1991]. In C3H10T1/2 cells, induction of adipogenesis was observed without any change in cell proliferation [Asahina et al., 1996]. In rat marrow stromal cell cultures, the expression of bone-related proteins was found to follow a temporal and spatial relationship associated with the proliferative activity [Malaval et al., 1994]. The differences in cell responses to BMPs might be due to variation of cell proliferative activity and to the degree of cell maturity when treatment was initiated. None of these studies has demonstrated a direct correlation between proliferative and differentiation functions by BMPs.

The published information on stromal cell differentiation has been derived from experiments performed in serum containing media. Serum contains a variety of hormones and growth factors. The concentration of these components in serum depends on the available lots. In BMS2 cells, Gimble et al. [1995], noticed the lot of fetal calf serum affected the extent of adipocyte induction. The effect of any specific hormone or growth factor can be altered or masked by the interaction of the other components in the serum and thus generate inconsistent or biased results.

In the present study, a serum-free medium was used to examine the effects of BMP-7/OP-1 on the adipogenic and osteoblastic potential of BMS2 cells. Treatment of BMP-7/OP-1 was performed at two different maturation stages in culture to link its proliferative activity with its differentiation function.

MATERIALS AND METHODS

Cell Line

Mouse bone marrow stromal cells line (BMS2) was a generous gift from J. M. Gimble (Oklahoma University Health Science Center) with the permission of Oklahoma Medical Foundation. The cell line was obtained from 5-flurouracil treated mouse bone marrow culture and found to support lymphocyte growth, undergo adipogenesis and display certain osteoblast gene markers [Pietrangeli et al., 1988; Gimble et al., 1989; Dorheim et al., 1993]. Cells were grown in high glucose Dulbecco's minimum essential medium (DMEM) containing 1 mM Na pyruvate supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM/ml glutamine (GIBCO/BRL, Gaithersburg, MD) and 10% fetal bovine serum (Hyclone, Logan, UT). Cells were plated at a density of 4,000/cm^2

and replaced with serum-free medium (RenCyte fibroblast medium, made from a propietary formula by Medi-Cult, Jyllinge, Denmark) containing 10 mM of β−glycerophosphate and 300 μM of ascorbate phosphate, magnesium salt (Waco, Osaka, Japan) for adipocyte induction and BMP-7/OP-1 treatments. The stock adipocyte induction cocktail (INC) contains 0.5 M methylisobutylxanthine, 0.5 mM hydrocortisone and 60 mM indomethacin (Sigma). The reagents were dissolved in dimethylsulfoxide (DMSO) and added to the medium to be diluted into 1/1,000 of the original concentration. BMP-7/OP-1 was made 100-fold concentrated in 1 mg/ml of bovine serum albumin (vehicle, Sigma, immuno-assay grade) and added to reach final concentrations ranging from 0.03 to 30 nM at the initiation of adipocyte differentiation. After three days, medium was replaced with the RenCyte medium with INC only for additional two days. This was followed by two more days of incubation in RenCyte medium without INC before examining markers of cell differentiation. In some experiments, when non-induced cells were needed, cells were treated following the same protocol except for replacing the INC with DMSO vehicle.

Primary Culture

Young male C57BL/6j mice (4 weeks old) were obtained from Jackson Laboratory (Bar Harbor, ME). They were euthanized by cervical dislocation according to protocols approved by the Institution Use and Care Committee. Femora and humora were removed aseptically and bone marrow was flushed out with a 26 gauge needle and 5 cc syringe in αMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml of fungizone, 2 mM/ml glutamine, 1 mg/ml of BSA, 10 mM of HEPES buffer (pH 7.0). After mechanical dispersion of the marrow into cell suspension, the number of nucleated cells was counted after incubating and disruption of the cells in zapoglobin (Coulter, Miami, FL) to lyse the red blood cells. They were plated at a density of $2 \times 10^7/25$ cm^2 flask in the same medium containing 1/10 of fungizone and 10% fetal bovine serum, (Hyclone). After 4 days, the non-adherent cells were removed by aspiration of the medium followed by two washes with phosphate-buffered saline. The adherent cells were either treated with vehicle or 3 nM of BMP-7/OP-1 for 10 days before extraction of total RNA for Northern blot analysis.

Alkaline Phosphatase Histochemical and Oil-Red-O Staining

Cells in 24-well plates after treatments were fixed in 10% neutral formalin for 5 min followed by dual-staining for alkaline phosphatase activity and lipid. After extensive washing in water, they were first stained for alkaline phosphatase activity using the leukocyte alkaline phophatase diagnostic kit from Sigma (No. 85). The positive cells with the enzyme activity stained blue as a result of the reaction product coupled with fast blue dye. The cells were then stained for lipid by incubating with the oil-red-O stain (1% oil-red-O made in 67% isopropanol/ 33% water) for 30 min. After washing with 50% of isopropanol and water, the cells were counter stained for 10 min in Meyer's hematoxylin to reveal the nuclei.

Alkaline Phosphatase Assay

Cells were plated in 24-well plates and treated as described previously. They were harvested and sonicated in 10 mM Tris buffer (pH 7.4)/ 0.1% Triton X-100 (200 μl/well). Aliquots were taken for assay of the enzyme activity using p-nitrophenyl phosphate as substrate [Chen et al., 1991]. A separate aliquot was taken from each sample for assay of protein concentration using the bicinchoninic acid method (Pierce, Rockford, IL). A unit of enzyme activity is defined as the conversion of 1 μmol of substrate into *p*-nitrophenol per mg of protein in 30 min.

DNA Measurement

DNA was measured by its emission of fluorescence when incubated with Hoechst 33258 dye [Labarca and Paigen, 1980]. Five to 25 μl of the cell lysate was incubated with a saline buffer (10 mM Tris, 1 mM Na_2EDTA, 0.1 M NaCl, pH 7.4) containing 0.1 μg/ml of the dye. Fluorescence was read in a Turner fluoremeter at an excitation wavelength of 365 nm and emission wavelength of 458 nm against a standard curve.

RNA Blotting Analysis

Total RNA was extracted from 25 cm^2 T flasks after treatments using Medi- RNA Isolation kit ordered from Quiagen according to the manufacturer's description. For Northern blot analysis, approximately 15 μg of RNA/lane was

separated by a formadehyde agarose gel after denaturation. RNA from the gel was blotted to a nylon membrane (Hybond, Amersham/Pharmacia). Prehybridization (6–16 h) was followed by hybridization (16–24 h) at 42°C with [^{32}P]dCTP (Amersham/ Pharmacia) labeled cDNA probes following the standard method. Membranes were rinsed twice and washed once for 10 min with 2X SSC, 0.1% SDS at room temperature. This was followed by two washes with 0.1X SSC, 0.1% SDS at 42°C. Membranes were exposed to a Phosphorimager (Molecular Dynamics) cassette overnight before scanning for the bands. The following cDNA probes were used: rat LPL (Gene accession no. NM012598, courtesy of S Azhar, VA Palo Alto Health Care System), mouse adipsin (Gene accession no. XO4673, courtesy of JM Gimble, Zen Bio) [Wilkison et al., 1990], mouse collagen α2 (I) (gene accession no. X58251, courtesy of JM Gimble, Zen Bio) [Rosen et al., 1988], mouse osteocalcin (gene accession no. XO4142, courtesy of A. Celeste, Genetics Institute, Cambridge, MA) [Celeste et al., 1986], human GAPDH (gene accession no. AF261085, courtesy of J-W Liu, VA Palo Alto Health Care System) [Liu et al., 1997].

Hormone Sensitive Lipase (HSL) Assay

Cells cultured in 6-well plates after treatments were sonicated in TES buffer (0.25 M sucrose, 1 mM EDTA, 2 μg/ml leupeptin and 50 mM Tris-HCl, pH 7.0) and supernatants from 12,000*g* (15 min. at 4°C) were used for the HSL assay. HSL activity was assayed by its ability to hydrolyze cholesteryl-[1-^{14}C]oleate as previously described [Kraemer et al., 1993]. One unit of the enzyme activity is expressed as 1 nM of cholesteryl oleate hydrolyzed /mg protein /h.

Immunoblotting

Aliquots of cell lysates from the same samples prepared for HSL activity assay were taken for the detection of the expression of HSL protein, peroxisome proliferative activating receptor-γ2 protein (PPARγ2) and the osteoblast-specific transcription factor, Cbfa1/Runx2, by immunoblotting. An equal amount of protein (15–20 μg, same amount within one experiment) was loaded onto each gel lane for all assays. The procedures for the HSL detection were followed exactly as described previously [Shen et al., 1999]. For the PPARγ2 detection, the blotted membranes containing separated proteins were incubated with 1:5,000 dilution with rabbit anti-PPARγ2 (Santa Cruz Biotech, Santa Cruz, CA) overnight at 4°C after 1 h blocking at 37°C (in PBS with 5% milk and 0.05% Tween 20). The membranes were washed twice with PBS containig 0.05% Tween, and incubated with secondary antibody (1:10,000 dilution of donkey anti-rabbit IgG, horseradish peroxidase conjugated, Amersham) at room temperature for 1 h. The immuno-reactive bands were visualized using ECL kit (Amersham/Pharmacia, Piscataway, NJ) after two more washes with PBS with 0.05% Tween. For the detection of Cbfa1/Runx2, membranes were probed with an antiserum (1:300 dilution) obtained from S. W. Hiebert [Meyers et al., 1996] which has been shown to crossreact with the rat osteoblast protein following the method by Chen et al. [1998] and Ji et al. [1998] at M. Centrella and T. L. McCarthy's laboratory. Molecular weight of the immuno-reactive protein was determined according to standard molecular weight markers from Bio-Rad (Richmond, CA).

Data Analysis

Data on DNA content and ALP activity on growth studies are presented as mean ± SEM of three to five independent samples from representative experiments. For HSL activities, immunoblotting and RNA blotting assays, duplicate samples from each treatment group were analyzed. In all studies, at least three similar experiments were performed for each type of experiment. Comparisons between control and treatment groups were analyzed by the use of StatView software package. After one-way ANOVA, a Bonferroni/Dunn test was used for multiple comparison post tests. Statistical significance is defined as $P < 0.01$.

RESULTS

BMP-7/OP-1 Stimulation of Cell Proliferation and Alkaline Phosphatase Activity

Cells were treated with BMP-7/OP-1 at varying concentrations either early at 30–40% confluence (Day 3) or late at 90–100% confluence (Day 5) and DNA content/culture and ALP activity were assayed. The results, as illustrated in Figure 1, indicate that BMP-7/OP-1 increased the DNA content in the BMS2 cells in a dose-dependent manner. The stimulatory effect was much greater when cells were treated early (Fig. 1A) than late (Fig. 1B). With

early treatment, the stimulation was first observed at 0.1 nM and reached a peak at 3 nM, where nearly ten fold more DNA/culture was seen as compared with the control, untreated cultures (Fig. 1A). In the late treatment, where the DNA content/culture of the control reached 10 μg, only slight stimulation occurred (Fig. 1B). In the absence of the adipocyte induction cocktail (INC), the level of DNA/culture in the control cultures was much higher than that in the presence of INC (comparing Fig. 1A and C). Under these conditions, the DNA content/culture increased with BMP-7/OP-1 concentration only above 3 nM.Although ALP activity has been reported to appear in preadipocytes [Satomura et al., 2000], the basal level of this enzyme was undetectable in BMS2 cells (Fig. 1A, B, and C). It was significantly stimulated by BMP-7/OP-1 under all three conditions (Fig. 1A, B, and C) but only at concentration above 3 nM. Putting together these results, it demonstrates that BMP-7/OP-1 enhances growth of BMS2 cells and that this effect was best shown in early treatment cultures when growth was suppressed by the presence of factors to induce adipocyte differentiation. Moreover, the ability of BMP-7/OP-1 to induce ALP, a marker of osteoblast differentiation required higher concentrations than needed for growth stimulation.

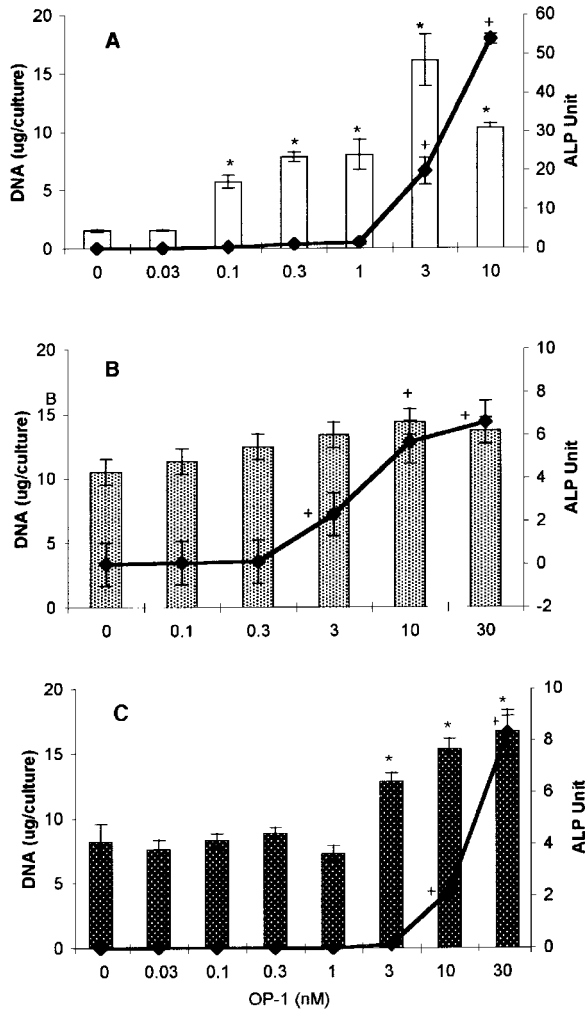

Fig. 1. BMP-7/OP-1 increased cell proliferation and ALP activity in BMS2 Cells. BMS2 cells were treated with or without adipocyte induction cocktail (INC) in the presence of increasing concentrations of BMP-7/OP-1 in a serum free medium containing ascorbic acid phosphate and β–glycerophoshate for 4 days and the medium was replaced with no addition of INC. Two days later, cells were lysed and assayed for ALP, protein and DNA as described in the Materials and Methods. The ALP unit represents μmole of p-nitrophenol produced/mg protein/30 min. Cultures were treated at day 3 after the beginning of the culture for the early treatment and day 5 for the late treatment. **(A)** Early treatment with INC and BMP-7/OP-1; **(B)** Late treatment with INC and BMP-7/OP-1; **(C)** Early cultures treated with BMP-7/OP-1 only. Values are mean +/− SEM of 3–5 cultures from a representative experiment. Bars represent DNA content/culture and lines represent ALP activity. * $P < 0.01$ for DNA; + $P < 0.01$ for ALP.

BMP-7/OP-1 Stimulation of Adipocyte and Osteoblast Differentiation

When BMS2 cells were treated with INC and BMP-7/OP-1 simultaneously, there was a biphasic effect. At low concentrations, the number of adipocytes increased with increasing concentrations of BMP-7/OP-1 but at high concentrations osteoblasts appeared. This is particularly obvious when BMS2 cells were treated early in the culture where the number of adipocytes started to increase at 0.03 nM, reached a peak at 0.3 nM (Fig. 2A, panels 1 and 2) and showed a reduction at 3 and 10 nM. At these high concentrations, the adipocytes were much larger than those exposed to lower concentrations of BMP-7/OP-1 and were surrounded by layers of cells heavily stained with alkaline phosphatase (Fig. 2A, panels 3 and 4). When cells were treated with BMP-7/OP-1 later in culture, adipocyte number was also increased by low doses of BMP-7/OP-1 (Fig. 2B, panels 1 and 2). However, with late exposure to BMP-7/OP-1, high concentrations of BMP-7/OP-1 continued to increase the number of small adipocytes further in spite of the appearance of alkaline phosphatase positive cells (Fig. 2B, panels 3 and 4). Low levels of ALP activity started to appear at 0.3 nM of BMP-7/OP-1;

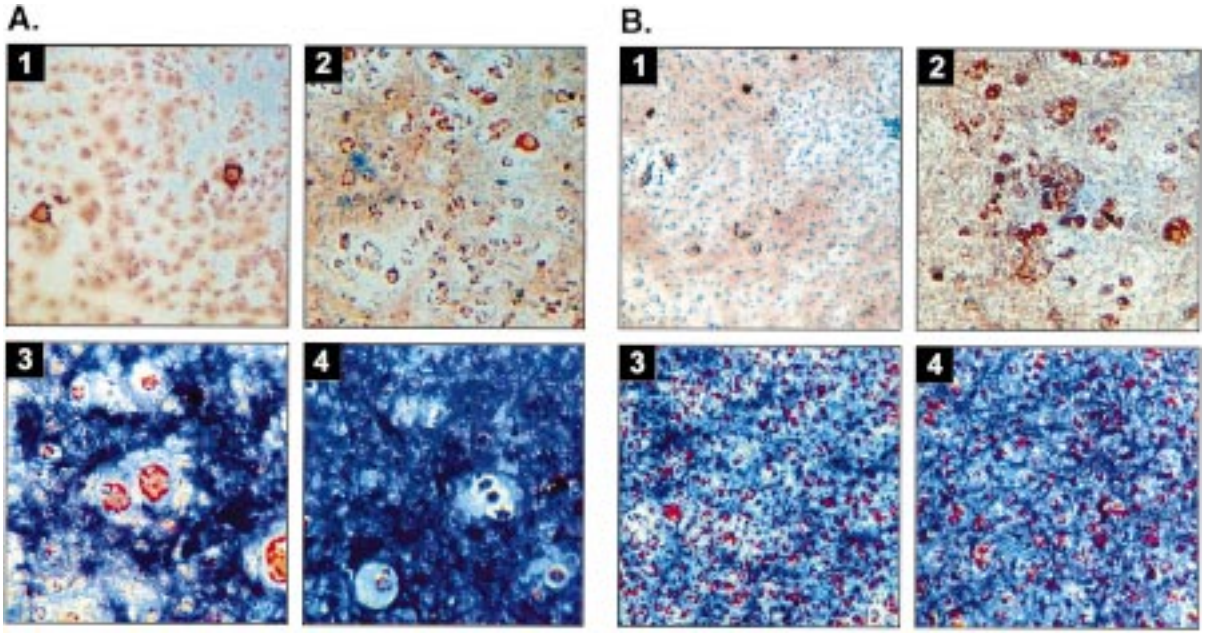

Fig. 2. BMP-7/OP-1 increased the number of adipocytes in a biphasic manner. The cultures were treated with INC and varying concentrations of BMP-7/OP-1 as described in Fig. 1. They were fixed and dual-stained for ALP activity and lipid at the end of experiment as described in the Materials and Methods. Cultures were photographed using an inverted microscope at 100X magnification. Adipocytes are shown as distinct oval-shaped cells containing red lipid droplets while osteoblasts positive for ALP activity stained as blue fibroblastic-looked cells appearing in area surrounding the adipocytes (panels 3,4 in A and B). **A.** Culture treated early on Day 3. **B.** Culture treated late on day 5: In both A and B: 1, control; 2, 0.3 nM of BMP-7/OP-1; 3, 3 nM of BMP-7/OP-1 and 4, 10 nM of BMP-7/OP-1.

most of it distributed in cells without lipid droplets, although some of the adipocytes also stained positive for ALP. BMP-7/OP-1 did not induce the differentiation towards chondrocyte lineage as it failed to induce positive staining of Alcian blue (data not shown), a specific stain for sulfated glycoaminoglycans, which occurs in ATDC5 cells [Asahina et al., 1996].

Expression of Gene Markers in BMP-7/OP-1and INC Treated BMS2 Cells by RNA Blotting

RNA blots were performed to examine the expression of several specific gene markers for adipocyte and osteoblast differentiation in BMP-7/OP-1 and INC treated cultures. For adipocyte markers, we examined the levels of lipoprotein lipase (LPL), an early differentiation marker, and adipsin, a mid-late differentiation marker. Type I collagen expression was used as the marker for osteoblast expression. Basal levels of LPL appeared in control cultures of both early and late cultures, with a higher expression level in the early cells. When treated with OP-1 early, there were dose-dependent increases in the expression of all three genes up to 3 nM followed by a decrease at 10 nM (Fig. 3A). When the cells were treated late, only the expression of LPL increased gradually with the BMP-7/OP-1 concentration. There was no adipsin expression and the expression of collagen was not significantly altered by BMP-7/OP-1 (Fig. 3B).

Characterization of BMS2 Cells-Comparison With Primary Culture of Bone Marrow Stromal Cells

Under the current conditions, BMS2 cells could not be further differentiated to become mature osteoblasts or to form calcified nodules, despite a long term 21-day culture period in serum containing medium containing ascorbic acid and β−glycerophosphate without INC.

To further characterize BMS2 cells, parallel studies were performed on BMS2 cells and primary bone marrow stromal cells (BMSP), which form calcified nodules in culture in serum containing medium. In addition to LPL and collagen expression, we examined the expression of osteocalcin, a gene marker for mature osteoblasts. In BMSP cells, the basal level of osteocalcin expression was low, and expression

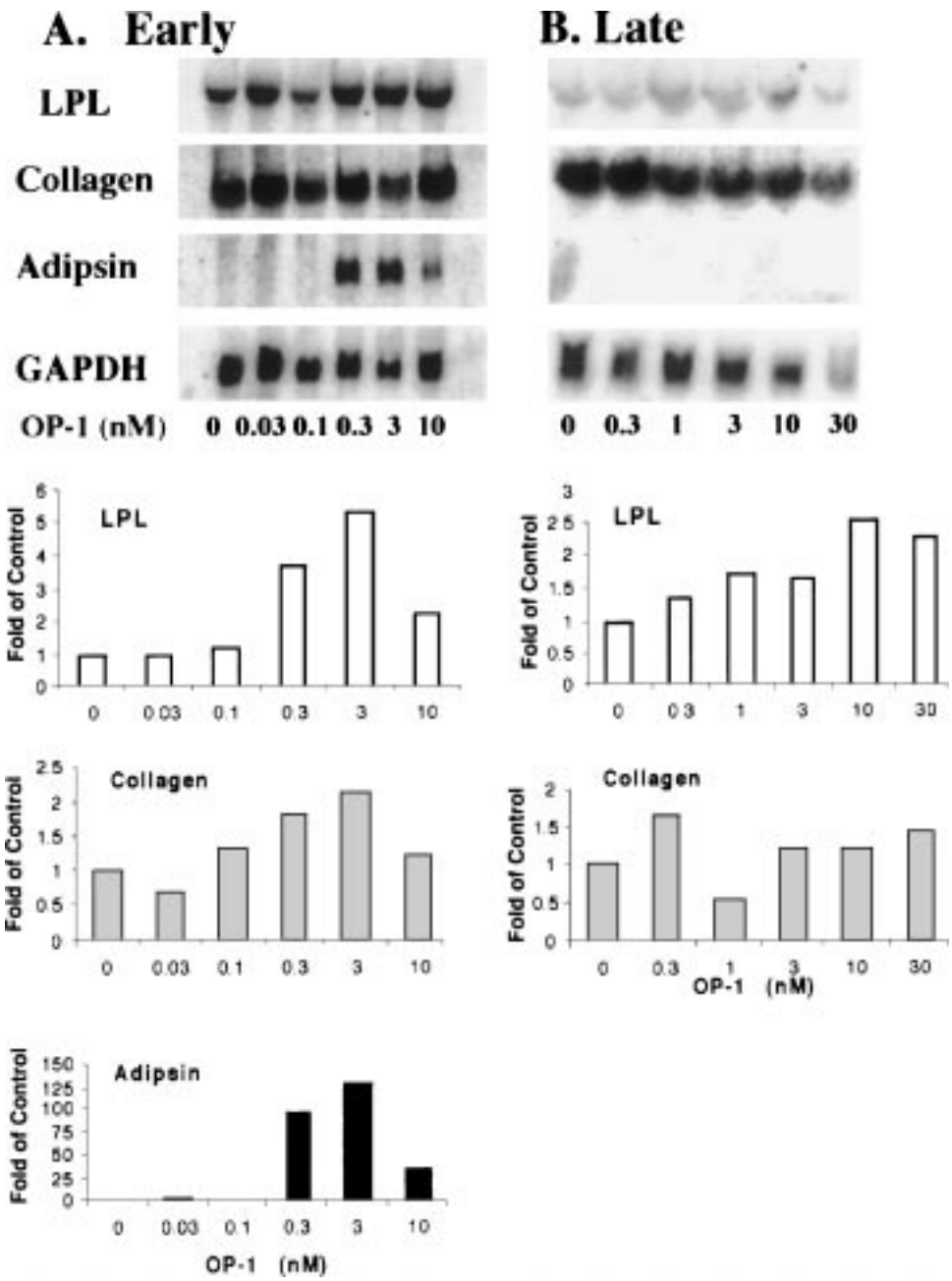

Fig. 3. BMP-7/OP-1 concentration-dependent changes in mRNA expression. Northern blot analysis was performed using total RNA from INC induced cultures treated with increasing concentration of BMP-7/OP-1 as described in Figure 1. Cells were treated with BMP-7/OP-1 at early or late growth stages in culture and total RNA was extracted for separation and blotting. After hybridizing with labelled cDNA probes, the radioactive image on the filter was captured by a phosphoimager and analysed for its density using a image-analysis quantitation software and calculated as fold of control value after normalizing by the level of GAPDH expressed. Data are illustrated as bars. **A.** Early, day 3 treatment; **B.** Late, day 5 treatment.

was greatly enhanced by BMP-7/OP-1 treatment (Fig. 4, lanes 3 and 4). In contrast, there was no osteocalcin expression in BMS2 cells with or without BMP-7/OP-1 treatment (Fig. 4, lanes 1 and 2). BMS2 also did not express bone sialoprotein, a gene marker for calcification matrix (data not shown). BMSP cells expressed lower levels of LPL compared with BMS2 cells. In both cells, LPL levels were not changed with the BMP-7/OP-1 treatment, while type I collagen was increased by BMP-7/OP-1 (Fig. 4, lanes 1–4).

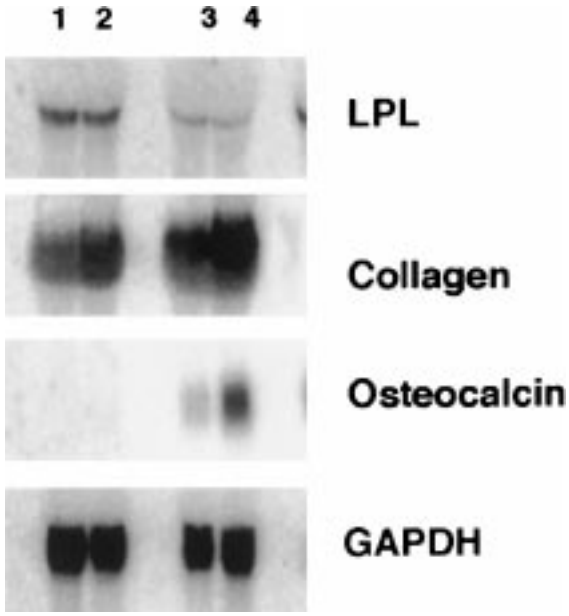

Fig. 4. BMP-7/OP-1 effects for non-induced BMS2 cells and primary bone marrow cell cultures (PBMS). BMS2 cells **(lanes 1,2)** and PBMS cells **(lanes 3, 4)** at early growth stage were treated with vehicle (lanes 1, 3) or 3 nM of BMP-7/OP-1 (2,4) for 10 days and Northern blot analysis was performed on LPL, collagen, osteocalcin and GAPDH.

Effects of BMP7/OP-1 on the Levels of HSL, PPAR γ2, and Cbfa1/Runx2

To assess adipocyte differentiation at the protein level, we performed immunoblotting analysis on HSL and PPARγ2 in BMS2 cell extracts of INC treated cultures. PPARγ2 is considered to be an early marker and HSL is a late marker of adipocyte differentiation [MacDougald and Lane, 1995]. The basal levels of these two markers were non-detectable in early cultures and increased with time in late cultures. In addition, the expression patterns were different between these two markers in response to increasing concentrations of BMP-7/OP-1 (Fig. 5). While the expression of HSL followed the same biphasic pattern as the formation of adipocytes (compare with Fig. 2), this was less obvious for PPARγ2. In cells exposed to BMP-7/OP-1 early, although there was a progressive increase in PPARγ2 with increasing concentrations of BMP-7/OP-1, the inhibition at high concentration of BMP-7/OP-1 was not as strong as for HSL, which was significant at 10 nM (Fig. 5A). Similarly, in late cultures, the inhibition of HSL occurred above 10 nM but no obvious difference of PPARγ2 was seen among the control and the BMP-7/OP-1 treated cultures (Fig. 5B).

The osteoblast-specific transcription factor, Cbfa1/Runx2, was assayed to detect osteoblast differentiation. This transcription factor has been considered to be the differentiation switch for the osteoblast lineage [Banerjee et al., 1997; Ji et al., 1998; Gori et al., 1999; Lecka-Czernik et al., 1999]. The expression of Cbfa1/Runx2 was enhanced in early cultures at 3 and 10 nM of BMP-7/OP-1 (Fig. 5A) correlating with expression of high ALP activity (Figs. 1 and 6). BMP-7/OP-1 did not have any significant effect on Cbfa1/Runx2 in late cultures (Fig. 5B).

Biphasic Responses of HSL Activity to BMP-7/OP-1 Treatment

When HSL activity was assayed, we found a similar pattern as seen for the expression of HSL protein (Fig. 6). HSL activity was increased by low concentrations of BMP-7/OP-1 and inhibited at high concentrations. Cells exposed to BMP-7/OP-1 early during differentiation were more sensitive (maximum induction at 0.3 nM) to BMP-7/OP-1 than cells treated late (maximal induction at 1 nM). In both groups of treatments, a decline in the activity appeared at high concentrations beyond 3 nM, where ALP activity was markly stimulated.

DISCUSSION

BMP-7/OP-1 stimulated cell proliferation (Fig. 1) and differentiation (Fig. 2) in BMS2 cells. Both effects can be demonstrated in a serum-free medium with defined constituents containing both adipocyte induction cocktail (INC) and osteoblast induction agents-ascorbic acid and β−glycerophosphate. As opposed to the antagonistic effects of BMPs on adipogenesis reported by Gimble et al. [1995], under the current culture conditions, BMS2 cells displayed a biphasic response to BMP-7/OP-1: stimulation of adipocyte differentiation at low concentrations which was inhibited at high concentrations. A decrease of mature adipocyte number and an increase in the number of ALP positive osteoblast-like cells occurred only at high BMP-7/OP-1 concentration.

The differentiation function of BMPs does not necessarily parallel their effects on stimulation of cell proliferation. In BMS2 cells, proliferation and differentiation are both stimulated by

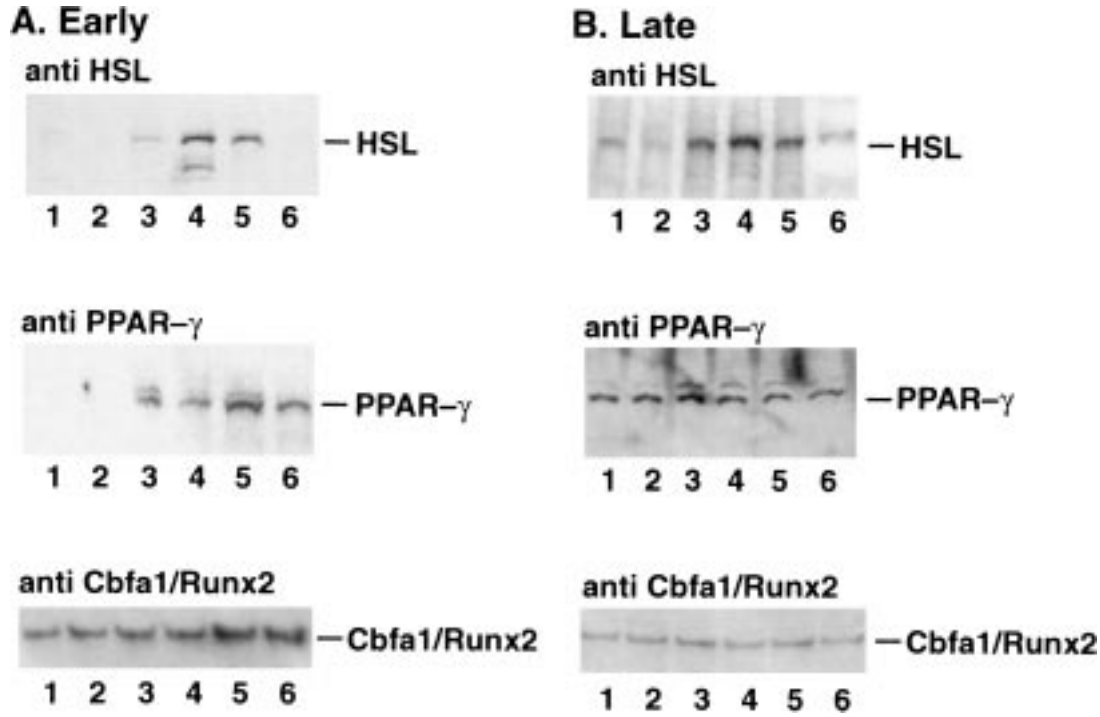

Fig. 5. BMP-7/OP-1 concentration-dependent changes in HSL, PPAR-γ2 and Cbfa1/Runx2 protein expression. Immunoblotting was performed in cell extracts of BMS2 cells following INC and BMP-7/OP-1 treatments. Cells were harvested, sonicated and spun down in TES buffer. Aliquots of sample containing equal amount of protein were subjected to SDS/PAGE, blotted onto nitrocellulose paper and incubated with antibodies as described in the Materials and Methods. The molecular weight of the bands are: HSL, 84 kDa; PPAR-γ2, 56 kDa; Cbfa1/Runx2, 55 kDa. **A:** Early, day 3 treatment: **lane 1**, control; **lanes 2–6**, varying concentrations BMP-7/OP-1. The concentrations are: lane 2, 0.03 nM; lane 3, 0.1 nM; lane 4, 0.3 nM; lane 5, 3 nM; lane 6, 10 nM. **B:** Late, day 5 treatment: **lane 1**, control; **lanes 2–6**, varying concentrations BMP-7/OP-1. The concentrations are: lane 2, 0.3 nM; lane 3, 1 nM; lane 4, 3 nM; lane 5, 10 nM; lane 6, 30 nM.

BMP-7/OP-1. The stimulation of proliferation was the greatest when the cells were treated early in the presence of INC which by itself suppresses cell growth (Fig. 1A). The early treatment group was more sensitive than the late treatment group to BMP-7/OP-1 effects for cell differentiation, as indicated by the lower concentrations of BMP-7/OP-1 required to induce adipocyte or osteoblast markers (Figs. 2, 5, 6). Since the treatment schedules for cell growth and differentiation were identical, the stimulation of BMP-7/OP-1 on cell proliferation and the differentiation of both adipocyte and osteoblast lineages occurred concurrently. The effects of BMP-7/OP-1 in BMS2 are therefore different from the conventional studies where cell confluence is required before differentiation into adipocytes [MacDougald and Lane, 1995] or osteoblasts [Stein et al., 1996] occurs. It is not possible to determine from the data whether the proliferative effects of BMP-7/OP-1 are restricted to fully undifferentiated cells or whether BMP-7/OP-1 might stimulate proliferation of cells that have already committed to a specific differentiated lineage.

During the early course of the study, we found that both the basal level of adipocyte differentiation and the effect of BMP-7/OP-1 varied among batches of serum. We found that by switching to a serum-free chemically defined medium, our results were consistent for each experiment performed. As opposed to serum containing medium, the basal level of adipocyte appearance in the INC treated cultures was extremely low (<1%) and responded robustly to BMP-7/OP-1 stimulation (Fig. 2). The biphasic responses to BMP-7/OP-1 in our cultures are different from the inhibitory effects of other BMPs on adipocyte differentiation of BMS2 cells [Gimble et al., 1995]. In our hands, the responses of BMS2 cells to BMP-2 and BMP-4 are similar to that of BMP-7/OP-1 (data not shown). The difference between our results and previous findings is more likely due to the presence and absence of serum in the culture conditions rather than the types of BMP used.

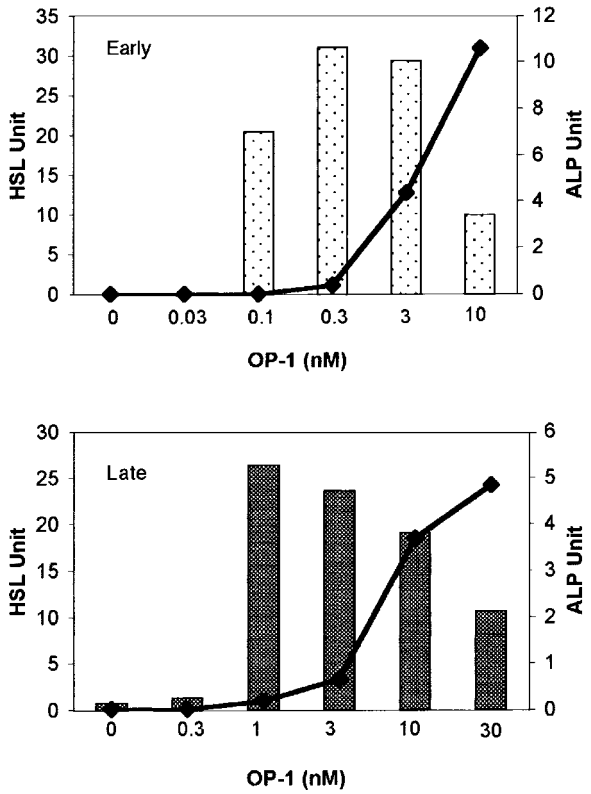

Fig. 6. BMP-7/OP-1stimulation of HSL activity with corresponding changes in ALP activity in INC treated BMS2 cells. BMS2 cells were treated with INC in the presence of increasing concentrations of BMP-7/OP-1 at early and late treatment as describe in Figure 1. Cells were harvested, sonicated and spun down in TES. Aliquots were taken for assay of HSL and ALP activity and protein concentration buffer as described in the Materials and Methods. Data are mean of duplicate cultures. Bars are HSL activity and lines represent ALP activity. Similar experiments were performed three times.

We found that the maturation stage in culture influenced the ability of BMP-7/OP-1 to induce cell differentiation. This is particularly obvious for adipocyte differentiation. The basal level of LPL mRNA and its response to BMP-7/OP-1 were lower in the late growth stage compared to the early treated cells, although the changes at both growth stages followed a similar pattern (Fig. 3). In sharp contrast to the early treated cells where a significant amount of adipsin was induced by BMP-7/OP-1, there was no trace of adipsin expression when cells were treated with BMP-7/OP-1 late. Interestingly, this phenomenon correlates with the failure of BMP-7/OP-1 to increase the size of adipocytes when these cells were treated late with high concentrations of BMP-7/OP-1, in contrast to the large adipocytes generated following early exposure to BMP-7/OP-1 (Fig. 2).

The expression and activation of PPARγ2 are considered necessary for the terminal differentiation of adipocytes [Spiegelman and Flier, 1996; Wu et al., 1996; Shao and Lazar, 1997]. In contrast, the appearance of PPARγ2 negatively regulates the osteoblast maker Osf2/Cbfa (Cbfa1/Runx2) and osteoblast-like activity [Lecka-Czernik et al., 1999]. In BMS2 cells, activation of this receptor by its ligand, thiazolidinediones, induces adipogenesis [Gimble et al., 1996]. We found that changes in the level of PPARγ2 in BMS2 cells treated with BMP-7/OP-1 correlated with the induction of adipocyte formation particularly when treated early in culture (Fig. 5). However, when high concentrations of BMP-7/OP-1 induced an osteoblast phenotype, PPARγ2 expression did not decrease as significantly as was seen with HSL, another adipocyte differentiation marker. Other results have shown in a conditionally immortalized human marrow stromal cell line, hMS (2–6) cells, that BMP-2 failed to alter PPARγ2 mRNA level [Gori et al., 1999]. Therefore, the relationship between PPARγ2 expression and BMP stimulated osteoblast differentiation is not clear and can apparently vary among cell lines.

Transient enhancement in the expression of Cbfa1/Runx2 is essential for the differentiation of cells towards osteoblast lineage [Gori et al., 1999; Lee et al., 1999]. The transient upregulation of this transcription factor coincided with subsequent increased expression of ALP, type I collagen and osteocalcin. Cbfa1/Runx2 expression was increased by both BMP-2 and TGF-β in a myoblast cell line, C2C12, to suppress myogenesis. While the treatment of BMP-2 was followed by the expression of osteoblast makers, this did not occur in the TGF-β treatment. Although the expression of Cbfa1/Runx2 may be the turning switch for the osteoblast lineage, additional factors are necessary for the expression of fully differentiated osteoblasts. This is supported by a recent report indicating that the levels of Cbfa1/Runx2 transcript remained the same over time in culture among different murine stromal cell lines with heterogenous differentiation potential [Satomura et al., 2000]. BMS2 cells express constitutively high level of Cbfa1/Runx2. Similar to the earlier finding of BMP-2 effects in C2C12 cells [Lee et al., 1999], BMP-7/OP-1 in the early cultures of BMS2 cells increased the Cbfa1/Runx2

expression which coincided with the increases in ALP and type I collagen (Figs. 1, 3, 6).

Multiple bone marrow stromal cell lines have been established recently from mice [Chen et al., 1998; Diascro et al., 1998; Dennis et al., 1999; Lecka-Czernik et al., 1999] and human sources [Gori et al., 1999]. The nature of these cell lines and their responses to adipocyte inducing and osteoblast-inducing factors are varied. In addition to differences in the maturation stage of the stem cells in vivo when the cells were first isolated, the time of treatment during the culture period may be an additional factor for this variation based on our observation in BMS2 cells.

In previous studies, it has been shown that BMS2 cells possess many preosteoblast and osteoblast gene markers, including ALP, type I collagen and osteocalcin [Dorheim et al., 1993]. In our hands, the basal levels of ALP and osteocalcin were undetectable. ALP activity appeared after high concentrations of BMP-7/OP-1 (Figs. 1 and 6), where the adipocyte markers were low (Figs. 2, 4–6). Although some lipid containing adipocytes also expressed ALP activity, the majority of the ALP positive cells induced by BMP-7/OP-1 are osteoblast-like and do not contain lipid droplets (Fig. 2). The mRNA for osteocalcin was not detectable in BMS2 cells despite our ability to detect its appearance in the primary bone marrow culture where the expression of the gene was greatly enhanced by BMP-7/OP-1 (Fig. 4). The reason for the discrepancy in osteocalcin expression is not clear. Despite the enhancement of ALP and type I collagen expression (Figs. 1–3, 4, 6), BMP-7/OP-1 at high concentrations was not able to differentiate BMS2 cells into mature osteoblasts. Serum is not a determinant factor, since results were identical with or without serum in the culture medium (data not shown). The failure of BMS2 cells to express additional osteoblast makers such as osteocalcin and bone sialoprotein suggests it might not be an ideal cell line for studying osteoblast differentiation.

Our data indicate that BMP-7/OP-1, a conventional growth factor for the induction of bone growth and osteoblast differentiation, can be a potent adipocyte differentiation factor in a murine bone marrow stromal cell culture, BMS2 cells. The effects of BMP-7/OP-1 are concentration dependent-low concentrations induce adipocyte. This effects dwindles at high concentrations with the appearance of heavily stained ALP positive osteoblast-like cells. The stage of cell maturity in culture can also contribute to the extent of the BMP-7/OP-1 differentiation effects. Further experiments are necessary to explore the mechanisms for these responses.

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