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Bone Morphogenetic Proteins Inhibit Adipocyte Differentiation by Bone Marrow Stromal Cells

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Abstract The bone morphogenetic proteins were originally identified based on their ability to induce ectopic bone formation in vivo and have since been identified as members of the transforming growth factor-β gene superfamily. It has been well established that the bone morphogenetic cytokines enhance osteogenic activity in bone marrow stromal cells in vitro. Recent reports have described how bone morphogenetic proteins inhibited myogenic differentiation of bone marrow stromal cells in vitro. In vivo, bone marrow stromal cells differentiate along the related adipogenic pathway with advancing age. The current work reports the inhibitory effects of the bone morphorphogenetic proteins on adipogenesis in a multipotent murine bone marrow stromal cell line, BMS2. When exposed to bone morphogenetic protein-2, the pre-adipocyte BMS2 cells exhibited the expected induction of the osteogenic-related enzyme, alkaline phosphatase. Following induction of the BMS2 cells with adipogenic agonists, adipocyte differentiation was assessed by morphologic, enzymatic, and mRNA markers. Flow cytometric analysis combined with staining by the lipophilic fluorescent dye, Nile red, was used to quantitate the extent of lipid accumulation within the BMS2 cells. By this morphologic criteria, the bone morphogenetic proteins inhibited adipogenesis at concentrations of 50 to 500 ng/ml. This correlated with decreased levels of adipocyte specific enzymes and mRNAs. The BMS2 pre-adipocytes constitutively expressed mRNA encoding bone morphogenetic protein-4 and this was inhibited by adipogenic agonists. Together, these findings demonstrate that bone morphogenetic proteins act as adipogenic antagonists. This supports the hypothesis that adipogenesis and osteogenesis in the bone marrow microenvironment are reciprocally regulated.

Key words: adipocytes, bone morphogenetic proteins, differentiation, bone marrow stromal cells, transforming growth factor β

The bone morphogenetic proteins (BMP) were first discovered based on their ability to induce ectopic bone formation at intra-muscular sites in vivo [Urist, 1965]. The genes encoding these cytokines have recently been cloned and identified as members of the transforming growth factor-β (TGF-β) gene superfamily [Wozney et al., 1988; Lyons et al., 1989; Ozkaynak et al., 1990]. Within the bone marrow microenvironment, bone morphogenetic proteins induce multipotential stromal cells to differentiate along the osteoblast pathway; this is associated with the induction of tissue specific genes such as alkaline phosphatase [Chen et al., 1991; Hiraki et al., 1991; Paralkar et al., 1991; Rickard et al., 1994; Rosen et al., 1990; Thies et al., 1992; Vukicevic et al., 1989; Yamaguchi et al., 1991]. However, BMPs can also act as negative regulators of stromal cell differentiation. Stromal cell clones have been derived from the rat which can display an osteoblast, chondrocyte, adipocyte, and/or myoblast phenotype in vitro [Grigoriadis et al., 1988; Yamaguchi and Kahn, 1991]. Although these cells spontaneously displayed a myoblast-like phenotype upon reaching confluence, this was directly inhibited by recombinant BMP-2 in a concentration dependent manner, with maximum effects observed between 100 to 1,000 ng/ml [Yamaguchi et al., 1991]. In a similar manner, bone morphogenetic protein has been reported to block differentiation of skeletal muscle-derived murine myoblasts in vitro [Murray et al., 1993].

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Myogenic development is not normally observed within the bone marrow microenvironment. However, with advancing age, marrow stromal cells do differentiate along a related mesodermal pathway, undergoing adipogenesis [Owen, 1988; Beresford, 1989; Gimble, 1990]. The role of bone marrow adipocytes remains unclear. These cells may serve as an energy reserve or occupy space no longer required for lympho-hematopoiesis [Owen, 1988; Beresford, 1989; Gimble, 1990]. It is postulated that an inverse relationship exists between the adipocyte differentiation and the osteogenic capacity of bone marrow stromal cells [Beresford et al., 1992; Dorheim et al., 1993]. In vitro, adipocyte differentiation by stromal cells is accompanied by a loss of gene markers consistent with an osteoblast-like phenotype, including osteocalcin, type I and type III collagen, and osteopontin [Beresford et al., 1992; Dorheim et al., 1993]. It has been suggested that osteogenic inductive cytokines may act as negative regulators of adipogenesis [Bennett et al., 1991; Beresford et al., 1992].

The current work tests this hypothesis using the BMS2 cell line as a multi-potent bone marrow stromal cell model [Pietrangeli et al., 1988; Gimble et al., 1989, 1990; Dorheim et al., 1993]. These cells support lympho-hematopoiesis, undergo adipocyte differentiation, and constitutively display gene markers consistent with an osteoblast phenotype.

MATERIALS AND METHODS

Unless noted, all materials were obtained from Sigma Chemical Co., St. Louis, MO, or Fisher Scientific, Dallas, TX.

Alkaline Phosphatase Assay

The BMS2 cells were plated at 3×10^4 cells per 35 mm dish, incubated overnight in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco/BRL, Gaithersburg, MD) supplemented with 2% fetal bovine serum (Hyclone, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin, with or without β-glycerophosphate (10 mM) and ascorbate (50 μM). After 24 h, cells were treated with recombinant human bone morphogenetic protein-2 (BMP-2) at concentrations of 0.5 to 50 ng/ml for 3 days. Cells were harvested in phosphate-buffered saline/0.1% Triton X-100 (800 μl/plate) and assayed for alkaline phosphatase activity using p-nitrophenyl phosphate as substrate [Hausamen et al., 1967; Dorheim et al., 1993]. Enzyme activity was normalized relative to the protein concentration as determined using the bicinchoninic acid method (Pierce, Rockford, IL). Each study was performed in duplicate and each data point was assayed in duplicate.

Adipocyte Cell Culture

The BMS2 cells were grown to confluence in DMEM supplemented with 10% fetal bovine serum, 1 mM Na pyruvate, 50 μM 2-mercaptoethanol, 100 mg/ml streptomycin, 100 U/ml penicillin [Pietrangeli et al., 1988; Dorheim et al., 1993]. Quiescent cultures of confluent cells were treated for 72 h with 0.5 mM methylisobutylxanthine, 0.5 μM hydrocortisone (Elkins-Sinn, Cherry Hill, NJ), and 60 μM indomethacin to induce adipocyte differentiation. When present, recombinant human bone morphogenetic proteins −2, −4, and −6 (Genetics Institute, Cambridge, MA) were added at concentrations of 0.5 to 500 ng/ml at the initiation of adipocyte differentiation. At the end of the 72 h period, fresh medium without the adipocyte inducing agents was used to replace the entire culture volume; the bone morphogenetic proteins were maintained at their initial concentrations. Cells were examined for adipocyte differentiation 6 days after the induction of adipocyte differentiation.

Fluorescence Activated Cell Sorting

Cells grown in 24-well plates (Corning, Corning, NY) were fixed with 0.5% paraformaldehyde and stained with Nile red (1 mg/ml DMSO stock diluted 1:100 in phosphate-buffered saline immediately prior to use) at a final concentration of 1 μg/ml [Smyth and Wharton, 1992; Dorheim et al., 1993]. Cells were analyzed on a FACScan (Becton-Dickinson, San Jose, CA) multiparameter flow cytometer. Fluorescence emissions were detected between 564 and 604 nm using a 582/42 bandpass filter. The median channel number (fluorescence intensity) was held constant for uninduced (pre-adipocyte) cell populations between samples. Between 7.5×10^3 and 10^4 cells were analyzed per data point. All studies were conducted in quadruplicate.

Lipoprotein Lipase Assay

Cell culture supernatants in the presence of heparin (10 U/ml) were harvested and assayed for lipoprotein lipase (LPL) activity as previ-

ously described [Nilsson-Ehle and Schotz, 1976; Gimble et al., 1994]. Activities were normalized relative to the level expressed by adipocyte BMS2 cells incubated in the absence of BMPs. Each study was conducted in quadruplicate and assayed in duplicate.

Northern Blot Analysis

Total RNA was isolated from cells according to published methods [Chomczynski and Sacchi, 1987]. Formaldehyde agarose gels (1%) were electrophoresed with approximately 10 μg of total RNA per lane, transferred to MSI-NT membranes (MSI, Westboro, MA) [Thomas, 1980], and cross-linked by UV light exposure [Church and Gilbert, 1984]. The Northern blots were probed with random primer labeled probes [Feinberg and Vogelstein, 1983] for adipsin (courtesy B. Spiegelman, W. Wilkison, Dana Farber Cancer Center, Boston, MA) [Wilkison et al., 1990], CHO-B (courtesy R. Wall, UCLA, Los Angeles, CA) [Harpold et al., 1979], complement C3 (ATCC 63097/63098) [Domdey et al., 1982], and lipoprotein lipase (ATCC 63117) [Gimble et al., 1992], or with single stranded riboprobes (Promega Riboprobe Gemini II System, Madison, WI) for BMP-2, BMP-4 (Genetics Institute) [Wozney et al., 1988] and BMP-6/Vgr-1 [Lyons et al., 1989]. Blots were exposed with an intensifying screen for 1 to 7 days at −70° and the autoradiographs were developed.

Statistics

Data were analyzed using the student *t*-test and the SigmaStat software package (Jandel, San Rafael, CA).

RESULTS

Effects of BMP-2 on BMS2 Stromal Cell Differentiation

It is well established that bone marrow derived stromal cells respond to bone morphogenetic proteins with increased expression of the osteoblast marker, alkaline phosphatase. The BMS2 cells were cultured in low serum concentrations (2%) to reduce their constitutive expression of alkaline phosphatase. With subsequent exposure to recombinant BMP-2, the BMS2 cells increased their alkaline phosphatase enzyme activity by 3-fold (Table I). This response was independent of the presence of β-glycerophosphate and ascorbate, agents which enhance the expression of alkaline phosphatase and the osteoblastic phenotype.

To determine the effect of BMP-2 on bone marrow stromal cell adipogenesis, BMS2 cells were induced to undergo adipocyte differentiation in the absence or presence of increasing concentrations of BMP-2 (Fig. 1, Table II). Confluent, quiescent cultures of BMS2 cells were treated with adipogenic agonists (0.5 mM MIBX, 0.5 μM hydrocortisone, 60 μM indomethacin: MHI) for an initial 3-day period. When present, BMP-2 concentrations were maintained throughout the 6-day period. At this time, the BMS2 cell morphology was determined by Nile red staining. The fluorescence emission profile of this lipophilic dye is altered when placed in a lipid environment [Smyth and Wharton, 1992]. Flow cytometric analysis was used to quantitate the degree of adipocyte differentiation; a representative experiment is shown in Figure 1. In the absence of BMP-2, up to 40% or more of the cell population displayed an increased Nile red fluorescence intensity consistent with lipid accumulation and adipocyte differentiation. With increasing concentrations of BMP-2, the adipocyte population was diminished. The relative inhibition of adipogenesis by BMP-2 is displayed in Table II; at concentrations of 50 to 500 ng/ml, BMP-2 reduced adipocyte numbers by 50% or more.

Additional measures of adipocyte differentiation supported these morphologic observations. Over the same BMP-2 concentrations, BMS2 expression of the enzyme lipoprotein lipase (LPL) decreased by up to 60% (Table III). This corre-

TABLE I. Induction of BMS2 Alkaline Phosphatase Expression by BMP2*

	BMP2 (ng/ml)			
	0	0.5	5	50
Control	1.23 ± 0.11	1.77 ± 0.28	2.65 ± 0.35	3.43 ± 0.24
β-GLY/ASC	1.89 ± 0.33	2.73 ± 0.12	3.10 ± 0.41	5.27 ± 0.20

*Cells were plated at a density of 3×10^4 in control media or media supplemented with 10 mM β-glycerophosphate and 50 μM ascorbate in the absence or presence of BMP2. Alkaline phosphatase expression is expressed as nmol substrate hydrolyzed/μg protein/min. Values are the mean ± S.D. (representative of two individual experiments conducted in quadruplicate).

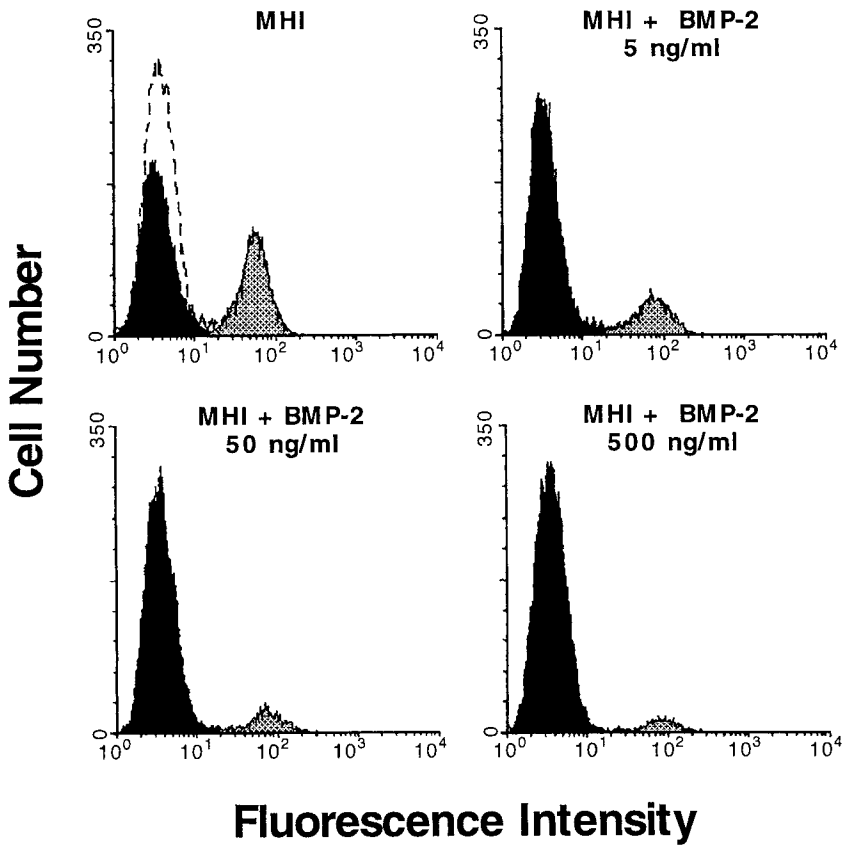

Fig. 1. Quantitation of stromal cell adipogenesis by FACS: Effects of BMP-2. Confluent cultures of BMS2 pre-adipocytes were induced with adipogenic agonists (0.5 mM MIBX, 0.5 μM hydrocortisone, 60 μM indomethacin: MHI) in the absence or presence of BMP-2 at 0, 5, 50, or 500 ng/ml. Culture medium was replaced after 72 h without MHI present. Cells were harvested on day 6 for flow cytometric analysis after staining with the lipophilic dye, Nile red, which increases its fluorescent emission when placed in a lipid environment. Dotted lines represent the fluorescence emission of pre-adipocyte control cells which were not treated with adipogenic agonists and cultured for an equivalent period of time. Black shading indicates that population of the induced cells which retained a fibroblast morphology. Stippled shading indicates those induced cells which exhibited an increased Nile red emission, consistent with an adipocyte morphology.

TABLE II. Adipocyte Induction in the Presence of BMP-2†

	BMP-2 (ng/ml)				
BMP-2 (ng/ml)	0	0.5	5	50	500
% Maximum induction	100	94 ± 4	76 ± 13	50 ± 13*	34 ± 21*

†Confluent BMS2 cells were induced for 3 days with adipogenic agonists (0.5 mM MIBX, 0.5 μM hydrocortisone, 60 μM indomethacin) in the absence or presence of BMP-2. Cells were maintained for an additional 3 days at the same concentration of BMP-2 and analyzed by FACS analysis for lipid accumulation. Values are expressed as a percentage of the maximum adipocyte differentiation observed in the absence of BMP-2 and defined as 100% (the actual percentage of total cells containing lipid ranges from 19 to 42% within individual experiments). Values are the mean ± S.D. (n = 5 each conducted in quadruplicate). *P value < 0.01 relative to control.

lated directly with the mRNA levels for LPL (Fig. 2). On Northern blots prepared with total RNA and hybridized with a radiolabeled LPL probe, increasing concentrations of BMP-2 decreased the LPL signal intensity. Likewise, the presence of BMP-2 reduced the mRNA signal for adipsin (equivalent to complement factor D), another gene induced with adipogenesis.

Effects of BMP-4 and BMP-6 on Stromal Cell Adipogenesis

The effects of other bone morphogenetic proteins on BMS2 adipocyte differentiation were determined. Cells were induced with adipogenic agonists in the absence and presence of either BMP-4 or BMP-6. With increasing concentra-

TABLE III. Lipoprotein Lipase Expression in the Presence of BMP-2*

	BMP-2 (ng/ml)				
BMP-2 (ng/ml)	0	0.5	5	50	500
LPL (% maximum)	100	95 ± 14	93 ± 13	71 ± 19	40 ± 6

*Confluent BMS2 cells were induced with adipogenic agonists in the absence or presence of BMP-2 as described in Table II. After 6 days in the presence of BMP-2 at the indicated concentrations, cells were incubated at 37°C in fresh medium containing 10 U/ml heparin and the supernatants collected after 1 h. The lipoprotein lipase activity in the supernatant was assayed and expressed relative to the maximum level observed in cells induced in the absence of BMP-2. Values are the mean ± S.D. of eight individual assays.

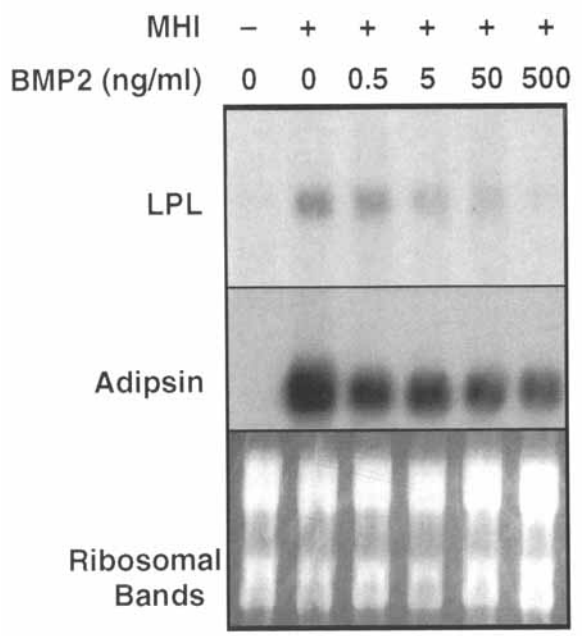

Fig. 2. Northern blot analysis of adipocyte-specific gene markers: Effects of BMP-2. Confluent cultures of BMS2 pre-adipocytes were induced with adipogenic agonists (MHI) in the absence or presence of BMP-2 (0 to 500 ng/ml) as described in Figure 1. Cells were harvested for total RNA on day 6 after the initiation of adipogenesis. Northern blots prepared with approximately 10 μg total RNA per lane were successively hybridized with probes for lipoprotein lipase (LPL), and adipsin (equivalent to complement factor D). The ribosomal bands of the Northern blot are shown to demonstrate equal loading between lanes.

tions of either cytokine, BMS2 adipocyte differentiation decreased by 40–60% relative to control levels (Table IV). The concentration dependence and magnitude of the BMP-4 and BMP-6 effects were similar to those observed with BMP-2. No statistically significant differences were observed between the percentage of inhibition at equal concentrations of the different bone morphogenetic proteins.

Expression of BMP-4 mRNA by BMS2 Cells

Since bone morphogenetic proteins are synthesized within the bone marrow microenvironment, the production of BMP mRNAs by the

TABLE IV. Adipocyte Differentiation in the Presence of BMP-4 and BMP-6†

Concentration (ng/ml)	BMP-4 (% maximum induction)	BMP-6 (% maximum induction)
0	100	100
0.5	91 ± 4	97 ± 2
5	77 ± 10	91 ± 1
50	60 ± 13	78 ± 5*
500	54 ± 10*	50 ± 19

†Confluent BMS2 cells were induced with adipogenic agonists for 3 days in the presence or absence of BMP-4 or BMP-6 at the indicated concentrations for 3 days. The concentrations of BMP-4 or BMP-6 were maintained for an additional 3 days at which time adipocyte differentiation was determined by FACS analysis. Data was normalized relative to the maximum adipocyte differentiation observed in the absence of exogenous BMP-4 or BMP-6, defined as 100% (actual percentages range from 38 to 78% within individual experiments). Values are the mean ± S.D. (n = 3, each conducted in triplicate or quadruplicate). *P value < 0.01 relative to control.

BMS2 stromal cells was assessed (Fig. 3). Using total RNA from BMS2 pre-adipocytes incubated in the presence of varying agents, Northern blots were prepared and hybridized with riboprobes for BMP-2, BMP-4, and BMP-6. While no signal was observed for either BMP-2 or BMP-6 (data not shown), BMP-4 was constitutively expressed in control BMS2 cells. In the presence of adipogenic agonists (MHI) or hydrocortisone, the BMP-4 signal intensity was reduced. These data indicate that BMS2 stromal cells have the potential to express one of the bone morphogenetic proteins. Similar observations have been made in the multipotent CH3/10T1/2 fibroblast cell line [Gazit et al., 1993].

DISCUSSION

In vitro, the bone morphogenetic proteins have been found to regulate differentiation along multiple mesodermal pathways. It is well established that these cytokines induce osteoblastic differentiation and bone formation [Urist, 1965;

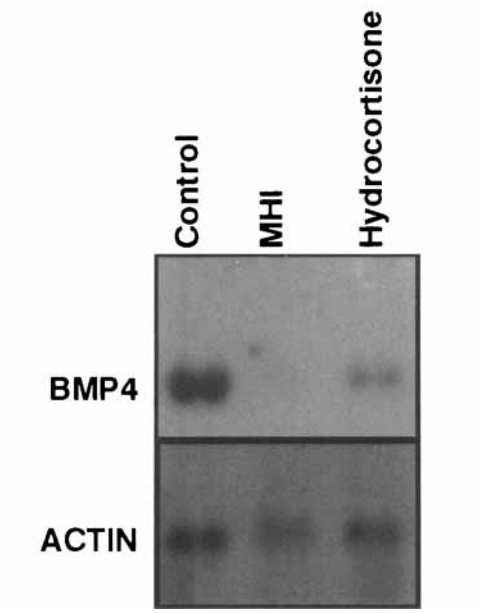

Fig. 3. Expression of BMP-4 mRNA by pre-adipocyte BMS2 cells: Effects of exogenous agents. Confluent cultures of BMS2 pre-adipocytes were treated for 3 days with hydrocortisone (0.5 μM) or MHI (0.5 mM MIBX, 0.5 μM hydrocortisone, 60 μM indomethacin). Northern blots were hybridized with probes for BMP-4 or actin as a control for equal loading between lanes.

Wozney et al., 1988; Ozkaynak et al., 1990; Paralkar et al., 1991]. In selected multipotential mesodermal cell lines, the presence of bone morphogenetic proteins inhibited myogenesis in favor of osteogenesis [Yamaguchi et al., 1991]. Similarly, bone morphogenetic proteins inhibited myotubule formation by committed C2C12 myoblasts in a concentration dependent manner [Murray et al., 1993]. The current work extends these findings, demonstrating that recombinant human bone morphogenetic proteins inhibit adipogenesis in the murine derived, multipotential BMS2 stromal cells based on morphologic, enzymatic, and mRNA criteria. In the absence of bone morphogenetic proteins, BMS2 cells rapidly undergo adipocyte differentiation in response to agonists. Based on FACS analysis, between 30 to 70% of the BMS2 cells accumulate lipid vacuoles within a single experiment. The extent of adipocyte induction can be related to the lot of fetal calf serum employed and the passage number of the cell line; later passages (>25) are often less responsive. Cell sorting experiments have determined that those BMS2 cells which exhibit a fibroblast phenotype following a single adipocyte induction retain the ability to undergo adipogenesis with subsequent inductions (Gimble and Morgan, unpublished observations). Using confluent BMS2 cells induced with adipogenic agonists for the first time, the inhibitory actions of the bone morphogenetic proteins were concentration dependent. At concentrations similar to those required to antagonize myogenesis [Yamaguchi et al., 1991; Murray et al., 1993], adipogenesis was inhibited by 50 to 60% relative to controls; however, complete blockage of adipocyte differentiation was not observed. Heterogeneity within the BMS2 cell population may explain this finding. Individual BMS2 cells may vary in their expression of bone morphogenetic protein surface receptors and/or in their expression of the cytoplasmic proteins necessary for signal transduction. Just as cell passage number may influence adipocyte differentiation, this could also effect the bone morphogenetic response. Alternatively, these murine derived cells may display greater sensitivity to the native murine, as compared to the human recombinant, bone morphogenetic proteins. Exposure to appropriate concentrations of the native murine proteins may inhibit a higher percentage of the adipogenic events.

Based on Northern blot analysis, the pre-adipocyte BMS2 stromal cells constitutively expressed mRNA for bone morphogenetic protein 4, and this was reduced following treatment with adipogenic agonists. This suggests the possibility that bone morphogenetic proteins may act in an autocrine and/or paracrine manner. It remains to be determined if the various bone morphogenetic proteins act in synergy in vitro or in vivo. Nevertheless, within the context of the bone marrow microenvironment, these data support the hypothesis that a reciprocal relationship exists between osteogenesis and adipogenesis; commitment to the osteoblast lineage may occur only at the expense of adipocyte formation [Beresford et al., 1992; Dorheim et al., 1993]. The relationship between bone morphogenetic proteins and the mesodermal cell lineages is summarized in Figure 4.

While the bone morphogenetic proteins belong to the TGFβ gene superfamily, TGFβ actions are similar but not identical to the bone morphogenetic proteins with respect to mesodermal cell differentiation. In vitro, TGFβ has been found to antagonize myogenesis in the C2C12 cell line in a manner similar to the bone morphogenetic proteins [Massague et al., 1986; Murray et al., 1993]. Likewise, TGFβ inhibited adipocyte differentiation in a number of murine cell lines, including BMS2, 3T3-L1, and 3T3 T [Ig-

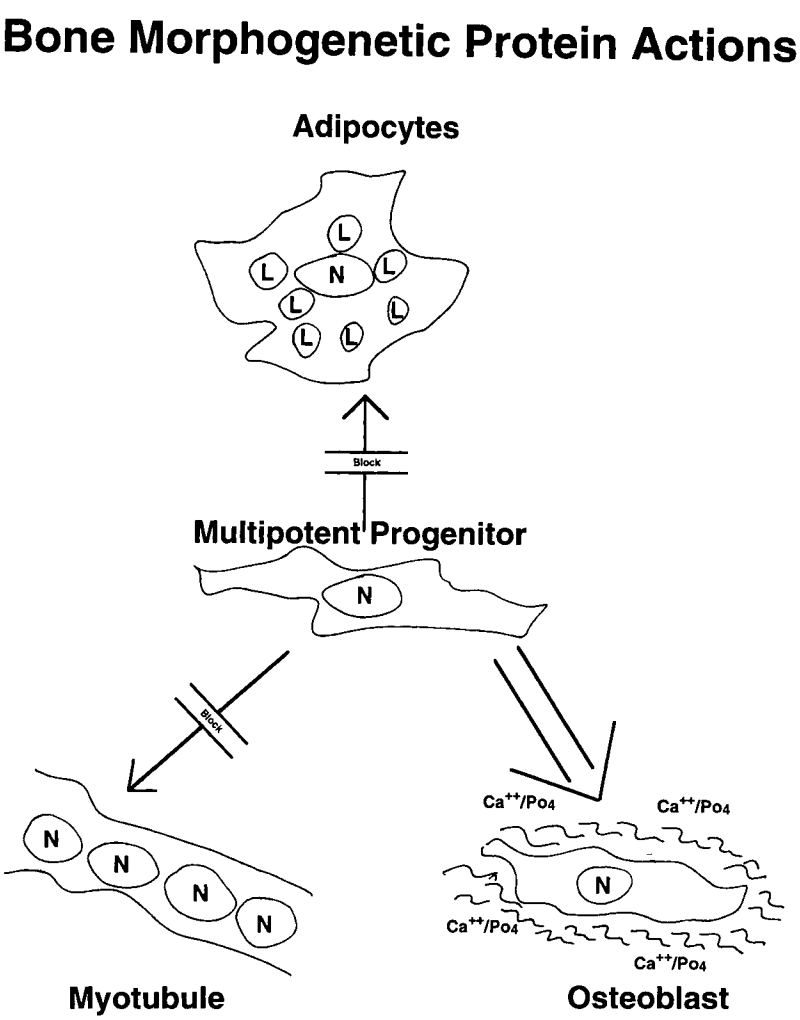

Fig. 4. Model of bone morphogenetic protein regulation of differentiation along the mesodermal lineages. Ca^{+}/PO_4 = hydroxyapatite crystals; L = lipid vacuole; N = nucleus.

notz and Massague, 1985; Sparks and Scott, 1986; Gimble et al., 1989; Sparks et al., 1992; Smyth et al., 1993; Bortell et al., 1994]. The presence of TGFβ during adipocyte induction permitted adipocyte precursor cells to continue to express some bone extracellular matrix proteins (fibronectin, type I collagen) not normally expressed by mature adipocytes [Bortell et al., 1994]. Based on these parallels, it is likely that the bone morphogenetic proteins would inhibit adipocyte differentiation in the 3T3 cell models although this remains untested at present. However, unlike bone morphogenetic proteins, TGFβ does not induce bone formation in vivo. In vitro, TGFβ has consistently failed to induce alkaline phosphatase expression by mesodermal cells such as BMS2 and, in some cases, has inhibited this enzyme activity [Rosen et al., 1990; Chen et al., 1991]. Thus, the cellular responses to these individual cytokines are distinct.

A number of laboratories are actively pursuing the receptors for TGFβ, the bone morphogenetic proteins, and related factors [Mathews and Vale, 1991; Attisano et al., 1992; Lin et al., 1992; He et al., 1993; Ebner et al., 1993; Yamaji et al., 1993; Estevez et al., 1993; Bassing et al., 1994; ten Dijke et al., 1994a–c; Wrana et al., 1994; Koenig et al., 1994]. A general model describing signal transduction by the TGFβ superfamily cytokine receptors is emerging [ten Dijke et al., 1994d; Wrana et al., 1994]. Receptor complexes consist of heterodimers of a type I and type II protein; both proteins exhibit the serine/threonine kinase activity [Wrana et al., 1994]. Receptor interactions are initiated by binding of the cytokine to the type II protein. This, in turn,

recruits a cytokine-specific type I protein to the complex. Distinctive features in the extracellular domains of the Type I receptors are believed to be responsible for conferring ligand binding specificity to the receptor complex. Following ligand binding of specific TGFβ gene superfamily cytokines, autophosphorylation of the receptor complex initiates signal transduction [Wrana et al., 1994]. Subsequent phosphorylation of cytoplasmic proteins by the activated receptor complex may then serve to propagate this signal. It is possible that bone morphogenetic proteins and TGFβ activate a common pathway which may account for their shared inhibition of adipogenesis and myogenesis in mesodermal derived cells. However, bone morphogenetic protein receptors may be unique in activating osteogenic signalling pathways.

Recently, the type I receptor protein binding to bone morphogenetic proteins-2, -4, and -7 has been identified as the human ALK-3 gene; its murine homolog has been named BRK-1 [ten Dijke et al., 1994c; Koenig et al., 1994]. Ongoing studies have detected the ALK-3 homolog mRNA in BMS2 cells (Wu et al., unpublished observations). Future studies will focus on the BMS2 bone morphogenetic protein receptor complex to explore the mechanism underlying the unique actions of these important cytokines.

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