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Selective synthesis of bone morphogenetic proteins-1, -3, -4 and bone sialoprotein may be important for osteoinduction by Saos-2 cells

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Abstract An ability to induce new bone formation at a required site would represent a considerable advance in bone repair and tissue engineering. It has been shown that the healing of critical-size bone defects in rats can be augmented by extracts of Saos-2 cells. These human osteosarcoma cells uniquely contain a bone-inducing activity, whereas other human osteosarcoma cells, e.g., U-2 OS cells, cannot replicate the osteoinductive capacity. To understand the necessary components of the Saos-2 bone-inducing activity, this study compared osteoinductive Saos-2 cells with non-osteoinductive U-2 OS cells with respect to the synthesis of bone morphogenetic proteins (BMPs)-1, -2, -3, -4, -5, -6, and -7 and the non-collagenous matrix proteins bone sialoprotein (BSP), osteonectin (ON), osteopontin (OPN), and osteocalcin (OC). The main differences were abundant synthesis of BMP-1/tolloid, BMP-3, -4, and BSP by Saos-2 cells, but absence or reduced synthesis in U-2 OS cells. BMP-2 and -7 were present in low amounts in both cell types, while BMP-5 and -6 were more abundant in U-2 OS cells, suggesting that these BMPs were of lesser importance for the osteoinductivity of Saos-2 cells. However, a relatively high expression of BMP-3 and -4, together with BMP-1/tolloid, may be important for the osteoinductive capacity of Saos-2 cells. The inability of U2-OS cells to induce bone, despite expressing most of the BMPs, may be due to an insufficiency of tolloid, BMP-3 or -4, BSP, and/or other unknown factors. A better understanding of the necessary components of the Saos-2 cell bone-inducing agent may, in future, lead to clinically useful Saos-2 cell products for bone repair and tissue engineering.

Key words bone morphogenetic proteins · bone sialoprotein · osteoinduction · tolloid · Saos-2 cells

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Introduction

There has been considerable interest in factors that induce new bone formation, ever since the discovery that ectopic bone could be induced in muscle tissue after the implantation of cultured human amniotic cells [1] or demineralized bone matrix [2]. The importance of the bone morphogenetic proteins (BMPs), members of the transforming growth factor-β superfamily [3], has been well established. BMPs are synthesized as precursor forms, which are then cleaved at the carboxy-terminal regions to yield mature proteins of around 130 amino acids. Active BMPs are dimers, either homodimers or heterodimers, of two different BMPs. These dimers bind to one of two types of serine/threonine kinase receptor [4,5] located in the plasma membrane, which results in intracellular signaling by Smad proteins, ultimately leading to changes in the transcription of specific target genes [6].

All BMPs, except BMP-1, have a high amino-acid sequence identity in the carboxy-terminal region, sharing a conserved pattern of seven cysteine residues [3]. Based on this structural similarity, BMPs fall into several groups, which may also reflect their functional similarities. BMP-2 and -4 are 92% identical in the 7-cysteine region and have almost identical functions, in that either or both can initiate the commitment of mesenchymal cells to osteogenic-chondrogenic precursor cells [7,8]. BMP-5, -6, and -7 share an 89% amino-acid sequence identity. BMP-3 and -3b form another group. BMP-1 and its spliced variant, tolloid, are not members of the TGF-β superfamily, but have procollagen C-proteinase activity [9]. Nevertheless, BMP-1/tolloid may be important for BMP-induced bone formation, because they can activate BMPs [10,11].

With the availability of recombinant human BMPs, several BMPs have been used to enhance bone regeneration in animal models, especially BMP-2 and -7 (OP-1) (reviewed in reference [12]). However, recombinant

BMPs are still expensive, and 10 to 1000-fold higher concentrations of recombinant than of native BMPs are required to produce the same osteoinductive effect [13,14]. In addition, there are considerable variations in the responses to BMPs, depending on the species in which BMPs are implanted, the implantation site, and the type of BMP implant [14–16].

The question of which combination of BMPs, possibly interacting with other factors, is required for consistent and repeatable bone induction is not yet solved. To identify the optimal combination of BMPs required for efficient bone induction, the present study utilized the fact that devitalized cells, extracts, or secretions from one type of osteosarcoma cell, the Saos-2 cell line, are uniquely capable of inducing new bone formation when implanted subcutaneously in Nu/Nu mice [17–19]. On the other hand, U-2 OS and TE85 human osteosarcoma cells, and UMR-109 and ROS 17/2.8 rat osteosarcoma cells were not osteoinductive when tested in parallel with Saos-2 cells [17,18]. The bone-inducing capacity was also found in secretions of Saos-2 cells in conditioned culture media [19,20]. Bone formation, induced by implants of Saos-2 cell products, recapitulated skeletal embryonic development in the same manner as the implantation of demineralized bone matrix, with cartilage arising at 7 days post-implantation from a blastema-like aggregation of mesenchymal osteoprogenitor cells, and with trabecular bone and marrow replacing the cartilage by 14 days [17]. After 3 to 4 weeks, the osteoinductive stimulus was exhausted. Resorption exceeded formation, and the induced bone was completely resorbed after about 10 weeks, unless measures were taken to replenish the osteoinductive stimulus or to inhibit osteoclastic activity [21].

We hypothesized that osteoinductive Saos-2 cells differentially expressed an optimal admixture of BMPs and other factors when compared with non-osteoinductive U2-OS cells. Previous studies compared the gene expression of BMP-1 through -7, as well as TGFβ1, in Saos-2 versus U2-OS cells [22]. Both types of cells expressed significant mRNA levels of BMP-2, -4, and -6, and TGF-β1. The non-osteoinductive U-2 OS cells also expressed BMP-5 and -7, whereas the Saos-2 cells expressed higher levels of BMP-1 and -3 mRNAs. The absence of osteoinductivity in U-2 OS, despite the expression of several BMPs, could have resulted either from a lack of translation of selective mRNAs into BMP proteins, or from the lack of processing of the propeptides into the active proteins. Therefore, in the present study, Western blot protein analyses were carried out on Saos-2 versus U-2 OS cells to determine whether BMP synthesis followed gene expression, and to assess relative levels of protein expression. We also analyzed relative BMP protein levels in the subcellular, particulate retentate fraction of Saos-2 conditioned media. Proteins extracted from these microsome-sized Saos-2 media particles, which are retained by a 0.45-μm filter (hence the name, "retentate"), had recently been shown to exhibit greater osteoinductive ability per milligram of protein than did freeze-dried Saos-2 cells or conditioned media filtrate proteins [19]. In addition, we compared levels of the non-collagenous matrix proteins bone sialoprotein (BSP), osteonectin (ON), osteocalcin (OC), and osteopontin (OPN) in Saos-2 cells and retentate versus U-2 OS cells.

Our results show that Saos-2 cells and retentate exceeded U2-OS cells in synthesized amounts of BMP-1/tolloid, BMP-3, and BMP-4, and BSP, while U-2 OS cells appeared to contain higher levels of BMP-5 and -6. Extracts from the particulate retentate fraction of Saos-2 cell-conditioned media consistently showed higher levels of BMP-1/tolloid, BMP-3 and BMP-4, and BSP, than did either Saos-2 or U-2 OS whole cell lysates. These differences may account for the selective ability of Saos-2 cells and media to induce ectopic bone.

Materials and methods

Cell culture

Saos-2 and U-2 OS cells were grown in Dulbecco's minimal essential medium (DMEM) or McCoy's 5A medium (for U-2 OS cells), plus 10% fetal calf serum [17]. At confluence, the cultures were washed once in Hank's balanced salt solution (BSS) and then maintained on serum-free medium for 48 h before harvest. Confluent Saos-2 and U-2 OS cells were harvested and lysed by scraping with rubber policemen, then lyophilized, and stored at −20°C prior to bioassay.

Serum-free Saos-2 cell-conditioned medium was passed through 0.45-μm pore-size filters after precentrifugation at 1000 RPM for 20 min to remove and discard large cell fragments. Microsome-sized cell fragments retained by 0.45-μm filters ("retentate") were extracted into 6 M urea for 7 days at 4°C. The extract was dialyzed against H_2O to remove urea, and lyophilized prior to storage at –20°C. The proteins of the "retentate" had previously been shown to be more osteoinductive per milligram of protein than were Saos-2 whole cell lysates [19].

Bioassay for osteoinduction

Ten-milligram pellets of freeze-dried, fragmented Saos-2 cells or U-2 OS cells were implanted subcutaneously for 14 days in Nu/Nu mice to bioassay osteoinductive ability, as previously described [17]. To construct media protein implants for bioassay, 1–3 mg of sample protein was mixed with 2 mg of purified bovine collagen in 40 μl of 0.01 N HCl. After the mixing, and the addition of

300 µl of 0.1 M, pH 7, sodium phosphate buffer to reach a neutral pH, the samples were lyophilized, compressed into tablet form in a tuberculin syringe, and stored at –20°C until implantation into Nu/Nu mice. All implants were placed subcutaneously, adjacent to the latissimus dorsi muscles of the backs of anesthetized Nu/Nu mice (Charles River Laboratories, Boston, MA, USA) and the skin was closed by stainless steel staples. The Animal Care and Use protocol used (ACUP94-06-0100) was reviewed and approved by the Kansas University Medical Center (KUMC) Institutional Animal Care and Use Committee.

At 14 days, the Nu/Nu mice were anesthetized with ether and Killed by cervical dislocation. The implants were removed and bisected, and one-half of each implant was fixed for 24 to 48 h in 10% phosphate-buffered formalin, embedded in paraffin, sectioned, and stained by conventional histologic methods, with or without acid decalcification. Light-microscopic sections were evaluated for the presence of cartilage, bone, and marrow. The other half of the implant was homogenized in deoxycholate for the measurement of protein [23] and alkaline phosphatase. The latter was assayed by measuring the rate of release of inorganic phosphate from *p*-nitrophenylphosphate at room temperature, spectrophotometrically at 410 nm [24].

Western blotting for BMPs and non-collagenous matrix proteins

Freeze-dried, fragmented Saos-2 cells, U-2 OS cells, or Saos-2 media retentate (labeled S-R) were dissolved in 1 µg protein/µl sample buffer (0.06 M Tris-HCl, pH 6.8, containing 10% glycerol, 2% sodium dodecylsulfate (SDS), 5% 2-mercaptoethanol, and 0.5% bromphenol blue) and heated at 95°C to denature. Samples, 15 to 30 µg each, were then electrophoresed at 200 V (25–50 mA) for 30 min in SDS running buffer (25 mM Tris, pH 8.3, 190 mM glycine, and 0.1% SDS), using 12% Biorad (Richmond, CA, USA) precast minigels and electrophoretically transferred at 100 V (200–250 mA) to nitrocellulose membranes in transfer buffer (25 mM Tris base, pH 8.3, 190 mM glycine, 0.1% SDS, and 20% methanol). After three washes in TBS-Tween-20 buffer (10 mM Tris, pH 8.0, 0.15 M NaCl, 0.005% Tween-20) (TBS-T), the membranes were blocked with 10% non-fat milk in TBS-T, washed in TBS-T, incubated in primary antibody for 1 h (at the dilutions indicated below), washed again in TBS-T, incubated in secondary antibody labeled with horseradish peroxidase (HRP) for 20 min to 1 h, and washed again in TBS-T. The bound HRP was immunodetected by enhanced chemiluminescence (ECL), using reagents and procedures obtained from Amersham Life-Science. The following primary antibodies were used: anti-BMP-1/tolloid rabbit antibody (1:1000), a polyclonal antibody from Dr. Paul Reynolds, University of Rochester (New York); anti-BMP-2 (W 12), anti-BMP-3 (W 22) and anti-BMP-7 (W 32) (1:100), polyclonal antibodies provided by Dr. I.K. Moutsatsos, Genetics Institute; anti-BMP-4 (1:100) a monoclonal IgG antibody from Dr. K. Masuhara, Osaka University [25]; anti-BMP-5 (1:100), a polyclonal antibody from Santa Cruz Biotechnology, Santa Cruz, California; anti-BMP-6 (1:250), a polyclonal antibody for the precursor form of BMP-6, provided by Dr. S. Gitelman, University of California, San Francisco [26]; and anti-BSP, ON, OPN, and OC, polyclonal antibodies provided by Dr. Larry W. Fisher, National Institutes of Health (NIH), National Institute of Dental and Craniofacial Research (NIDCR) [27].

Fluorescence immunohistochemistry for BMPs

Cultured monolayer cells were rinsed with phosphate-buffered saline (PBS), then fixed in 60% cold acetone for 5 min and air-dried at room temperature (RT). The cells were blocked with normal goat serum for 20 min at 37°C. After being thoroughly washed in PBS, the cells were incubated with a 1:200 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit or anti-mouse immunoglobulin, as a secondary antibody, for 1 h at RT. The cells were then washed thoroughly, mounted in 90% glycerol and 10% PBS solution, and viewed with a fluorescence microscope. As controls, cells were treated with non-immune goat, rabbit, or mouse serum, or with the secondary antibody alone.

Immunohistochemistry for BMPs

Paraffin sections from 4% paraformaldehyde-fixed 7-day old implants of freeze-dried Saos-2 cells were deparaffinized in xylene, rehydrated in 95% ethanol, and permeabilized in 0.3% Triton-X100. Endogenous peroxidase was inhibited by incubation in 3% hydrogen peroxide. The sections were then blocked with Dako (Carpinteria, CA, USA) blocking solution, reacted with appropriate dilutions of primary antibody overnight in a humidified chamber, and incubated in Dako link solution. Secondary antibody, labeled with streptavidin peroxidase was applied for 15 min, followed by Dako 3,3′-diaminobenzidine chromogen solution, washing in distilled water, and counterstaining with 0.5% toliudine blue in 10% ethanol.

Results

Bone-inducing effects of Saos-2 cell products

Seven days after the in-vivo implantation of devitalized Saos-2 cell fragments, positive BMP-4 immunostaining

was still present in the Saos-2 cell debris (on the left in Fig. 1A). After 14 days, an ossicle of new bone had formed in the muscle (Fig. 1B), and BMP-4 was present in responding host mesenchymal cells undergoing chondro-osseous differentiation (Fig. 1B). No staining was seen in negative controls (Fig. 1C).

Synthesis of BMP-2 in both Saos-2 and U-2 OS cells, but diminished BMP-3 and absence of BMP-4 in U-2 OS cells

Moderate levels of BMP-2, at an expected molecular weight (Mr) of 18 kDa [28], were observed in the Saos media retentate (S-R), Saos-2 cells (S), and U-2 OS (U) cells, at about equal intensities (Fig. 2A). High levels of BMP-3, at an expected Mr of 16 to 18 kDa [29], were detectable in Western blots in Saos-2 cells, and increased amounts were present in the "retentate" (Fig. 2B). Although some BMP-3 was present in U-2 OS cells, the levels were low, and several bands appeared on the Western blots, suggesting alternatively spliced products (Fig. 2B). BMP-4, at an expected Mr of 17 to 18 kDa [25], appeared as two bands, at approximately 17 to 18 kDa, with an especially strong 18-kDa band in the Saos-2 media retentate, and just a trace in Saos-2 cells (Fig. 2C, Fig. 4C). BMP-4 protein was not detected at all in U-2 OS cells.

Comparison of BMPs-5, -6, and -7 in Saos-2 and U-2 OS cells

BMP-5, an apparent dimer at approximately 30 kDa [30], was more intense in U-2 OS cells than in Saos-2 cells or retentate (Fig. 2D). Higher levels of the precursor form of BMP-6, at an expected Mr of 69 kDa [26], were detected in U-2 OS cells. It is noteworthy that both BMPs-5 and -6 were more abundant in the non-osteoinductive U-2 OS cells than in Saos-2 cells. Interestingly, the more osteoinductive retentate fraction contained lower levels of BMP-5 and -6 than did the Saos-2 cells. BMP-7, at an expected Mr of 16 kDa [31], was detected in low amounts in both Saos-2 and U2 OS cells. Apparent dimeric BMP-7, at approximately 30 kDa, was moderate in Saos-2 cells, with a trace detectable in U-2 OS cells. Immunoreactive bands were seen at unexpected molecular weights in some of the Western blots. BMP-2 and -7 showed an additional weak band at approximately 34 kDa (Fig. 2A,F). These may represent dimeric forms.

Relative abundance of BMP-1/tolloid in Saos-2 cells and absence of tolloid in U-2 OS cells

BMP-1 was present, at an expected Mr of approximately 85 kDa [10], in Saos-2 retentate and Saos-2 cells, and a trace was present in U-2 OS cells (Fig. 3). Tolloid, a 100-kDa protein which shares considerable homology with BMP-1, with an expected Mr of approximately 100 kDa [11], was absent from U-2 OS cells. Tolloid was, however, moderate in Saos-2 cells and further enriched in the retentate fraction of Saos-2 cell-conditioned medium (Fig. 3).

The presence of BMP-1/tolloid protein in Saos-2 cells was confirmed by immuno-fluorescence, which revealed BMP-1/tolloid in the cytoplasm of Saos-2 cells (Fig. 4A). U-2 OS cells, on the other hand, did not stain for BMP-1/tolloid (Fig. 4B). The presence of BMP-4 in Saos-2 was also confirmed by immunofluorescence (Fig. 4C).

Table 1 compares the relative abundance of tolloid and BMPs-1 through -7 in Saos-2 "retentate" versus whole Saos-2 cells and U-2 OS cells. The "retentate" fraction of Saos-2 conditioned media contained more abundant tolloid and BMP-1 and -4 than did freeze-dried Saos-2 cells. On the other hand, the levels of BMP-5 and -6 were reduced in the "retentate" compared with findings in freeze-dried Saos-2 cells and U-2 OS cells, in which BMP-5 and -6 were abundant. Both Saos-2 cell media retentate and Saos-2 cells contained more BMP-1, -3, and -4 and tolloid than did U-2 OS cells. The "retentate", which is composed of microsome-sized vesicles and other subcellular elements, has shown consistently higher osteoinductivity when implanted into Nu/Nu mice [19].

Synthesis of bone sialoprotein (BSP), osteonectin (ON), osteopontin (OPN), and osteocalcin (OC)

Saos-2-conditioned media retentate contained high levels of BSP, at approximately 80 kDa (Fig. 5) and ON at approximately 40 kDa (data not shown), versus only trace amounts of these proteins in Saos-2 cells and U-2

Table 1. Relative abundance of bone morphogenetic proteins (BMPs) estimated by Western blots in Saos-2 cell-conditioned culture medium retentate (microsomal particulate fraction) versus Saos-2 and U2-OS cells

	Saos-2-conditioned media retentate	Saos-2 cells	U-2 OS cells
BMP-1	+++	++	+
Tolloid	++	+	−
BMP-2	+	+	+
BMP-3	+++	++	+
BMP-4	+++	+	−
BMP-5	+	++	+++
BMP-6	+	++	+++
BMP-7	+	+	+

+++, Maximal; ++, moderate; +, trace; −, no protein

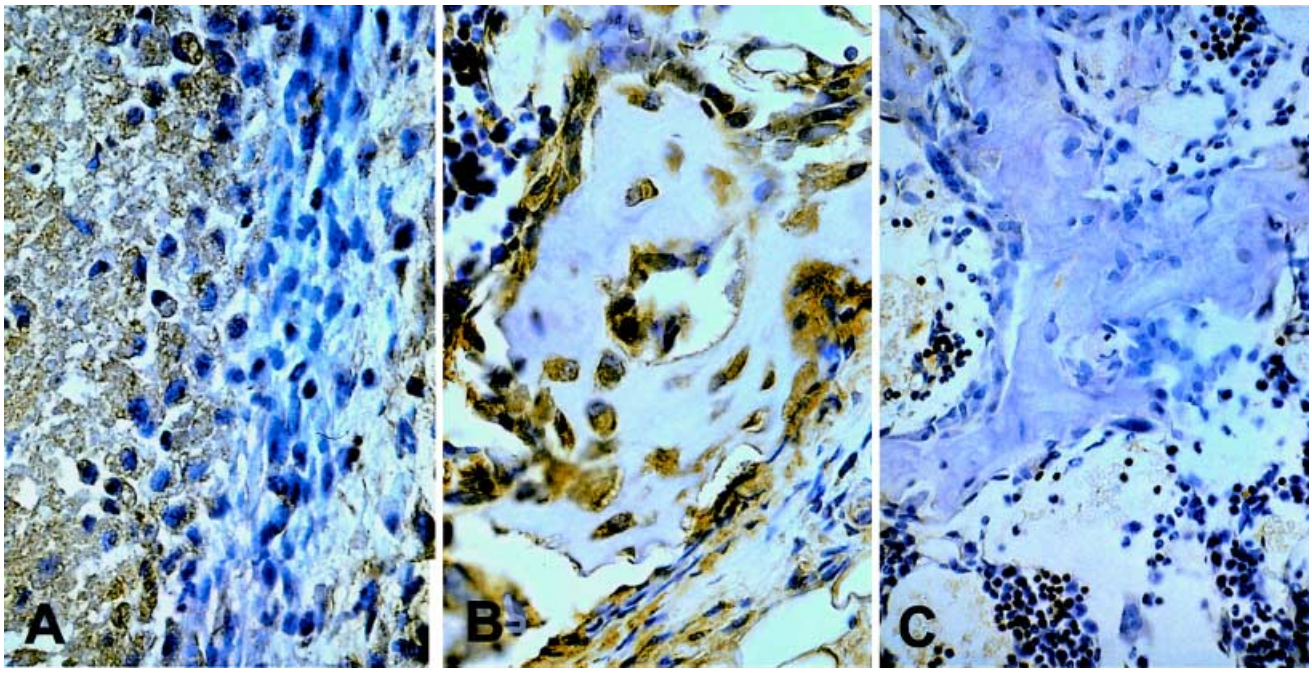

Fig. 1. Immunohistochemical stains for bone monphogenetic protein-4 (BMP-4) in sections from implants of freeze-dried Saos-2 cells. **A** Implant after 7 days. *On the left*, BMP-4 staining is moderate in the devitalized Saos-2 cell debris. Mesenchymal cells, which will undergo chondro-osseous differentiation, at the perimeter of the Saos-2 implant (*right*), also show a trace of BMP-4 staining in their cytoplasm. **B** Ectopic bone and marrow formed at the perimeter of a 14-day Saos-2 cell implant. Osteoblasts and osteocytes stain positively for BMP-4. **C** Nonimmune serum negative control of 14-day implant. ×625

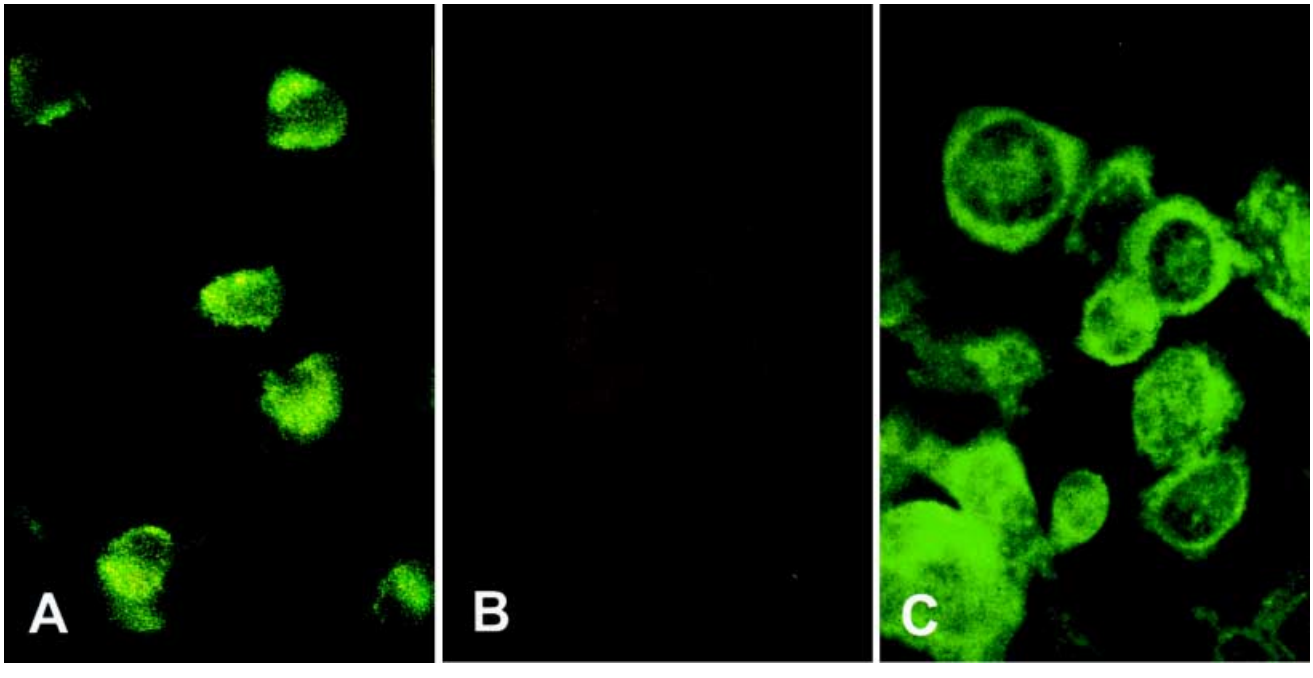

Fig. 4. Immunofluorescent cytochemistry for BMP-1 and -4 in monolayers of Saos-2 and U-2 OS cells. **A** Saos-2 cells, stained for BMP-1/tolloid, show predominantly cytoplasmic fluorescence. **B** U-2 OS cells, stained for BMP-1/tolloid, lack detectable staining. **C** Saos-2 cells, stained for BMP-4, also show predominantly cytoplasmic staining. ×1000

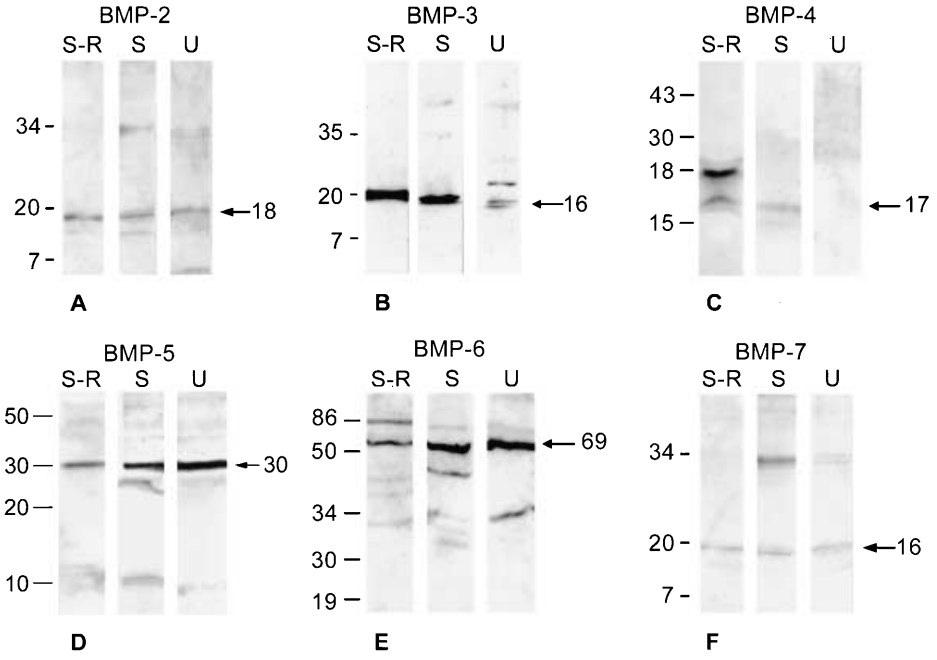

Fig. 2. A–F Western blots for BMP-2, -3, -4, -5, -6, and -7 in the Saos media retentate (*S-R*), Saos-2 cells (*S*), and U-2 OS (*U*) cells. The expected approximate molecular weights (in kDa) are indicated with an *arrow* on the right of each blot; the *numbers* on the left are molecular weight markers. The greatest differences between the nonosteoinductive U2-OS cells and the Saos-2 cells were in the amounts of BMP-3, -4, -5 and -6, with BMP-3 and -4 being particularly high in the more osteoinductive Saos-2 culture media "retentate"

Table 2. Relative abundance of noncollagenous matrix proteins, bone sialoprotein, osteonectin, osteopontin, and osteocalcin, estimated by Western blots, in the Saos-2 cell particulate media fraction versus Saos-2 and U-2 OS cells

	Saos-2-conditioned media retentate	Saos-2 cells	U-2 OS cells
Bone sialoprotein	+++	+	±
Osteonectin	+++	−	+
Osteopontin	++	++	++
Osteocalcin	±	++	+

+++, Maximal; ++, moderate; +, trace; ±, faint trace

OS cells (Table 2). OPN was detected at 60kDa in all groups (Table 2). Only a faint trace of 8kDa OC was visible in the Saos-2-conditioned media retentate and U-2 OS cells a while a moderate amount of OC was detectable in Saos-2 (Table 2).

Discussion

An important question with respect to bone repair or bone tissue engineering is whether a combination of BMPs will be the more cost-effective in clinical practice than a single recombinant BMP. Although individual BMPs, especially recombinant BMP-2 [3,32,33] or recombinant BMP-7 [34], have been used successfully to accelerate bone regeneration in large defects, the concentrations needed for a specific recombinant BMP are up to 1000-fold higher than those needed for the native BMP complex [13]. This suggests that the right combination of several BMPs might be more cost-effective for enhancing new bone formation than individual recombinant human BMPs.

By comparing the differential synthesis of BMPs and non-collagenous proteins in osteoinductive Saos-2 cells versus non-osteoinductive U-2 OS cells, the present study was able to identify some possible components of the osteoinductive agent of Saos-2 cells that are relatively highly synthesized by Saos-2 cells.

Possible involvement of BMP-1/tolloid and BMP-2, -3, and -4 in Saos-2 cell induction

Higher intracellular levels of BMP-1/tolloid and BMP-3 and -4 correlated with osteoinduction by Saos-2 cells. BMP-4 was synthesized by Saos-2 cells, and its quantity was increased in the more highly osteoinductive Saos-2 cell "retentate", whereas this protein was not detectable in U-2 OS cells, even though previous work had shown that U-2 OS cells expressed the mRNAs for BMP-4 [22,35]. BMP-4 was also highly expressed by Dunn

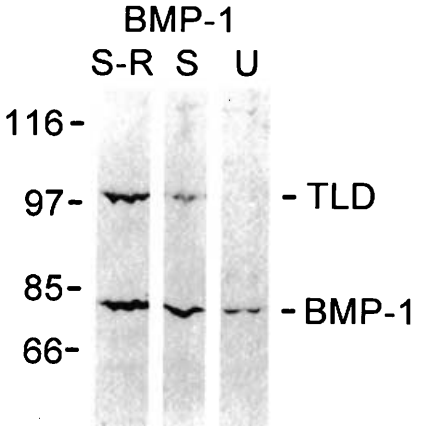

Fig. 3. Western blot for BMP-1/tolloid. BMP-1, at an expected molecular weight (Mr) of approximately 85 kDa, and tolloid (*TLD*), at approximately 100 kDa [10], in Saos media retentate (*S-R*), Saos-2 cells (*S*), and U-2 OS cells (*U*). Nonosteoinductive U-2 OS cells demonstrated moderate levels of BMP-1 protein, but apparently lacked tolloid, a protein that shares considerable homology with BMP-1 [11]. Tolloid also was strongly stained in the particulate retentate fraction of Saos-2 cell-conditioned medium and Saos-2 cells

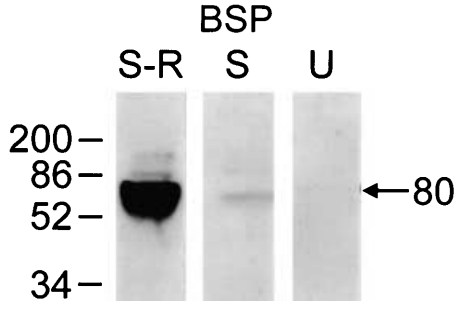

Fig. 5. Western blot for bone sialoprotein (*BSP*). A high level of bone sialoprotein, at an expected Mr of approximately 80 kDa [71], was present in the particulate Saos-2 media retentate (*S-R*). Only a trace of BSP was detected in Saos-2 cells (*S*), and none was detected in U-2 OS cells (*U*)

murine osteosarcoma cells, which induced bone when implanted into co-isogenic mice [36]. These findings suggested that BMP-4 synthesis was an important component of the osteoinductive capacity of Saos-2 cells.

BMP-2, on the other hand, was also synthesized by the non-osteoinductive U-2 OS cells, yet this did not confer osteoinductive capacity. In previous studies, BMP-2 alone has been shown to be sufficient for osteoinduction [37] and recombinant BMP-2 is the most widely used BMP in preclinical and clinical trials [32,33,37–42].

One possible reason for the non-osteoinductivity of U2-OS cells may be the complete absence of tolloid and the reduced synthesis of BMP-1 in U-2 OS cells compared with Saos-2 cells. BMP-1/tolloid belong to the astacin family of metalloproteinases [11,43,44], which have pro-collagen C-proteinase activity [9,43], i.e., they can cleave the pro-peptide of pro-collagens I, II, and III to yield the mature triple helix. However, the function that is most relevant in the present context is the ability of BMP-1/tolloid to promote the activity of some BMPs. BMP antagonists, such as chordin and noggin, are usually synthesized together with BMP-2 or -4, and prevent BMP-receptor interaction [45–47]. Cleavage of these antagonists by BMP-1/tolloid can liberate the active BMPs [48,49]. It is possible that the lack of osteoinduction in U-2 OS cells in spite of the presence of BMP-2 could be related to a lack of BMP-inhibitor removal, due to the absence of tolloid and/or the reduced presence of BMP-1.

BMP-3 was present in large amounts in Saos-2 cells and "retentate", whereas the Western blot for BMP-3 of U-2 OS cells showed several faint bands, suggesting differences in post-translational modification or alternatively spliced products. Previous work has shown that BMP-3 can be osteoinductive in vivo [50,51]. Also, BMP-3 inhibited proliferation, and stimulated differentiation of cultured marrow stromal cells [52]. Taken together, these data suggest that BMP-3 synthesis by Saos-2 may enhance osteogenic differention. However, a recent report by Daluiski et al. [53] indicates that BMP-3 can inhibit the osteogenic effect of BMP-2 in cell culture, and that BMP-3 is a negative regulator of postnatal bone density. Furthermore, a recent study by Takao et al. [54] did not detect osteoinductive activity in recombinant human BMP-3b. Thus, there is conflicting evidence regarding the ability of BMP-3 to stimulate bone formation. It is possible that BMP-3 may have either a positive or a negative effect on bone development, as is the case with BMP-4, depending upon the experimental or developmental conditions. Although BMP-4 is widely accepted to be an osteoinductive agent, during morphogenesis, BMP-4 can promote the apoptosis of interdigital mesenchymal cells as a part of digital development [55], and, recently, BMP-4 has been shown to inhibit ductal budding and branching during morphogenesis of the prostate [56]. Thus, BMPs, such as BMP-3, may have dual roles in bone development.

BMP-5, -6, and -7 are unlikely to be crucial for osteoinduction by Saos-2 cells

Although BMP-5, -6, and -7 were synthesized by Saos-2 cells, these proteins were more abundant in the non-osteoinductive U-2 OS cells. This lack of association between osteoinductivity and BMP-5, -6 and -7 suggested that these BMPs were not required for the osteoinductive capacity of Saos-2 cells, although their

presence may still contribute to the formation of new bone. The low quantities of BMP-7 in Saos-2 cells and retentate was of particular interest, because recombinant BMP-7 has previously been used to induce new bone [57] or accelerate fracture healing [34]. In some experimental models of osteoinduction, BMP-7 can substitute for BMP-2/4 in their absence [31].

Significance of bone sialoprotein for osteoinduction

The media retentate fraction was also found to be an abundant source of non-collagenous bone matrix proteins, especially bone sialoprotein (BSP) and osteonectin (ON) (Fig. 5 and Table 2) [58]. This new finding is consistent with recent reports indicating that Saos-2 cells show advanced osteoblastic differentiation, characterized by the secretion of a phenotypic bone matrix containing type I collagen [59], and by the release of mineral-initiating matrix vesicles [60].

Our finding of diminished BSP in U-2 OS cells, as contrasted with strong expression by Saos-2 retentate and, as previously observed, strong expression in maturing hypertrophic chondrocytes, osteoblasts, and odontoblasts during bone and tooth development [61], suggests that the U-2 OS cells are less mature than Saos-2 cells, i.e., that U-2 OS cells are not as far along the pathway as Saos-2 cells to full osseous differentiation. Bone sialoprotein (BSP) is known to promote bone matrix mineralization [62,63]. Recently, BSP has been linked to the promotion of osseous differentiation and bone repair [64–66]. Thus, the above demonstration of selective BSP expression by osteoinductive Saos-2 cell media retentate may indicate that BSP has a positive function in Saos-2 cell bone induction and bone morphogenesis.

Optimal admixture of BMPs and other factors for osteoinduction

The present study suggested that the combination of BMP-1/tolloid, BMP-3 and -4, and BSP was important for the osteoinductive capacity of Saos-2 cells. It is possible that Saos-2 cells contain other, as yet unrecognized, non-BMP-related osteogenic factors or cofactors that are required for bone induction. An example of such a factor could be sonic hedgehog, a non-BMP protein that interacts with BMPs during embryonic development [67], and that was shown to be capable of initiating in-vivo bone formation without the addition of BMP [68].

In future, it may be possible to isolate and utilize an effective combination of BMPs and other proteins that are present in extracts of cultured Saos-2 cells to promote the clinical healing of bone defects [69] and/or to generate new bone for tissue engineering. There are several advantages in using this source of the bone-inducing agents. The Saos-2 cells can be grown in mass culture, virtually indefinitely, to produce large quantities of an active combination of osteoinductive factors at a fraction of the cost of the individual recombinant proteins. Although Saos-2 cells are osteosarcoma cells, they are non-tumorigenic and do not survive after implantation into immunosuppressed hosts [70]. Moreover, living Saos-2 cells are not required or even optimal for bone induction. Attempts to purify the osteoinductive activity of Saos-2 cells have so far been only partially successful, possibly because the activity resides not in a single factor, but in an optimal combination of factors, which includes tolloid, BMP-1, -3, and -4, and BSP, as well as other unknown components.

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