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THE BONE-INDUCING AGENT IN SAOS-2 CELL EXTRACTS AND SECRETIONS

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Abstract

An extractable bone-inducing agent (BIA) is present in lysates of Saos-2 cultured human osteosarcoma cells. Recently it was observed that: (1) Saos-2 cells secrete BIA into their conditioned medium as well as retaining it intracellularly, and (2) Saos-2 cells express bone morphogenetic proteins (BMPs)-1, 2, 3, 4 and 6. Any or all of these BMPs (plus possible unknown factors) may explain Saos-2 cell bone induction. However, we also found that U20S human osteosarcoma cells (comparable to Saos-2) also express high levels of BMPs, but are non-osteoinductive. These data suggest that Saos-2 cells may express a unique combination of morphogenetic factors and/or previously unidentified osteoinductive molecule(s).

Key Words: Bone-induction, bone morphogenetic proteins (BMP), cartilage differentiation, osteogenesis, Saos-2 cells.

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Background

Our research group recently has uncovered a bone-inducing agent (BIA), obtained from cell lysates and extracts of Saos-2 cultured human osteosarcoma cells (Anderson, 1994; Anderson *et al*., 1992). Saos-2 cell-derived BIA can induce endochondral bone formation in the soft tissues of recipient animals where bone does not normally occur. Cartilage appears at approximately 1 week after subcutaneous implantation of BIA-containing Saos-2 cell products, with bone and marrow replacing the cartilage at 10 to 14 days (Fig. 1). Electron microscopy of induced ectopic cartilage and bone shows normal chondrocytes and osteoblasts with prominent rough endoplasmic reticulum and Golgi complexes. The induced matrix is a biphasic admixture of components, some with randomly dispersed small collagen fibrils and proteoglycan granules typical of cartilage (Fig. 2), and some, with broader collagen fibrils typical of bone (Fig. 3). Many clusters of matrix vesicles showing early stages of apatite deposition within and at their surfaces, are present in both the osseous and cartilaginous matrices (Figs. 2 and 3). Thus, bone induction, initiated by Saos-2 cell products, recapitulates the multifactorial process of endochondral bone formation as seen in embryonic limb development (Anderson and Reynolds, 1973), and at fracture repair sites.

The osteoinductive ability of Saos-2 cells is unique. None of the other cultured human or rodent osteosarcoma cell lysates we tested, including U20S, MG-63 and TE85 (of human origin) or UMR-106 (of rat origin), were osteoinductive when bioassayed in the Nu/Nu mouse (Anderson *et al*., 1992; Raval *et al*., 1996a).

Characteristics of the Saos-2 osteosarcoma cells are as follows: (1) They were originally cultured from an osteosarcoma of an 11 year old female human patient (Fogh and Trempe, 1975); (2) they can be propagated in mass culture using methods already available; (3) they have a hyperdiploid to hypotetraploid karyotype and do not form tumors in nude mice (Fogh and Trempe, 1975; Scotlundi *et al*., 1993); (4) they are osteoinductive either as cell lysates or as extracts (Anderson *et al*., 1992); (5) they are enriched in BIA as compared to the trace amount of bone mor-

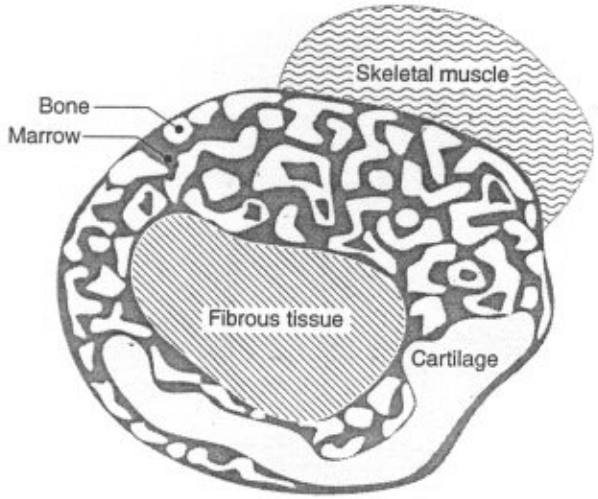

Figure 1. Fourteen day old intramuscular implant of a GuHCl extract of Saos-2 BIA, (microscopic image drawn to scale). The newly formed ossicle is composed mostly of trabecular bone and interspersed marrow. An area of persistent cartilage is present in the lower part of the field. The central area, where most of the BIA extract has been resorbed, is composed of non-osseous, fibrous tissue. [Reprinted from Anderson *et al.* (1995)].

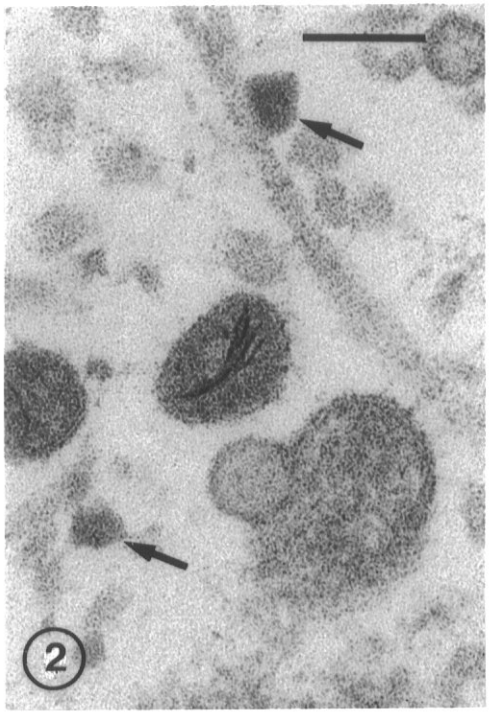

Figure 2 (*at right*). Matrix vesicles in induced cartilage-like matrix showing early intravesicular deposition of needle-like, electron-dense, apatitic mineral. Small, randomly dispersed collagen fibrils are present with adherent droplets of homogenous, electron-dense proteoglycan (indicated by arrows). Electron micrograph; stained with uranyl acetate and lead citrate. [Reprinted from Anderson *et al.* (1992), p. 49]. Bar = 10.5 µm.

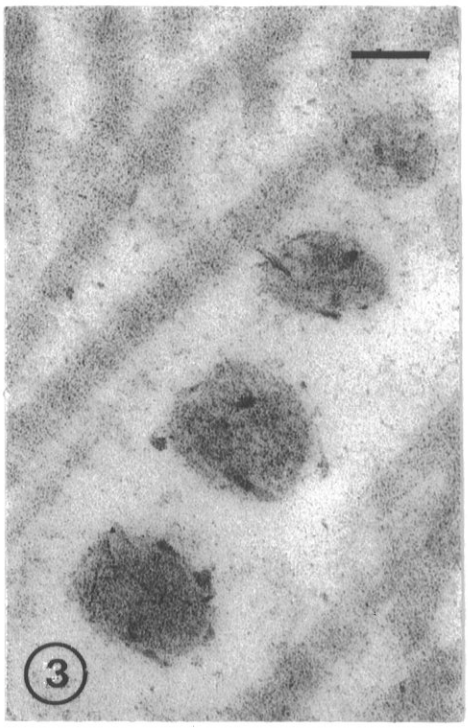

Figure 3 (*at right*). Matrix vesicles in induced osteoid. The vesicle sap contains a homogenous electron-dense, amorphous material, plus faintly visible needle-like apatite deposits. Larger diameter collagen fibrils are evident. Electron micrograph; stained with uranyl acetate and lead citrate. [Reprinted from Anderson *et al.* (1992), p. 49]. Bar = 10.5 µm.

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phogenetic protein (BMP) obtainable from bovine bone (Wozney and Rosen, 1993), only 1.5 mg of GuHCl extract or 10 x 10^6 freeze-dried Saos-2 cells is required for essentially 100% bone induction in nude mice, (Fig. 4); and (6) Saos-2 cells or their extracts are non-toxic and apparently non-carcinogenic in recipient animals. As will be described below, Saos-2 cells express significant levels of several BMPs (Raval *et al.*, 1996a,b).

In recent years, a number of purified bone morphogenetic proteins have been isolated, sequenced, and produced by recombinant technology using transfected animal tissue culture cells. These molecules are designated bone morphogenetic proteins (BMPs)-1 through 13, or,

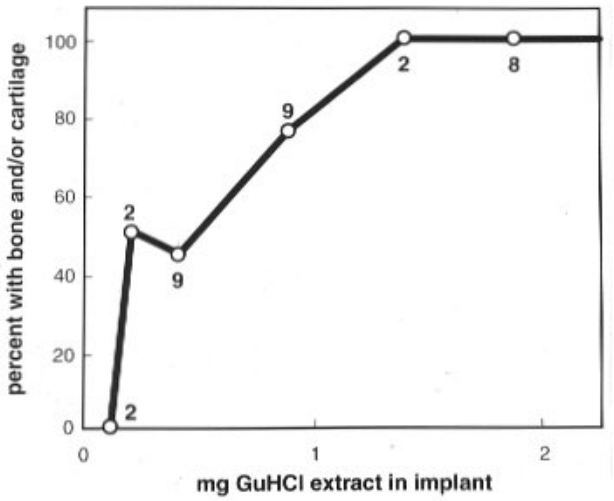

Figure 4. Percent of animals showing ectopic bone and/or cartilage, after receiving an increasing dosage of Saos-2 cell extract, from 0.1 to 2.0 mg per subcutaneous implant. Number of implants tested per dose level are given adjacent to each dosage data point.

alternately, "osteogenin" which is identical to BMP-3 (Luyten *et al*., 1989) and "osteoinductive protein-1" (OP-1) which is identical to BMP-7 (Sampath *et al*., 1990). All the BMPs (except BMP-1) have molecular structures similar to transforming growth factor-beta (TGF-β). Most of the BMPs (except BMP-1, 12 and 13) were reported to be osteoinductive when implanted subcutaneously in combination with extracted, decalcified bone matrix. The latter is an incompletely defined collagenous substance which is believed to function as a slow release vehicle for BMP. However, the possibility remains that trace amounts of one or more unknown osteoinductive cofactors may persist in the extracted bone matrix and interact with exogenously added single BMPs to enhance bone induction (Anderson, 1994). Indeed, some experimental evidence suggests that mixtures of multiple bone growth factors are more active as bone inducers than are single BMPs. The "osteoinductive factor" (OIF), isolated from decalcified bovine bone, turned out to be a naturally occurring heterodimer of BMP-2 and BMP-3, which could only induce heterotopic bone if combined with TGF-β (Bentz *et al*., 1991). The following mixtures of two BMPs were reported to be more active in bone induction than were homodimeric preparations of single BMPs: BMP-2 plus BMP-3 heterodimers (Hammonds *et al*., 1991) and heterodimers of BMP-2/7 (Sampath *et al*., 1990), or BMP-2/5, 2/6 or 4/7 (Israel *et al*., 1996). Also, combinations of BMPs with non-BMP growth factors have been advocated as being therapeutically effective (for a review, see Anderson, 1994). Thus, significant experimental evidence points to a mechanism of bone induction which utilizes a sequential, or cascade-like interaction requiring more than one bone growth factor.

Several of the BMPs are expressed at specific tissue sites and at specific stages of embryonic development. Early in embryogenesis, BMP-1 and BMP-4 are expressed in the central mesoderm and appear to play a role in dorsoventral patterning (Dale *et al*., 1992; Fukagawa *et al*., 1994; Harland *et al*., 1994; Suzuki *et al*., 1995). BMP-2, 3, 4, 6 and 7 mRNAs and proteins have been localized in the craniofacial mesenchyme, in developing limb buds and in tooth germs of mouse embryos (Elima, 1993; Francis-West *et al*., 1994; Heikinheimo, 1994; Jones *et al*., 1991; Lyons *et al*., 1990; Vukicevik *et al*., 1994), suggesting an important role for these BMPs in cartilage and bone formation during embryogenesis. In fracture healing, BMP-4 mRNA is expressed by pre-osseous bone repair cells in early fracture healing (Nakase *et al*., 1994). Therefore, it is logical to predict that one or more of the BMPs could be utilized in future therapy to augment bone development or repair.

Bone-inducing agent (BIA) is secreted by Saos-2 cells

Recent experiments from our lab indicate that Saos-2 cells not only retain bone-inducing activity, but they also secrete BIA into their serum-free conditioned culture medium (Anderson *et al*., 1996). Secreted BIA is present in both the aqueous and particulate fractions of the conditioned culture medium. However, the particulate fraction, which is retained by 0.45 micron pore-size filters, (the "retentate") has a higher specific activity of osteoinductivity than does the soluble filtrate fraction. This retentate, on electron microscopy examination, consists of a mixture of vesicles, resembling matrix vesicles of dentin, cartilage and bone (Anderson, 1995), plus granules resembling ribosomes, and 10 nm microfilaments consistent with cytoskeleton and/or collagen microfilaments (Fig. 5). The presence of vesicles in the osteoinductive retentate raises the possibility that such extracellular matrix vesicles may function as transporters of morphogenetic information as was suggested several years ago by Slavkin *et al*. (1972) in a study of embryonic dentin and enamel development.

The discovery that Saos-2 cells release BIA into their culture media suggests that the conditioned medium from mass cultures could be utilized for large-scale production of BIA, employing methods already developed to produce recombinant proteins for therapeutic use. A continuous flow production system can be visualized in which the Saos-2 cells are stationary and medium is passed slowly over their surfaces for collection and isolation of BIA. We have conducted preliminary (unreported) experiments using gel filtration to concentrate osteoinductive activity from the soluble "filtrate" fraction of conditioned media. Bone-inducing activity was concentrated 5- to 10-fold in fractions containing proteins in the 20 to 70 kDa range (Hsu, unpublished results).

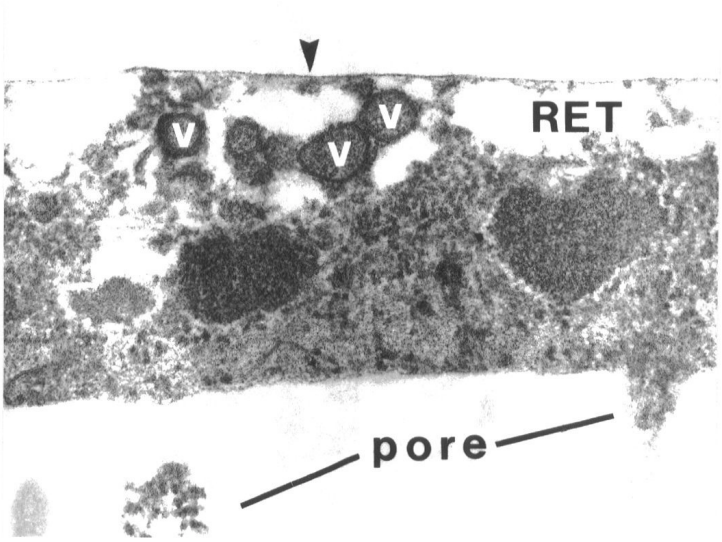

Figure 5. Overall appearance of parti-culate Saos-2 cell conditioned media retentate (RET) on the surface of a 0.45 μm filter. The retentate is composed of vesicles (V), consistent in size and structure with matrix vesicles of skeletal tissues, plus electron dense granules (resembling ribosomes and/or proteoglycan granules) and interspersed, approximately 10 nm diameter filaments (resembling cytoskeletal microfilaments and/or collagen micro-fibrils). An arrowhead indicates the exposed upper surface of the retentate. Photo width = 143 μm. [To be published in Anderson *et al.* (1999)].

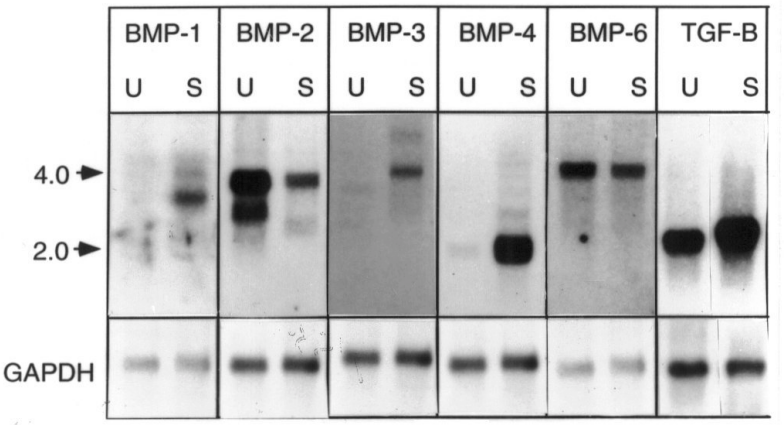

Figure 6. Northern blot analysis of expression levels of BMP-1 through BMP-6 and TGF-β mRNAs in con-fluent Saos-2 cells (S) versus confluent, non-osteoinductive U20S cells (U). Saos-2 cells exceeded U20S cells in expressing BMP-1, 3 and 4. The arrow-heads at left indicate the position of 4.0 and 2.0 kb size markers. The lower panel indicates the level of glyceral-dehyde-3-phosphate dehydrogenase (GAPDH) mRNA used as an invariant mRNA species for normalization of mRNA levels. [Reprinted from Raval *et al*. (1996b)].

Saos-2 cells express BMPs

Experiments were carried out by Raval *et al.* (1996a,b) to determine whether known osteoinductive factors, e.g., the BMPs and/or TGF-β, are expressed by Saos-2 cells. Northern blot analysis of mRNA from confluent cultures revealed strong expression of BMP-1, 2, 3, 4, 6 and TGF-β mRNA by Saos-2 cells (Fig. 6). However, we were surprised to find that U20S human osteosarcoma cells, which are not osteoinductive in Nu/Nu mice, also express mRNAs for BMP-2, 3, 4, 5, 6 and 7 and exceed Saos-2 cell expression of BMP-2, 5, 6 and 7 (Fig. 6).

Western blot analysis confirmed the presence of BMP-1, 2, 3, 4, 5, 6 and 7 proteins in Saos-2 cell lysates, extracts and in conditioned media retentate (Figs. 7 and 8) (Aguilera *et al*., unpublished results). All of the above BMP proteins (except BMP-1/tolloid) were also detectible at significant levels in the non-osteoinductive U20S cells (Fig. 7). Table 1 summarizes and compares relative expression levels of BMP proteins in Saos-2 cells vs. U20S cells. Overall, the levels of individual BMP mRNA and protein appear rather similar for both cell lines with Saos-2 expressing more BMP-1, 3 and 4 and U20S expressing more BMP-2, 5, 6 and 7. Only BMP-1 mRNA appears to be almost exclusively expressed by Saos-2 cells (Raval *et al*., 1996a).

Discussion

Parallels between embryonic bone development and Saos-2 induced bone

First, the process of Saos-2 cell bone induction is endochondral, and faithfully recapitulates the pattern seen in embryonic limb bud development, in which a central population of uncommitted mesenchymal stem cells are induced to chondrify, by inducer molecules released from adjacent cells of the apical epidermal ridge (AER) and zone of polarizing activity (Hogan, 1996).

Second, Saos-2 cells generate and secrete, several

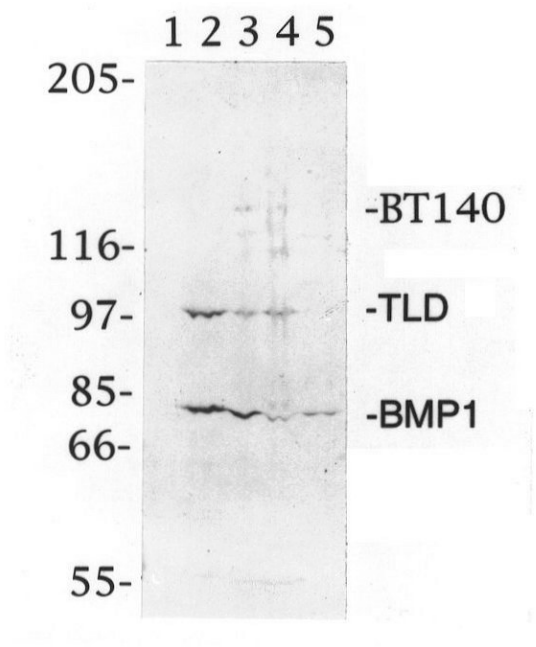

Figure 7. Western blot shows both BMP-1 (84 kDa) and tolloid (97 kDa) proteins in Saos-2 cell conditioned media retentate (lane 2), in GuHCl extract of Saos-2 cells (lane 3) and in acetone extract of Saos-2 cells (lane 4). Non-osteoinductive U20S cells (lane 5) show only a trace of BMP-1 protein while tolloid is undetectable. Molecular weights are indicated in kDa at the ordinate.

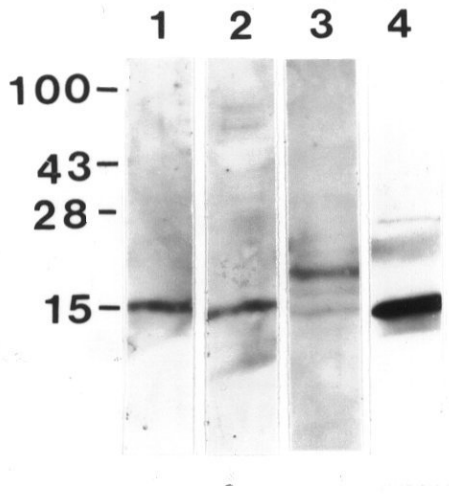

Figure 8. Chemiluminescent western blot shows most immunoreactive BMP-4 at approximately 15 kDa in a GuHCl extract of Saos-2 cells (lane 1), in a 0.45 µm pore size filtrate of Saos-2 conditioned media (lane 2), and in control recombinant human BMP-4 (lane 4). Saos-2 conditioned media retentate (lane 3) shows a prominent immunoreactive band at approximately 18 kDa plus a faint band at 15 kDa.

Table 1. Relative abundance of BMP proteins, estimated by Western blot in Saos-2 cell conditioned culture medium (microsomal particulate fraction) versus Saos-2 and U20S cell lysates.

	Saos-2 Conditioned Media	Saos-2 Cells Particles	U20S Cells
BMP-1	+++	++	++
BMP-2	++	++	++
BMP-3	+++	+++	+
BMP-4	+++	+	-
BMP-6	++	++	+++
BMP-7	++	++	++

+++ = maximal; ++ = moderate; + = trace.

BMPs that are known to be present and believed to function in limb development, facial development and dentinogenesis. The finding of a significant expression of BMP-2, 4 and 7 in the AER cells of limb bud just prior to mesenchymal chondrification has led to the suggestion that these BMPs may be involved in, or components of, the osteoinductive interaction involving AER and limb bud mesenchymal cells (Hogan, 1996). Interesting recent work indicates that BMP-2 and 4 are present and apparently interact with Sonic Hedgehog and fibroblast growth factor 4 (FGF-4) proteins in regulating early epithelial-mesenchymal cell interactions in embryonic tooth development (Thesleff *et al*., 1995). However, BMP alone apparently is not sufficient and must interact with other factors to induce tooth formation by neural crest mesenchyme.

Third, the secretion of BIA by Saos-2 cells resembles the paracrine mechanism of embryonic limb development in which AER cells interact with adjacent mesenchymal cells to initiate chondrogenic differentiation by the latter. With Saos-2 cells, the bone-inducing agent (whatever its essential components may be) is released into conditioned media where its activity may be enhanced or modified by as yet unknown regulatory factors.

A similar paracrine transmission of morphogenetic molecules also may occur *in vitro* among confluent populations of Saos-2 cells. Raval *et al*. (1996b) have shown enhanced BMP expression and osteoinductivity of Saos-2 cells after they reach confluence, with reduced BMP expression by proliferating, sub-confluent Saos-2 cells (Fig. 9). Long term culture of confluent Saos-2 cells leads to secretion of a calcifiable, bone-like extracellular matrix containing collagen type I, proteoglycans, osteonectin and bone sialoprotein (McQuillan *et al*., 1995). Confluent Saos-2

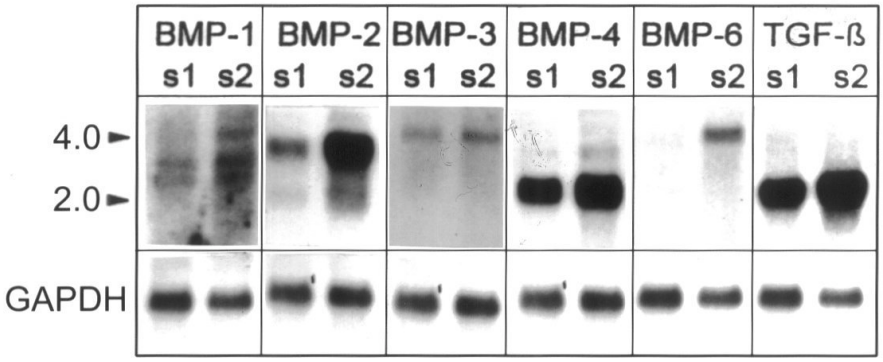

Figure 9. Expression of bone morphogenetic protein (BMP) mRNAs in subconfluent (S1) and confluent (S2) Saos-2 cells. The arrowheads on the left side of the figure indicate the position of 4.0 and 2.0 kb size markers. The lower panel in the figure indicates the level of GAPDH mRNA, used as an invariant mRNA species for normalization of mRNA levels. [Reprinted from Raval *et al.* (1996a), p. 605].

cells also release matrix-vesicle-like structures with the capacity to mineralize (Fedde, 1992). Finally, conditioned medium from post-confluent Saos-2 cell cultures stimulates ALP expression by cultured C3H1OT1/2 mesenchymal stem cells to an even greater degree than does recombinant human BMP-2 (Smith *et al*., 1997). The above findings all suggest that maturing Saos-2 cells when in close proximity, may stimulate each other toward greater osseous differentiation through the paracrine effect of released osteogenic molecules.

Mechanism of bone induction by Saos-2 cell products

It is likely that one or a combination of the BMPs that are expressed by Saos-2 cells are involved in the mechanism of ectopic bone induction. However, the fact that U20S human osteosarcoma cells, also express an array of BMPs, but are non-osteoinductive, suggests that other regulatory factors may play a role. The following are possible explanations for the unique osteoinductive ability of Saos-2 cells.

(1) Significant BMP-1/tolloid expression by Saos-2 cells, but little or none by U20S cells, may be correlated with the unique osteoinductivity of Saos-2 cells. However, pure recombinant BMP-1 is not known to induce bone formation by itself, and the actual biological function of this non-TGF-β-related molecule is uncertain. Both BMP-1 and tolloid cDNAs encode (i) a protease domain related to the astacin family of metallopeptidases, (ii) repeats of an epidermal growth factor (EGF)-like domain, and (iii) domains resembling the complement proteins C1r and C1s (Wozney *et al*., 1988). Kessler *et al*. (1996) have demonstrated that BMP-1 can function as a procollagen I c-propeptidase. Through its c-propeptidase activity, Saos-2 cell BMP-1 might digest, release and thus activate osteoinductive BMPs that are otherwise bound to the collagen of bone matrix. It has also been speculated that the peptidase domain of BMP-1/tolloid may directly or indirectly activate morphogenetic functions of the TGF-β-related BMPs (Fukugawa *et al*., 1994).

(2) Saos-2 cells may be capable of osteoinduction because they express a unique combination of BMPs. The higher levels of BMP-1, 3 and 4 seen in Saos-2 cells (possibly in combination with BMP-2 or -6) may fulfill the optimal mixture and quantity of BMPs required to activate bone induction.

(3) U20S cells may be incapable of inducing bone because, although they express sufficient bone morphogenetic factors, they may contain inhibitors of the BMPs, they may secrete inhibitory binding proteins, they may not process the BMPs correctly, or they may localize the BMPs inappropriately within cellular compartments, thus preventing secretion and osteoinduction by the extracellular matrix.

(4) Saos-2 cells (but not U20S cells) may contain and secrete as yet unknown, non-BMP molecules that are required to activate the osteoinductive cascade.

Overall, our studies support the concept that Saos-2 cells uniquely express an appropriate combination of osteoinductive factors, known and unknown. The fact that two similar human osteosarcoma cell lines (Saos-2 and U20S) express mRNA and proteins for several of the BMPs, but differ in their osteoinductive ability, indicates that expression of one or a few of the BMPs may be present but not necessarily sufficient to stimulate *de novo* bone formation and bone repair.

Experimental Objectives for the Future

Identification of the essential molecular components of Saos-2 cell BIA should take priority. This will require the stepwise purification of Saos-2 cell lysates and/or conditioned media using classical protein isolation methods similar to those used to initially purify the BMPs (Takaoka *et al*., 1993). If a single factor is responsible for Saos-2 cell osteoinductivity, then bone-inducing activity should increase with each successive purification step, until a single active molecule is isolated. If, on the other hand, multiple molecular factors from Saos-2 cells must interact to initiate osteoinduction, then it is likely that BIA activity will be lost when the requisite components of BIA are

separated from one another during progressive purification steps. If the mechanism is multifactorial, then it should be possible to reconstitute activity by recombining purified components of BIA.

After BIA purification is achieved, it will be desirable to map the cytologic distribution of BMPs and other necessary components of BIA, to the various cellular compartments of Saos-2 cells (e.g., cytoplasmic vs. cell membrane distribution). Histo- and cytolocalization of BIA components should also be carried out in *in vivo* implants at successive stages of ossification to determine which host cells give rise to cartilage and bone. This will allow identification of early osteoprogenitor cells in soft tissue, and a determination as to whether they arise from pericytes as has been suggested (Schor *et al*., 1995).

Another high priority objective will be to determine whether purified Saos-2 cell BIA can provide a practical source of a therapeutic bone-inducing agent which can be used clinically to promote bone repair. Our preliminary studies have shown that a semipurified, low molecular weight fraction of Saos-2 cell extracts can heal large femoral defects in adult rats (Hunt *et al*., 1996). However, a similar material was non-osteoinductive in beagle dogs (Griffon *et al*., 1996). Perhaps, greater purification of BIA than was possible at the time of the latter experiment, may remove contaminants that cause inflammation or otherwise impede bone healing in dogs. It is notable that even recombinant human BMP-2, under some conditions, inhibits bone healing in rabbits (Jeppsson and Aspenberg, 1996). Thus, the species in which a putative bone-healing agent is tested is quite important.

Mass culturing of Saos-2 cells may provide a convenient source of bone-inducing agent for clinical use. Methods of mass culture of Saos-2 cells, and large scale purification of BIA will require further development. There are several potential advantages to using cultured Saos-2 cells as a source of a bone repair agent. Such cells are of human origin, thus reducing the risk of sensitization to animal proteins when extracts are used therapeutically in humans. The Saos-2 cell line is permanently established, and can be grown indefinitely in mass culture to produce large quantities of BIA. As indicated above, bone-inducing activity appears to be quite concentrated in Saos-2 cells, and thus, may be more easily extracted from these cells than from decalcified bone matrix or transfected hamster cell tissue culture supernatant where the relative concentration of recombinant BMPs may be low. The addition of a poorly defined carrier such as GuHCl-extracted "bone matrix" is not required to support ossification induced by Saos-2 cell products. Extracts of Saos-2 cells can promote bone healing when combined with a chemically defined collagen carrier (Hunt *et al*., 1996). Nor is there a requirement for any other additive, such as TGF-β (Bentz *et al*., 1991), to support osteoinduction. The Saos-2 cells are already genetically programmed to produce an optimal mixture of native human proteins containing all that is required for effective bone induction.

Summary

(1) Saos-2 cultured human osteosarcoma cells express a bone-inducing agent (BIA) that stimulates ectopic endochondral ossification when implanted subcutaneously in Nu/Nu mice.

(2) The BIA of Saos-2 cells is recoverable not only in lysates or extracts of Saos-2 cells, but it is also secreted into conditioned culture media of confluent Saos-2 cells. In conditioned media, the bone-inducing activity is most concentrated in a particulate fraction of microsome-sized vesicles and other cell-derived particles. However, BIA also is released as an apparently soluble media protein fraction.

(3) Saos-2 cells express several bone morphogenetic proteins including BMP-1, 2, 3, 4 and 6, any or all of which may support bone induction. However, we also have found that U20S human osteosarcoma cells (comparable to Saos-2) express significant levels of BMP-2, 3, 4, 5, 6 and 7, but are non-osteoinductive in Nu/Nu mice. Our studies support the concept that Saos-2 cells uniquely produce an appropriate combination of osteoinductive factors, known and unknown. The fact that two similar human osteosarcoma cell lines (Saos-2 and U20S) express multiple BMPs, but differ in their osteoinductive ability, suggests that the mere expression of one or even several of the BMPs is not necessarily sufficient to stimulate bone formation or repair.

(4) Semipurified, low molecular weight extracts of Saos-2 cells effectively induced healing of diaphyseal, non-union defects in adult rat femurs.

(5) The bone-inducing agent produced by mass-culturing Saos-2 cells, has the potential to be used as a clinical agent, to promote bone repair in fracture non-unions and large bone defects, and possibly to enhance osteointegration of porous prosthetic implants.

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Discussion with Reviewers

R. Rajpurohit: Even though both Saos2 and U2OS are osteosarcoma cell lines, only Saos2 possesses the bone inducing activity. Does the mineralizing ability confer any biological advantage to the Saos2 cells in terms of its pathology?

Authors: If anything, the ability of Saos-2 cells to induce bone *in vivo* would appear to confer a negative biological advantage to these cells. These cells are unable to grow in an immunocompromized host, such as Nu/Nu mice (see Fogh and Trempe, 1975) and Scotlundi *et al*. (1993). Saos-2 cell non-tumorigenicity probably reflects a very advanced state of differentiation.

R. Rajpurohit: Is there any possible explanation for the loss of tumor inducing ability of the Saos2 in the immunologically compromised mice?
Authors: As indicated above, the Saos-2 cells appear to be at an advanced stage of osteoblastic differentiation. Such cell maturity would be uncharacteristic of a more aggressive, undifferentiated tumor cell.

R. Rajpurohit: The data indicates that in U2OS cells, the absence of BMP1 is clearly one of the limiting factors for the loss of bone inducing activity. Does supplementing the U2OS cell extract with BMP1 restore the bone formation activity?
Authors: The depleted expression of BMP-1 by U2OS cells could be a key factor in their inability to induce bone. To our knowledge, no one has yet attempted to enhance BMP-1 expression in U2OS cells to test whether this would confer osteoinductive ability, but this should be tried.

R.F. Valentini: Please explain the reason for differences in the Northern blot data and Western blot analysis for BMPs found in the two cell lines studied.
Authors: The differences in Northern and Western blot analysis for BMPs of Saos-2 and U2OS cells are not great, and for the most part, the mRNA and protein profiles compliment each other. One notable difference was the finding of undetectable BMP-1 in U2OS cells by Northern blot but not by western blot. The latter showed a trace of this protein in U2OS. However, even in western blots, there was a deficiency of toloid in U2OS cells. Toloid is very closely related in molecular structure to BMP-1, and might play a role in the selective bone-inducing ability of Saos-2 cells (Fukugawa *et al*., 1994).

R.F. Valentini: The osteoinductive activity of SAOS-2 retentate is described as being "highly enriched" several times in the test. Is this a fair comment considering that mg levels are required and orders of magnitude less (µg levels) of recombinant BMPs give the same effect?
Authors: The enrichment of bone-inducing agent in Saos-2 cells is considered relative to the amount of BMP that is extractable from decalcified bone matrix. It takes a kilogram of bovine bone to produce only one microgram of pure BMP (Wozney and Rosen, 1993).

R.F. Valentini: What is the history of tissue induced by U2OS vs. SAOS extracts *in vitro*. Are there any differences in angiogenesis?
Authors: While Saos-2 cell implants induce histotypic endochondral bone formation, similar devitalized cell implants of U2OS cells have yielded only fibrous connective tissue infiltrated with variable amounts of chronic inflammatory cells. We have not studied the process of angiogenesis in either type of implant.

R.F. Valentini: Is it possible to separate the potentially highly osteoinductive vesicles from the overall retentate?
Authors: This has not yet been attempted.

R.F. Valentini: Why do confluent cells show significantly higher osteoinductive capacity?
Authors: This may be related to observed higher expression levels of several of the BMPs by confluent mature Saos-2 cells versus proliferating Saos-2 cells (Raval *et al*., 1996b).

R.F. Valentini: The authors suggest that Saos-2 extract could be useful as a clinical product. Are there examples of tumor-derived cocktails being used for any human application? What is the FDA view on such products? Do all components of the product need to be identified before approval?
Authors: A possible example of a "tumor-derived product, foreseen as being potentially clinically useful," is recombinant human BMP itself. All forms of recombinant BMP now available are produced by transfecting Chinese hamster ovary (CHO) cells with the BMP gene affixed to a retrovirus promoter. The transfected CHO cells then secrete BMPs into their conditioned culture medium, from which the BMP is chemically purified. If purification is incomplete, there is the risk of implanting potentially sensitizing hamster proteins into human recipients. Furthermore, the CHO cells (unlike Saos-2 cells) are tumorigenic when implanted live into Nu/Nu mice (Shimizu *et al*., 1994). It is our understanding that a clinically useful, non-toxic, partially purified extract from Saos-2 cells, containing one or a few unidentified protein components, still could be evaluated by the FDA as a "biological product."

R.F. Valentini: What is the original histopathology of the various tumor cell lines? Did the original Saos tumor show massive amounts of bone compared with the other human and rat derived cells? This might be expected given the data.
Authors: Unfortunately, the histology of the primary osteosarcoma from which the Saos-2 cell was derived is not recorded in Fogh and Trempe's original 1975 description of the founding of the Saos-2 cell line.

R.F. Valentini: The issue of other active molecules in the Saos-2 mix are noted. Can the authors comment further on the nature of these molecules. Could retinoic acid, dexamethasone, or BMP heterodimers be present and what is known about their osteoinductive synergies with BMPs?
Authors: To date, our research findings are insufficient to answer the provocative question above.
R.F. Valentini: Have the authors ever mixed Saos and U2OS extracts to evaluate potential negative factors present in U2OS?
Authors: We have conducted one unreported experiment in which we bioassayed mixtures of devitalized Saos-2 and U2OS cells in various ratios. Although the data is preliminary, we had the impression that osteoinductivity was diluted by U2OS cells in proportion to the relative amount of U2OS cells present. There was no evidence of active inhibition of Saos-2 osteoinductivity by adding U2OS cells.

Additional Reference

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