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Recombinant human bone morphogenetic protein induces bone formation

(cartilage induction)

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ABSTRACT **We have purified and characterized active recombinant human bone morphogenetic protein (BMP) 2A. Implantation of the recombinant protein in rats showed that a single BMP can induce bone formation *in vivo*. A dose–response and time-course study using the rat ectopic bone formation assay revealed that implantation of 0.5–115 μg of partially purified recombinant human BMP-2A resulted in cartilage by day 7 and bone formation by day 14. The time at which bone formation occurred was dependent on the amount of BMP-2A implanted; at high doses bone formation could be observed at 5 days. The cartilage- and bone-inductive activity of the recombinant BMP-2A is histologically indistinguishable from that of bone extracts. Thus, recombinant BMP-2A has therapeutic potential to promote *de novo* bone formation in humans.**

The therapeutic potential for bone formation induced by demineralized bone or its extracts has long been recognized (1–4), but the definition of the factor(s) responsible has remained elusive. We previously described the molecular cloning of the genes for bone morphogenetic protein (BMP) 1, 2A, 2B, and 3, using peptide sequence information from a group of proteins purified from such an extract (5, 6). Each of these proteins was implicated in cartilage and bone formation by preliminary experiments which demonstrated *in vivo* cartilage induction at 7 days (5) in the rat ectopic bone-formation system (7). We now describe the purification and characterization of recombinant human BMP-2A, produced by a Chinese hamster ovary (CHO) cell line, and its activity in ectopic bone formation.

METHODS

Purification. CHO cells (line 2ΔD) were grown in Dulbecco's modified Eagle's medium (DMEM)/Ham's nutrient mixture F-12, 1:1 (vol/vol), supplemented with 10% fetal bovine serum. When the cells were 80–100% confluent, the medium was replaced with serum-free DMEM/F-12; medium was harvested every 24 hr for 4 days. Thirty-seven liters of conditioned medium was directly applied to an 80-ml heparin-Sepharose (Pharmacia) column. The resin was washed with 0.15 M NaCl/6 M urea/20 mM Tris, pH 7.4, and then developed with a linear gradient to 1 M NaCl/6 M urea/50 mM Tris, pH 7.4. Fractions were assayed for *in vivo* cartilage and bone formation after reconstitution of protein with a collagenous matrix (6, 7). The fractions with highest specific activity were pooled and concentrated by ultrafiltration with a YM10 membrane (Amicon). Conditioned medium from CHO cells not transfected with the BMP-2A gene was prepared similarly, except that a step gradient to 1 M NaCl was used.

Further purification was achieved by preparative NaDodSO$_4$/PAGE (8). Approximately 300 μg of protein was applied to a 1.5-mm-thick 12.5% gel; recovery was estimated by adding L-[^{35}S]methionine-labeled BMP-2A, purified over heparin-Sepharose as above. Protein was visualized by copper staining of an adjacent lane (9), appropriate bands were excised and extracted in 0.1% NaDodSO$_4$/20 mM Tris, pH 8.0, and then proteins were desalted on a 5.0 × 0.46-cm Vydac C$_4$ column (The Separations Group, Hesperia, CA) in 0.1% trifluoroacetic acid in acetonitrile (6).

Protein concentration was determined by amino acid analysis.

Immunological Methods. A fragment of BMP-2A (amino acids 130–396) produced in inclusion bodies in *Escherichia coli* (provided by John McCoy, Genetics Institute) was purified by NaDodSO$_4$/PAGE under reducing conditions (8), eluted from the gel, and used to immunize rabbits (antibody 130). Peptides (Applied Biosystems) were conjugated to thyroglobulin or bovine serum albumin with glutaraldehyde (10) and used to immunize turkeys. Animals were initially injected with 500 μg of protein mixed with complete Freund's adjuvant and then given biweekly booster injections with 250–125 μg of protein in incomplete Freund's adjuvant. Immunoblots (11) reacted with rabbit antiserum were visualized with ^{125}I-labeled protein A (New England Nuclear); immunoblots were incubated with turkey antisera in the presence of thyroglobulin or bovine serum albumin (100 μg/ml) and then visualized with ^{125}I-labeled rabbit anti-turkey IgG (12).

RESULTS

To achieve high levels of BMP-2A protein expression, the gene for BMP-2A was inserted into a mammalian expression vector, stably introduced into CHO cells, and amplified to high copy number by methotrexate selection of dihydrofolate reductase (13). Individual cell lines were selected for study after preliminary examination of levels of BMP-2A mRNA. Production of BMP-2A was analyzed by using antisera prepared against denatured BMP-2A produced in *E. coli* (referred to as antibody 130, made against a 30-kDa C-terminal fragment; Fig. 1*C*), a peptide of amino acids 103–115 (antibody 103, N-terminal region), or a peptide of amino acids 350–365 (antibody 350, C-terminal region). Direct assay of conditioned medium in the *in vivo* cartilage and bone induction assay was not reliable, probably because of the exogenous proteins present even in serum-free medium. The major secreted proteins in the conditioned medium from one CHO

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Abbreviation: BMP, bone morphogenetic protein.

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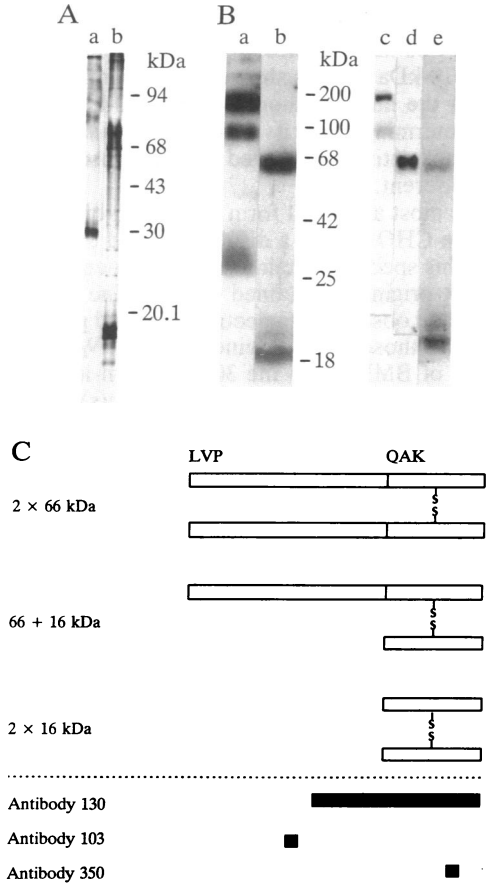

FIG. 1. Silver stain and immunoblot analysis of BMP-2A purified by heparin-Sepharose chromatography. (*A*) Silver stain (14) pattern of $NaDodSO_4$/12% polyacrylamide gel. Lane a: 1.1 μg of protein, nonreduced. Lane b: 1.1 μg of protein, reduced. Positions of molecular mass markers (kDa) are shown at right. (*B*) Immunoblot of 13.5% gel. Lanes a and b: 0.5 μg of protein, nonreduced (lane a) or reduced (lane b), incubated with antibody 130 (amino acids 130–396). Lanes c and d: 1.1 μg of protein, nonreduced (lane c) or reduced (lane d), incubated with antibody 103 (amino acids 103–116 coupled to thyroglobulin). Lane e: 4.6 μg of protein, reduced, incubated with antibody 350 (amino acids 350–365 coupled to bovine serum albumin; this antibody does not recognize nonreduced BMP-2A). Individual lanes from immunoblots were assembled in this figure. Molecular mass markers apply to lanes a–e. (*C*) BMP-2A subunit composition and antibody specificity. N-terminal sequences are indicated with one-letter amino acid symbols.

Table 1. Recovery and activity of 110-, 80-, and 30-kDa species of recombinant human BMP-2A

Molecular mass, kDa		N-terminal sequence(s)†	Protein recovered,‡ nmol	*In vivo* activity of 38 pmol§	
Obs.	Calc.*			Cartilage	Bone
110	84.1	^{24}LVPEL	0.14	±, 1	0, 0
80	55.1	^{24}LVPEL and ^{283}QAKHK	0.37	2, 1	3, 1
30	26.1	^{283}QAKHK	3.35	1, 1	2, 2

*Based on the BMP-2A cDNA sequence (5) and the N-terminal sequence.
†Positions in the cDNA-derived sequence are indicated.
‡Based on amino acid analysis.
§Protein was reconstituted as described (6) and implanted subcutaneously in rats for 10 days. Cartilage and bone were scored as follows: three nonadjacent sections were evaluated from each implant and averaged; ±, tentative identification of cartilage or bone; 1, >10% of each section was new cartilage or bone; 2, >25%; 3, >50%; 4, ≈75%; 5, >80%. Values are shown for duplicate implants.

cell line, 2ΔD, were products of the BMP-2A gene; BMP-2A containing the C-terminal region was enriched an additional 150-fold by chromatography on heparin-Sepharose. After $NaDodSO_4$/PAGE under nonreducing conditions, three protein bands of 110, 80, and 30 kDa reacted with antibody 130 (Fig. 1*B*, lane a); the 110- and 80-kDa bands also reacted with antibody 103 (lane c). Under reducing conditions, antibody 130 reacted with protein of 66 and 16–18 kDa (lane b); antibody 103 recognized only the 66-kDa band (lane d); antibody 350 recognized bands at 66 and 16–18 kDa (lane e). Protein detection by silver stain showed the major protein under nonreducing conditions to be the 30-kDa protein; the 80- and 110-kDa proteins were minor species (Fig. 1*A*, lane a). Under reducing conditions the major bands were at 16–18 and 66 kDa (lane b). From these data and other immunoblot and $NaDodSO_4$ gel analyses (I.M. and P.L., unpublished data), we concluded that the 30-kDa species was composed of two disulfide-linked 16- to 18-kDa subunits; the 80-kDa species of one 16- to 18-kDa subunit and one 66-kDa subunit; and the 110-kDa species of two 66-kDa subunits. Definitive

Table 2. Time and dose dependence of BMP-2A-induced cartilage and bone formation

Dose, μg	Activity	Score				
		Day 5	Day 7	Day 10	Day 14	Day 21
0	C	0	0	0	0	0
	B	0	0	0	0	0
	AP	2.6	6.3	7.8	2.8	15.1
0.46	C	0, 0, 0	1, 0, 2	1, 0, 0	±, 0, NR	0, 0, 0
	B	0, 0, 0	0, 0, 0	0, 0, 0	4, 4, NR	0, 3, 2
	AP	3.2	17.8	20.8	6.1	36.0
1.2	C	±, ±, 0	1, 2, 1	3, 2, 3	1, ±, 1	0, 0*
	B	0, 0, 0	0, 0, 0	1, 1, 1	3, 2, 3	4, 5*
	AP	1.5	3.1	24.6	12.4	27.0*
6.2	C	0, 1, 3	2, 2, 3	2, 3, 3	0, 0, 0	0, 0, 0
	B	0, 0, 0	0, 0, 1	3, 3, 3	4, 5, 4	4, 4, 4
	AP	2.2	172.7	125.0	44.1	111.3
12.0	C	3, 2, 3	3, 3, 3	1, 2, 2	0, 0, 0	0, 0, 0
	B	0, 0, 0	2, 1, 2	3, 2, 3	4, 4, 4	5, 5, 5
	AP	3.8	254.3	74.0	64.3	98.6
18.2	C	3, 2, 2	3, 3, 3	2, 2, 1	0, 0, 0	0, 0, 0
	B	0, 0, 0	4, 4, 2	2, 3, 3	4, 5, 4	5, 5, 5
	AP	4.3	288.5	300.0	61.2	98.5
24.0	C	3, 1, 1	4, 3, 3	2, 1, 1	±, 0, 0	0, 0, 0
	B	1, 0, 0	3, 1, 3	4, 4, 4	4, 4, 5	5, 5, 5
	AP	4.0	371.2	138.1	75.6	99.0
29.7	C	2, 2, 2	5, 3, 3	1, 1, 1	0, 0, 0	0, 0, 0
	B	0, 0, 0	2, 4, 4	4, 4, 3	5, 5, 5	5, 5, 5
	AP	3.6	283.3	133.7	64.6	98.6
115.3	C	2, 4	3, 2, NR		0, 0, 0	
	B	0, 2	5, 5, NR		5, 4, 5	
	AP	ND	378.1		70.6	

Partially purified BMP-2A was implanted as indicated. NR indicates that the implant was not recovered. The scores (as described for Table 1) of the individual implants (in triplicate, except for one time point for 1.2-μg and 115.3-μg doses) are tabulated to indicate assay variability, and the cartilage (C) and bone (B) scores are in corresponding order. The implant size itself was variable, and the protein content in replicate implants varied as much as 10-fold, although a range of 2 was more common. Alkaline phosphatase (AP) activity was measured as described (6) and is expressed as units/mg of protein; 1 unit is defined as 1 μmol/min at 37°C with *p*-nitrophenyl phosphate at pH 10.5. Protein concentration was determined by the Bradford method (18). ND, not determined.
*Implants removed at 17 days instead of 21 days and done in duplicate instead of triplicate.

assignation of structure was determined by N-terminal sequencing of each BMP-2A species under nonreducing and reducing conditions. The N terminus of the 66-kDa subunit begins at amino acid 24; the 16- and 18-kDa subunits, determined separately, begin with amino acid 283. The discrepancy between the calculated and observed molecular masses is most likely caused by glycosylation: there are four potential sites of N-glycosylation in the precursor molecule (5), and preliminary data indicate the presence of carbohydrate on both N- and C-terminal regions by N-Glycanase (Genzyme) digestion and binding to lectin columns (E.A.W., unpublished data). Thus, three disulfide-linked forms of BMP-2A have been purified from CHO cells by heparin binding; the structures and N-terminal sequences are shown in Fig. 1*C* and Table 1.

$NaDodSO_4$/PAGE was used to purify each of the three BMP-2A species to homogeneity. The overall recovery of BMP-2A protein after electrophoresis, desalting, and concentration was ≈30%, and 87% of the BMP-2A was the 30-kDa form (Table 1). All three forms of BMP-2A showed *in vivo* activity when assayed for cartilage and bone induction. The 30- and 80-kDa species were equivalent in this assay, while the 110-kDa species showed significantly less activity. Because of the limited amount of the 110-kDa species, we have not determined how its activity differs from that of the 30-kDa form: activity observed may be dose-related or inherently different.

Thus the most abundant form of heparin-binding BMP-2A produced in CHO cells is a disulfide-linked dimer of 16-kDa subunits; this species contains the tryptic peptide sequences of BMP-2A originally obtained from bovine BMP, and the calculated and observed isoelectric point and molecular mass are similar to those of the bovine BMP (6). We propose that processing of BMP-2A to the 30-kDa form involves dimerization of the proprotein through cysteine(s) in the mature domain, since there are no cysteines in amino acids 24–282, and removal of the N-terminal region in a manner analogous to the processing of a related protein, transforming growth factor β (15–17). Unlike transforming growth factor β, no difference in activity is observed if the protein is purified in

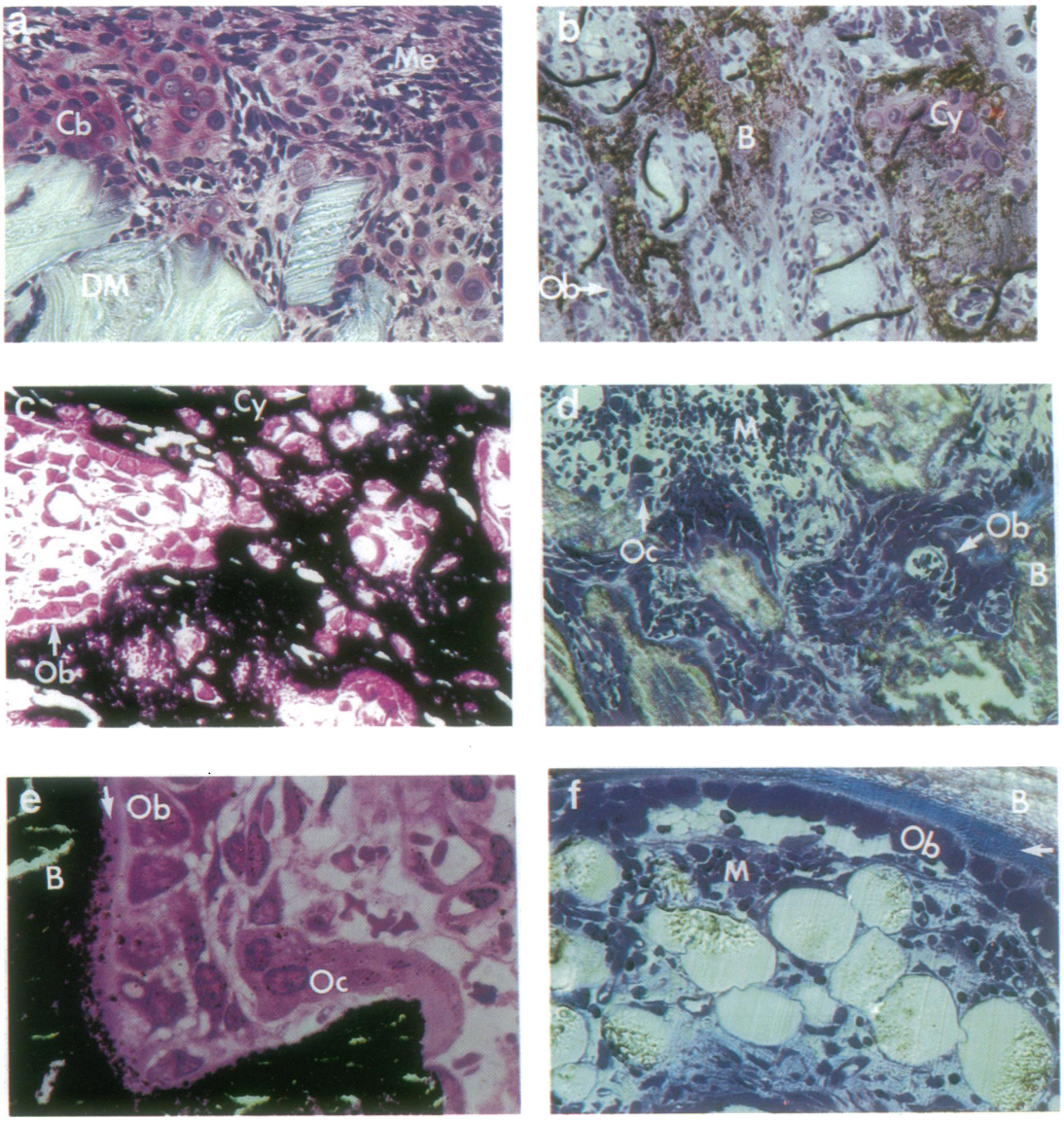

FIG. 2. Cartilage and bone formation induced by implantation of 12 μg of BMP-2A. DM, demineralized matrix carrier; Me, undifferentiated mesenchymal cells; Cb, chondroblasts; Cy, chondrocytes; Ob, osteoblasts; B, bone mineral; M, bone marrow; Oc, osteoclasts. (*a*) Five days after implantation. (Toluidine blue stain; ×140.) (*b*) Seven days after implantation. (Toluidine blue stain; ×140.) (*c*) Seven days after implantation. Black deposits represent *de novo* mineralization of cartilage and bone matrix. (von Kossa stain; ×280.) (*d*) Fourteen days after implantation. (Toluidine blue stain; ×140.) (*e*) Fourteen days after implantation. Arrow rests on the osteoid seam between osteoblasts and newly mineralized bone. (von Kossa stain; ×70.) (*f*) Twenty-one days after implantation. Arrow rests on the osteoid seam. (Toluidine blue stain; ×280.)

the absence of urea or any denaturants, although activation *in vivo* cannot be excluded.

Heparin-Sepharose-purified protein, which was ≈50% pure recombinant human BMP-2A, was implanted subcutaneously in rats. After 5–21 days the implants were evaluated histologically for newly formed cartilage and bone (6, 7) and enzymatically for levels of alkaline phosphatase, synthesized by both cartilage and bone cells. The time course of cartilage and bone development in response to 12.0 μg of BMP-2A is illustrated in Fig. 2 and summarized in Table 2. At 5 days, many immature and some hypertrophic cartilage cells were present in the BMP-containing implants, but no mineralizing cartilage was detected (Fig. 2*a*). After 7 days the chondrocytes were hypertrophic (Fig. 2*b*) and the cartilage was mineralized (Fig. 2*c*). Osteoblasts surrounded by osteoid were evident (Fig. 2 *b* and *c*). Vascular elements, including giant cells and bone marrow precursors, were seen and were most abundant in areas where calcified cartilage was undergoing remodeling. At 14 days (Fig. 2*d*) the removal of calcified cartilage was nearly complete and bone was widespread. Osteoblasts and osteoclasts were abundant. Implant vascularity had increased markedly, and we tentatively identified hematopoietic cell maturation. Twenty-one-day implants (Fig. 2*f*) showed increased maturity: the bone was highly organized with mature marrow spaces, and all remnants of matrix carrier had been removed. In contrast, matrix remained intact in control (no BMP-2A) implants (see Fig. 3*f*).

The effect of BMP-2A dosage on activity is illustrated in Fig. 3 and summarized in Table 2. Implantation of 0.46–115.3 μg of protein induced new bone formation. The lowest amount used, 0.46 μg, resulted in a minimally detectable response: at times longer than 7 days, cartilage and bone were observed in about half of the implants. This result may reflect a true threshold effect or an inconsistency in the detection of

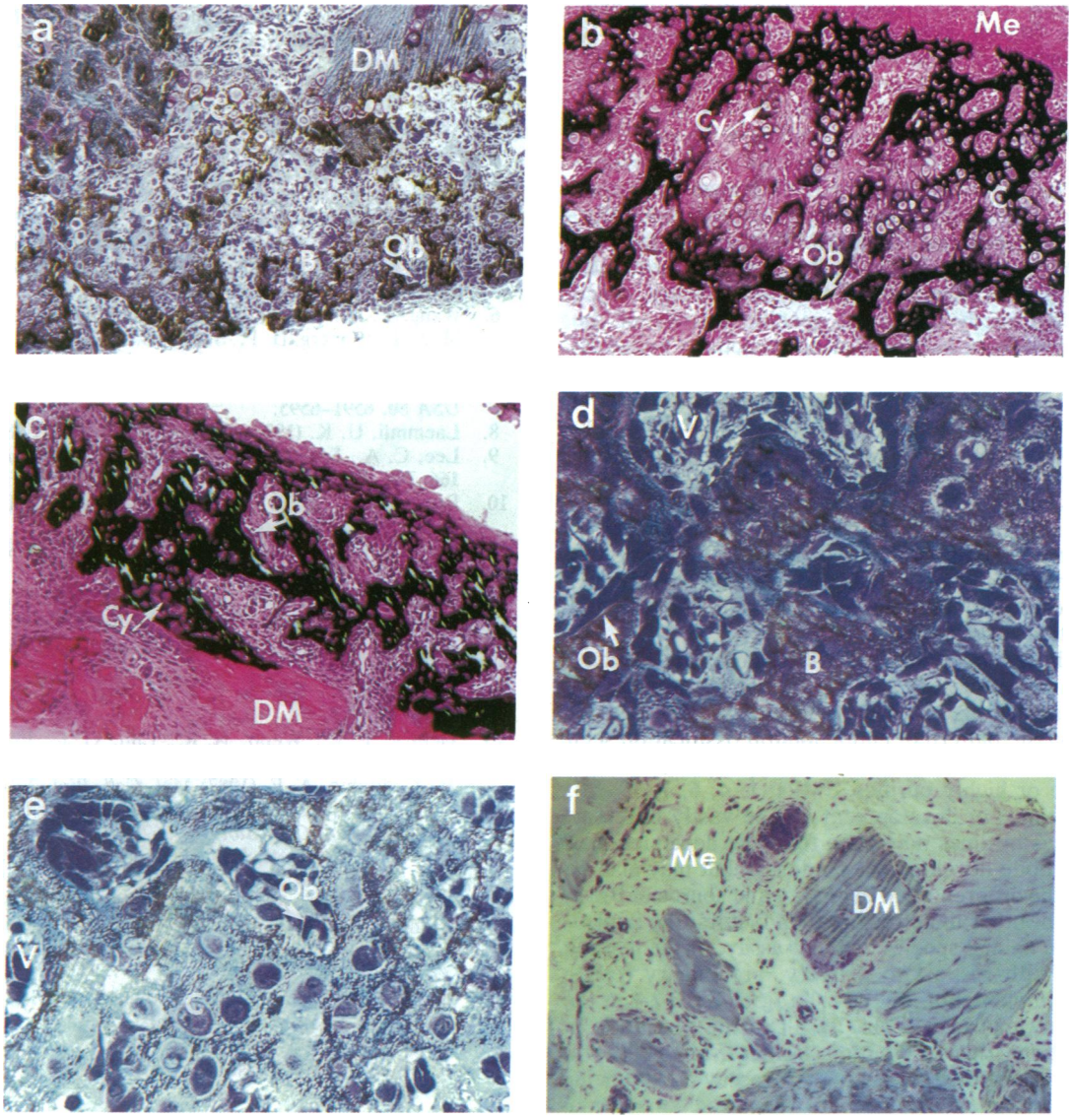

FIG. 3. Cartilage and bone formation with increasing doses of BMP-2A. DM, demineralized matrix carrier; C, cartilage; B, bone; Ob, osteoblasts; Me, mesenchymal cells; Cy, chondrocytes; V, vascular channel. (*a*) Seven days after implantation of 12 μg of BMP-2A. Arrow points to osteoblasts lining the surface of new bone. (Toluidine blue stain; ×70.) (*b*) Seven days after implantation of 24 μg of BMP-2A. Arrows point out chondrocytes (upper) and osteoblasts (lower) actively synthesizing new matrix. (von Kossa stain; ×70.) (*c*) Five days after implantation of 115 μg of BMP-2A. Both cartilage and bone matrix have begun to mineralize. (von Kossa stain; ×70.) (*d*) Five days after implantation of 115 μg of BMP-2A. (Toluidine blue stain; ×280.) (*e*) Same as for *d*, but an adjacent section. Note the simultaneous presence of newly formed cartilage and bone. (*f*) Seven days after implantation of matrix carrier with control protein: heparin-Sepharose-fractionated conditioned medium from a CHO cell line containing no human BMP-2A gene (approximately the equivalent of 3 ml of conditioned medium, which in the 2ΔD cell line would yield 1–3 μg of BMP-2A). (Toluidine blue stain; ×70.)

a small island of cartilage or bone in an implant. Higher doses of BMP-2A showed consistent amounts of cartilage and bone formation. At 7 days, the amount of newly formed cartilage reached a plateau at doses higher than 6.2–12.0 μg, whereas the amount of bone increased with increasing doses. For example, at 7 days, 24-μg implants (Fig. 3*b*) contained more bone that was mineralized as compared to 12-μg implants (Fig. 2*c*). Also, increasing doses decreased the time at which bone could first be observed. In two cases, high doses of BMP-2A resulted in the appearance of osteoblasts as early as 5 days after implantation (Fig. 3 *c–e*); both the bone and the cartilage matrix were heavily calcified (Fig. 3*c*).

DISCUSSION

We have purified and characterized recombinant human BMP-2A synthesized in CHO cells: processing of BMP-2A to its active form involves dimerization and cleavage analogous to the processing of transforming growth factor β and generates a basic dimeric molecule with properties similar to those of bovine bone-derived BMPs. The amount of BMP-2A required to consistently induce cartilage and bone formation is about 600 ng (assuming 50% purity), or ≈10 times more than the estimated 50 ng of nonrecombinant bovine BMPs required for a similar response (6). There are several possible reasons. The highly purified bovine BMP preparation was a mixture of several proteins that might act synergistically. Alternatively, recombinant human BMP-2A may differ from the bovine bone-derived BMP-2A because of posttranslational processing or aberrant proteolytic cleavage. Such changes could directly affect the specific activity of the recombinant protein or indirectly affect its activity by reducing its affinity for the rat collagenous matrix. Further, the presence of the incompletely processed forms of BMP-2A in this material may affect or even inhibit the biological activity. These possibilities are supported by the ability of nanogram amounts of partially purified BMP-2A from other CHO cell lines or transfected COS cells to direct cartilage formation; activity may also be affected by the purity of the implanted sample (5). Experiments with combinations of BMPs or other factors, extensive biochemical characterization of BMP-2A, and further description of the nonrecombinant BMPs should resolve questions about specific activity.

We have shown that a single BMP is sufficient to induce cartilage and bone formation in the rat ectopic model. We conclude from our histological analysis that BMP-2A can initiate the classic pattern of endochondral ossification seen with mixtures of factors found in demineralized bone matrix or with crude or highly purified BMPs (1, 4, 6, 7, 19–23), although the times of development can be vastly different. The differences in times may stem from the dependence of cartilage and bone development on dosage but may also be attributed to the differences in the purity and source of BMP, the presence of other undefined growth factors, the implantation site, the animal species used for assay, or the method of delivery. With respect to delivery, preliminary results indicate that implantation of 100–200 μg of BMP-2A in the absence of any matrix will also result in the formation of cartilage and bone; this observation clarifies the role of the rat matrix in increasing the sensitivity of the assay but not being a necessary component for *in vivo* cartilage and bone formation (E.A.W., unpublished data). The present availability of milligram quantities of BMP-2A has allowed study over a broad range. As noted previously with highly purified bovine BMP, a mixture of factors (6), implantation of increasing doses of BMP-2A appears to accelerate bone formation, or at least decrease the time that elapses before bone can be observed. Many questions on the actions and interactions of BMP-2A—the characterization of responsive cells and its effects on these cells, whether mitogenic, chemotactic, or differentiating, in ectopic bone formation, in normal bone development and maintenance, and in nonskeletal systems—remain to be answered. The described activity of recombinant human BMP-2A, the induction of cartilage and bone at an ectopic site, recapitulates the complex progression seen in fracture healing and embryonic long-bone development. Local, defined, and controllable bone formation induced by BMP-2A in conjunction with a suitable delivery system may be an important human therapeutic in applications requiring bone replacement and bone formation.

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