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Adenovirus-Mediated BMP2 Expression in Human Bone Marrow Stromal Cells

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Abstract Recombinant adenoviral vectors have been shown to be potential new tools for a variety of musculoskeletal defects. Much emphasis in the field of orthopedic research has been placed on developing systems for the production of bone. This study aims to determine the necessary conditions for sustained production of high levels of active bone morphogenetic protein 2 (BMP2) using a recombinant adenovirus type 5 (Ad5BMP2) capable of eliciting BMP2 synthesis upon infection and to evaluate the consequences for osteoprogenitor cells. The results indicate that high levels (144 ng/ml) of BMP2 can be produced in non-osteoprogenitor cells (A549 cell line) by this method and the resultant protein appears to be three times more biologically active than the recombinant protein. Surprisingly, similar levels of BMP2 expression could not be achieved after transduction with Ad5BMP2 of either human bone marrow stromal cells or the mouse bone marrow stromal cell line W20-17. However, human bone marrow stromal cells cultured with 1 μM dexamethasone for four days, or further stimulated to become osteoblast-like cells with 50 μg/ml ascorbic acid, produced high levels of BMP2 upon Ad5BMP2 infection as compared to the undifferentiated cells. The increased production of BMP2 in adenovirus transduced cells following exposure to 1 μM dexamethasone was reduced if the cells were not given 50 μg/ml ascorbic acid. When bone marrow stromal cells were allowed to become confluent in culture prior to differentiation, BMP2 production in response to Ad5BMP2 infection was lost entirely. Furthermore, the increase in BMP2 synthesis seen during differentiation was greatly decreased when Ad5BMP2 was administered prior to dexamethasone treatment. In short, the efficiency of adenovirus mediated expression of BMP2 in bone marrow stromal cells appears to be dependent on the differentiation state of these cells. J. Cell. Biochem. 82: 11–21, 2001.

Key words: bone morphogenetic protein 2; adenovirus vectors; bone marrow stromal cells

One of the most widely studied osteoinductive factors is bone morphogenetic protein 2 (BMP-2). BMP 2 is a member of the transforming growth factor beta superfamily and are one of a handful of proteins that are capable of stimulating heterotopic bone [Wang et al., 1990] and promoting bone defect healing [Zegzula et al., 1997]. Since this discovery, the BMPs have held great promise in various orthopedic applications including treatment of bone defects. BMPs play a critical role in embryonic development and pattern formation [Graff et al., 1994; Harland, 1994; Hawley et al., 1995; Kim et al., 1998; Merino et al., 1999], and also contribute post-embryonically in normal fracture repair [Bostrom, 1998; Reddi, 1998]. A number of clinical studies using recombinant BMP2 (rBMP2) have demonstrated the ability of this protein to augment fracture repair and induce bone union in segmental defect [Johnson et al., 1988; Heckman et al., 1991; Yasko et al., 1992; Bostrom et al., 1996]. Unfortunately, a large concentration of rBMP2 was required to be effective. Several factors have been postulated to compromise efficacy of rBMP2, including the limited diffusion of the injected protein and need for an adequate carrier.

To overcome the problems with rBMP2, Musgrave et al., 1999, demonstrated the formation of heterotopic bone using intramusclular injection of an adenovirus type 5 vector capable

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of expressing BMP2. Although bone was produced in both immunocompetent and immunocompromised animals, the amount of bone formed was very little in the former [Musgrave et al., 1999]. The authors speculated that immediate diffusion and rapid immune system clearance of the virus was responsible for the small picogram-nanogram quantities of BMP2 produced.

Many investigators have now shifted their attention towards cellular gene therapy where genes expressing osteoinductive factors, such as BMP2, are delivered to osteoprogenitor cells using various viral and non-viral vectors. The osteoprogenitor cells have been hypothesized to play a major role in skeletal bone healing. However, implantation of these cells alone does not reliably induce bone formation, presumably because of the lack of inductive signals necessary for osteoblastic differentiation. The fact that rBMP2 has been shown to induce osteogenesis in progenitor cells [Thies et al., 1992, Hanada et al., 1997; Lecanda et al., 1997], has led many investigators to attempt a cellular gene therapy approach. When either the C3H/10T1/2 mesenchymal cell line [Gazit et al., 1999; Lou et al., 1999] or W20-17 mouse bone marrow stromal cell line [Lieberman et al., 1998, Engstrand et al., 2000] are transduced to express BMP2, they express an osteoblastic phenotype followed by hetero- or -orthotopic bone formation in animals. Interestingly, the authors demonstrated that the transduced cells are cleared promptly by the receipient (within hours) suggesting that they act as a trigger of the bone formation process [Engstrand et al., 2000].

Work by Lieberman et al., 1999 advanced the cell gene therapy approach by demonstrating that administration of transduced bone marrow stromal cells to a long bone defect resulted in the formation of bone similar to the intact control. In this study these autologous BMP2-producing bone marrow stromal cells and rhBMP2 were compared for their ability to heal a critical-size defect in syngeneic rats. The results demonstrated that, although bone was formed in both cases, histologically and biomechanically the bone produced by the transduced cells more closely resembled skeletal bone [Lieberman et al., 1999].

Recently, Baltzer et al., 2000, directly administered Ad5BMP2 into a rabbit segmental defect model and demonstrated that the newly formed bone was biomechanically and histologically similar to skeletal bone.

These advances in the field of skeletal gene therapy have provided exciting possibilities for the future of orthopedics. However, technical refinement of the BMP2 gene therapy systems is required to improve their safety and efficacy. The aim of this study was to investigate transduction of bone marrow stromal cells with Ad5BMP2, determine the amount of BMP2 produced with respect to the differentiation state of these cells, and to compare biological activity of the adenovirally-produced BMP-2 with rhBMP-2.

MATERIALS AND METHODS

Cell Culture

Cell Lines. Human embryonic kidney cell line (293), and human lung carcinoma cell line (A549) were propagated as described by American Type Culture Collection in Dulbecco's Modified Eagles Medium (DMEM) (Biowhittaker, Walkersville, MD), supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Life Technologies Inc., Gaitersburg, MD) and grown in a humid chamber at 37°C and 5% CO_2. The W20-17 mouse stromal cell line (a gift from Genetics Institute, Cambridge, MA) was propagated as described by Thies et al., 1992. Briefly, the cells were grown in DMEM supplemented with 10% FBS, and antibiotics-antimycotics as described above and cultured at a subconfluent density in order to maintain their phenotype.

Primary Cells. Discarded human bone marrow products were obtained from.healthy donors in compliance with all state and federal regulations following Institutional Review Board approval. Mononuclear cells were isolated by gradient density centrifugation on Ficoll-Paque PLUS (Amersham Pharmacia Biotech, Piscatawy, NJ) and washed twice with phosphate buffered saline (PBS) (Life Technologies Inc, Gaithersburg, MD) prior to culturing. Cells were plated at a density of 5×10^6 cells/cm^2 [Jaiswal et al., 1997] in DMEM supplemented with 10% FBS and antibiotics-antimycotics as described above. Early fibroblasts appeared within two days of culture and after one week dead cells and debris were removed by washing with PBS and cells were passaged prior to confluence. Several vials of these cells were

frozen in Origen® dimethyl sulfoxide freeze medium (Igen International Inc., Gaithersburg, MD).

Infection of Cells with Adenovirus Vectors. Adenovirus infections were done as described previously [Davis et al., 1996]. Briefly, cells were infected at specified multiplicity in DMEM supplemented with 2% FBS and the antibiotic-antimycotic. The virus was allowed to adsorb overnight and then the media was replaced with either DMEM supplemented with 10% FBS, and antibiotic-antimycotic for the human bone marrow cells or in the reduced serum medium OPTI-MEM® I (Life Technologies Inc, Gaithersburg, MD) supplemented with 2% FBS, antibiotic-antimycotic for other cell types.

Differentiation of bone marrow fibroblasts. Bone marrow fibroblasts in culture were stimulated to differentiate by 1 μM dexamethasone (Sigma Chem Co, St Louis, MO) as described elsewhere [Jaiswal et al., 1997]. The cells were further treated with 50 μg/ml ascorbate to enhance their osteoblastic differentiation. The media was exchanged every third day to provide fresh ascorbic acid to the cells.

Production of Adenovirus

Construction of the Virus. A replication defective human type 5 adenovirus (Ad5) containing a cDNA for BMP2 in the E1 region of the virus was constructed by in vivo homologous recombination in 293 cells which constitutively produces E1 proteins. The human BMP2 cDNA was constructed by reverse transcription polymerase chain reaction using high fidelity Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA). The BMP2 clone was cloned into an adenovirus transfer plasmid, pCA14 (Microbix, Toronto, Canada) which has map units 0–1.4 and 9–16 of human adenovirus 5 and a deletion in E1 (1.4–9 map units). BMP2 was cloned into the E1 deleted region which contains an upstream human cytomegalovirus promoter, SV40 enhancer, and downstream SV40 polyadenylation sequence. This plasmid was then co-transfected by calcium phosphate precipitation (Promega, Madison, WI) along with the *Cla* I fragment of adenovirus DNA that contained a green fluorescent protein (GFP) marker gene as described by Davis et al., 1998. The appearance of clear plaques demonstrated recombination and several were selected for further plaque purification and screening, as described by Davis et al., 1998. Viral DNA was isolated [Davis et al., 1998] and viral lysates with the correct DNA structure were then tested for production of BMP2 protein by infection of A549 and cell extracts were then immunoblotted as described below (see Western blot detection of BMP2, Materials and Methods). A control virus was similarly constructed which contained a green fluorescent protein expression cassette in the E1 region.

Propagation and Purification of the Virus. Viral lysates that were positive for BMP2 protein expression were then expanded by infection of 293 cells as described in Davis et al., 1996. A crude lysate was generated by three cycles of freeze-thawing and then cellular debris pelleted by centrifugation. This lysate was used to infect one cell factory (Nalge-Nunc International, Denmark) of 293 cells. At maximal cytopathic effect the virus was harvested and again subjected to three cycles of freeze thawing. The virus was then banded on a series of two cesium chloride gradients [Davis et al., 1996] and then desalted using an Econo-Pac® 10 DG disposable size exclusion column (Bio-Rad Laboratories, Hercules, CA). The final viral stock titer is 5×10^9 pfu/ml on 293 cells. Infection of A549 cells indicated no contamination with wild type virus.

Western Blot Detection of BMP2

Cell extracts were generated from cells that had been infected with Ad5BMP2 and cultured for three days prior to lysis with 2X sample buffer (125 μM Tris-HCl pH6.8, 4% (w/v) sodium dodecyl sulfate, 20% glycerol, 0.05% (w/v) bromophenol blue, and β-mercaptoethanol (10% v/v) added just prior to gel electrophoresis). Conditioned media from cells were precipitated by addition of five volumes of cold acetone and pelleted by centrifugation for 10 min at 10,000 ×g [Constam and Robertson, 1999]. Next pelleted precipitated proteins were resuspended in sample buffer. Purified rhBMP2 (a gift from Genetics Institute, Cambridge, MA) was used as a positive control for protein detection. Proteins were separated on a Novex® Tris-Glycine gel 4–20% (Invitrogen Corp, Carlsbad CA) under denaturing conditions and transferred to supported nitocellulose membrane (Biorad). BMP2 was detected using a Western Breeze chemiluminescent kit (Invitrogen, Carlsbad, CA) with an anti-BMP2 monoclonal antibody (h3b2/17.8.1) (a generous gift from Genetics Institute, Cambridge, MA) and a

secondary anti-mouse IgG antibody conjugated with alkaline phosphatase. The signal was detected by incubation with CDP-star® chemiluminescent substrate and developed on Hyperfilm (Amersham Pharmacia Biotech, Piscatawy, NJ) and quantified by scanning with a laser densitometer (Molecular Dynamics, Sunnyvale, CA). Quantitations were performed, where indicated, in three independent experiments.

Determination of BMP2 Concentration

Known concentrations of hrBMP2 or known volumes of BMP2 produced from cells transduced by virus were subject to denaturing polyacrylamide gel electrophoresis and Western blotting as described above. The blots were scanned with a laser densitometer as described above, and a standard curve of BMP2 concentrations versus laser densitometer unit was generated by applying linear regression analysis to the data. Concentrations of the BMP2 in the media from cells transduced by virus were then determined by solving the equation for each line. Three independent standard curves were generated and therefore three independent concentrations for the amount of BMP2 synthesized by the transduced cells were determined. The average of these values and standard deviation ($n = 3$) are shown in Figure 2B.

Alkaline Phosphatase Assay

W20-17 cells were plated at subconfluent densities (5×10^4 cells/cm^2) prior to assay, and 24 h after plating the media was replaced with fresh media containing varying concentrations of rhBMP2. Cells were assayed for alkaline phosphatase activity three days later by a chemiluminescent procedure described by Blum et al., in press. Briefly, cellular alkaline phosphatase was extracted by washing the cells with PBS and then cells were lysed by three freeze-thaw cycles in 100 μl/cm^2 of 25mM Tris-HCl, pH 8.0, and Triton X-100. For detection of alkaline phosphatase 3–5 μl of the samples were added to 100 μl of CSPD® ready-to-use with Sapphire II enhancer (Tropix, Bedford, MA) in a luminometer tube, vortexed, and incubated at room temperature for 30 s. The light output from each sample was integrated for 10 s after a 2 s delay by the luminometer (TD-20/20, Turner Designs, Sunnyvale, CA). Alkaline phosphatase detection signal was recorded in relative luminescence units (RLU). Cellular alkaline phosphatase activities were normalized to protein content with the BCA assay using bovine serum albumin to derive a standard curve. Data was presented as percent induction above unstimulated basal control cells.

Statistical Analysis

All data were taken in triplicate and reported as means with standard deviation. A paired Student's t-test of unequal variances with 95% confidence interval ($P < 0.05$) was done between the untreated control and each experimental condition.

RESULTS

Recombinant Adenovirus Construction and Human BMP2 Expression

BMP2 expression mediated by Ad5BMP2 was tested by infecting A549 cells (human lung carcinoma) that are known to possess high levels of the coxackie B virus-adenovirus receptor (CAR) [Bergelson et al., 1997] for adenovirus type 5. Varying concentrations of the Ad5BMP2 (2,500 or 5,000 virus particles/cell; MOI 25 or 50) or a control virus Ad5GFP (5,000 particles/cell; MOI 50) were added to A549 cells and cultured for three days. Infection with both concentrations of virus resulted in production of mature BMP2 protein in a dose dependent manner (Fig. 1; lanes 2 and 3). Lane 4 depicts the amount of endogenous BMP2 expression in the A549 cells when mock infected with the Ad5GFP control virus. Although, there are several nonspecific proteins in cell extracts detected by the anti-BMP2 antibody, a 65 kDa potential precursor protein is detected in lanes 3 and 4, which is specific to the Ad5BMP2 infected cell extracts. Mature BMP2 also is present in the culture medium (lanes 5 and 6) demonstrating that the BMP2 expressed after viral infection can be both processed and secreted. Supernatant from cells infected with Ad5GFP does not contain detectable levels of BMP2 (lane 7), demonstrating that all BMP2 production is due to specific transduction with the Ad5BMP2 vector, rather than to cellular BMP2.

Comparison of rhBMP2 to Virally-Produced BMP2

In order to determine and compare activity of the BMP2 produced by viral infection with recombinant protein, alkaline phosphatase activity was measured in a mouse bone marrow

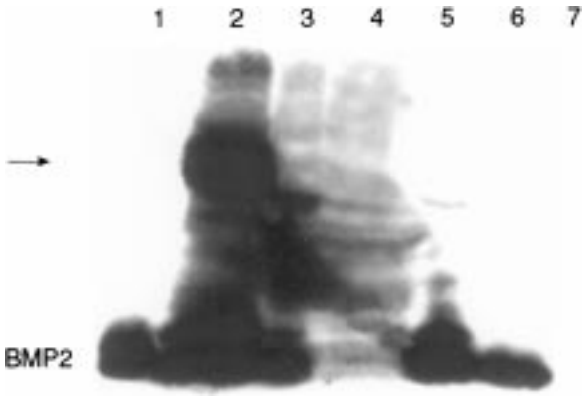
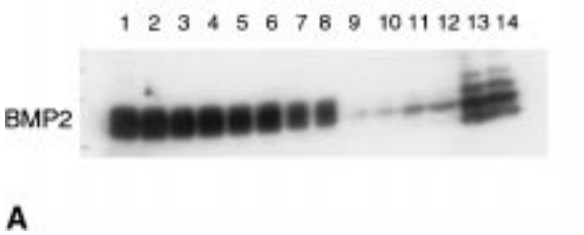


Fig. 1. Results of Western blot analysis for detection of BMP2 in cell extracts or conditioned media from A549 cells infected with either Ad5BMP2 or Ad5GFP. Recombinant BMP2 (50 ng) was used as a standard (**lane 1**). Cell extracts prepared from cells infected with 2,500 particles/cell (**lane 2**) or 5,000 particles/cell (**lane 3**) of Ad5BMP2 or 5,000 particles/cell Ad5GFP (**lane 4**) were electrophoresed under denaturing conditions and BMP2 detected using an anti-BMP2 monoclonal antibody (Genetics Institute, Cambridge, MA). Proteins from 500 μl of conditioned media from A549 cells infected with Ad BMP2 (2,500 particles/cell; **lanes 5**), Ad5BMP2 (5,000 particles/cell; **lane 6**), or Ad5GFP (5,000 particles/cell; **lane 7**) were precipitated and subjected to Western blot analysis. A 65 kDa protein specific to Ad5BMP2 infected cell extracts is marked by an arrow.

stromal cell line W20-17 [Thies et al., 1992]. Varying concentrations (25 ng, 50 ng, 75 ng, and 100 ng) of rhBMP2 were electrophoresed under denaturing conditions and immunoblotted as BMP2 concentration standards (Fig. 2A, lanes 1–4). BMP2 produced by viral infection along with cellular proteins in the medium were acetone precipitated from the culture supernatant and applied to the gel (Lanes 5–7). Control experiments (data not shown) using rhBMP2 show that this procedure results in quantitative recovery of BMP2. The amount of BMP2 in culture supernatant taken from A549 cells was determined as described in the Materials and Methods section. Figure 2A depicts one of these gels in which duplicate samples were run. As seen in Figure 2B, resultant concentrations of BMP2 produced as a result of viral infection, were 18.6 ng/ml, 34.4 ng/ml and 144 ng/ml culture supernatant, corresponding to infection of A549 cells with 1,000, 2,500, and 5,000 particles/cell Ad5BMP2 virus respectively. Each bar on the graph represents the average of the three samples and error bars depict the standard deviation amongst the samples.

BMP2 activity was assayed by culturing the W20-17 cells with either varying concentrations of rhBMP2 (3 ng/ml, 8 ng/ml, or 17ng/ml) or varying concentrations of culture supernatant (8 μl/ml, 50 μl/ml, and 85 μl/ml which corresponds to 1.2 ng/ml, 7.2 ng/ml or 12.2 ng/ml respectively) from A549 cells infected with Ad5BMP2 (5,000 particles/cell). BMP2 produced as a result of viral infection enhanced



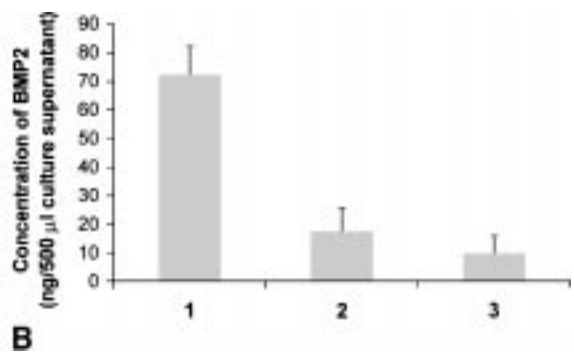


Fig. 2. Comparison of protein activities between recombinant BMP2 and BMP2 produced after Ad5BMP2 infection. **A:** Depiction of one of two independent experiments in which Western blot analysis to determine the protein concentration of the BMP2 produced after Ad5BMP2 infection of A549 cells. Duplicate samples of recombinant BMP2 (100 ng (**lanes 1** and **2**), 75 ng (**lanes 3** and **4**), 50 ng (**lanes 5** and **6**), and 25 ng (**lane 7** and **8**) were subjected to electrophoresis and immuno-detection in order to generate a BMP2 standard concentration curve. Protein in culture supernatant from A549 cells, which were infected with Ad5BMP2 at varying multiplicity of infection: 1,000 particles/cell (**lanes 9** and **10**); 2,500 particles/cell (**lanes 11** and **12**); and 5,000 particles/cell (**lanes 13** and **14**) were acetone precipitated and subjected to Western blot analysis. **B:** Estimated concentration of BMP2 from the experiments described in Figure 2A. The BMP2 concentration in conditioned media from A549 cells infected with 5000 particles/cell (bar 1), 2,500 particles/cell (bar 2) or 1000 particles/cell (bar 3) are depicted in the graph. The results were obtained by laser densitometric scanning of the Western blot in Figure 2A as well as a second independent experiment. The values depicted in this graph are the average laser densitometric units where $n = 3$ and the error is $\pm$ SD for $n = 3$.

the level of alkaline phosphatase activity 2.9–16 fold over the values obtained for the control cells (Fig. 3; bars 1, 2, 3, and 4) demonstrating that the protein was active. To determine if the virally expressed protein was as active as the rhBMP2 protein, the levels of alkaline phosphatase induction were compared between the two populations of BMP2 (Fig. 3; bars 2, 3, 4, 5, 6, and 7). The values obtained for alkaline phosphatase activity in samples where the cells were cultured with 7.2 ng/ml or 12.2 ng/ml were not significantly different, suggesting that alkaline phosphatase was maximally activated in these cells in response to the lower amount of protein. Therefore the values obtained for alkaline phosphatase activity from the sample in which the cells received 3 ng/ml of rhBMP2 was compared to the sample in which the cells received 1.2 ng/ml of BMP2 expressed after viral infection (Fig. 3; bars 2 and 5). For comparison between samples a specific activity of each sample was determined by defining a unit of BMP2 activity to be equivalent to 1,000 relative chemiluminescence units (RLU) (Table I). The results indicate that the protein expressed as a result of viral infection is not only as potent as the recombinant protein but produces a three fold greater induction in alkaline phosphatase activity (Table I).

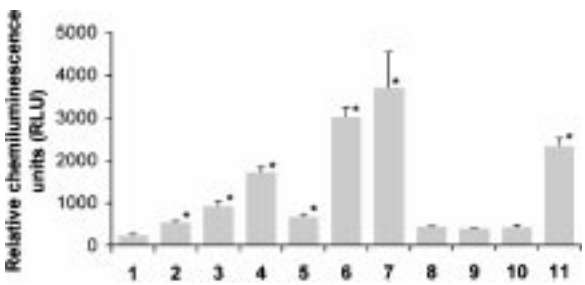


Fig. 3. Alkaline phosphatase activity in W20-17 cell with no additions (bar 1), 3 ng/ml (bar 2), 8 ng/ml (bar 3), and 16 ng/ml (bar 4) recombinant BMP2, 8 μl/ml (bar 5), 50 μl/ml (bar 6), and 85 μl/ml (bar 7) conditioned media from A549 cells infected with either Ad5BMP2 (5,000 particles/cell), 8 μl/ml (bar 8), 50 μl/ml (bar 9), and 83 μl/ml (bar 10) conditioned media from A549 cells infected with Ad5GFP (5,000 particles/cell), or conditioned media (83 μl) from A549 cells infected with Ad5GFP (5,000 particles/cell) and 10 ng of recombinant BMP2 (bar 11). Alkaline phosphatase activity is depicted as the average relative chemiluminescences units (RLU) where n = 3. Error bars represent means ± SD for n = 3. A student *t*-test was applied to demonstrate significance and all the samples demonstrated significance at the confidence interval $P < 0.05$ ($^*P < 0.01$).

W20-17 cells were also cultured with 8 μl, 50 μl, and 83 μl of culture supernatant from A549 cells infected with a control virus, Ad5GFP (5,000 particles/cell). The results showed a slight elevation in the samples cultured with the Ad5GFP culture supernatant as compared to the results obtained for the control W20-17 cells, demonstrating that adenovirus or A549 cells may secrete a factor that can enhance alkaline phosphatase activity (Fig. 3; bars 1, 8, 9, and 10). Further, cells were cultured with 83 μl of culture supernatant from A549 cells infected with Ad5GFP (5,000 particles/cell) along with 17 ng/ml of hrBMP2 protein (Fig. 3; bar 11) to determine if an inhibitor of BMP2 is present in the culture supernatants. The level of alkaline phosphatase induction in these samples was slightly elevated, similar to what was found for the culture supernatant alone, as compared to the level found in samples cultured with 17 ng/ml of rhBMP2 alone (Fig. 3; bars 4 and 11). The results demonstrate that the culture supernatants from cells infected with adenovirus do not contain inhibitors of this assay or BMP2.

Optimization of BMP2 Expression From Bone Marrow Stromal Cells

Knowing that the Ad5BMP2 construct could express active BMP2 protein, we next wanted to optimize this expression in bone marrow cells. The results of Western blot analysis of the W20-17 mouse bone marrow stromal cell extracts and precipitated supernatants showed the level of BMP2 protein, produced after viral infection of W20-17 cells to be below the level of Western blot detection by this method (< 5 ng/ml) (data not shown). These results were surprising since A549 cells infected with Ad5BMP2 at an equivalent MOI secreted a significant amount of BMP2 (Fig. 2). Also, since no BMP2 was detected in the W20-17 cell extracts it is unlikely that the W20-17 cells have sequestered the protein from the media.

Although levels of BMP2 produced after infection of W20-17 cells with Ad5BMP2 are too low for detection by Western blot, the more sensitive bioassay revealed that activity was present. Ad5BMP2 infection (5,000 particles/cell) of W20-17 cells led to a three fold elevation in their alkaline phosphatase activity, which is similar to the induction seen when the cells are cultured with 10 ng/ml hrBMP2 (Fig. 4A; bars 1 and 4). No such effect was seen with Ad5GFP.

TABLE I. Comparison of Specific Activities of Adenovirus Produced and hrBMP2

	Concentration (ng/ml)	Alkaline phosphatase activity(RLUs/ml)	BMP2 activity (units[a]/ml	Specific Activity[a] (units/ng
rhBMP2	3	175,600	175.6	58.5
Virally produced BMP2	1.2	212,300	212.3	176.9

[a]A unit of BMP2 activity is equal to 1000 RLUs of alkaline phosphatase activity.

This induction could be increased to 12 fold in the adenovirus infected W20-17 cells by extending the culture time from 3 days post infection to 6 days post infection (Fig. 4B, bars 1,2,3,4). Interestingly, the response to long term culture with hrBMP2 did not significantly change (Fig. 4A and B). This increase is not due to re-infection with the virus since the cells were washed and fresh media applied immediately following a 12 h adsorption of the virus. These data suggest that W20-17 cells upon infection do indeed synthesize BMP2 and that synthesis increases when culture periods are extended from 3 days (3 fold induction of alkaline phosphatase over controls) to 6 days (12 fold induction of alkaline phosphatase activity over controls).

BMP2 Production in Primary Bone Marrow Stromal Cells

Next we examined Ad5BMP2 mediated BMP synthesis in primary stromal cells. We first established primary cell culturtes of human bone marrow cells. Initial CFU-fs from the purified mononuclear cells were expanded for several days in culture, and were maintained at sub-confluent densities prior to experimentation. Equivalent cell numbers (5×10^4 cells/cm^2) were either immediately infected with Ad5BMP2 (5,000 particles/cell), cultured for 4 days in 1 μM dexamethasone prior to infection with Ad5BMP2 (5,000 particle/cell), or cultured for 4 days with 1 μM dexamethasone followed by 2 weeks in 50 μg/ml ascorbic acid prior to infection with Ad5BMP2 (5,000 particles/cell). In all cases cell extracts were collected 3 days after infection and immunoblotted for BMP2. Culture media was not analyzed due to the inclusion of serum (see Methods section for details). The results show the absence of BMP2 expression in undifferentiated cells (Fig. 5A; lane 2), but the presence of BMP2 in cells cultured with either dexamethasone or dexamethasone followed by ascorbic acid (Fig. 5A; lane 3 and 4). Lane 1; Figure 5 depicts the signal detected by Western blot analysis of hrBMP2 (50 ng). Cell extracts from uninfected cells cultured under the same conditions had no detectable BMP2 by immunoblotting. The data demonstrate that dexamethasone treatment of human bone marrow stromal cells results in expression of BMP2.

Then we infected primary bone marrow stromal cells with Ad5BMP2 (5,000 particles/

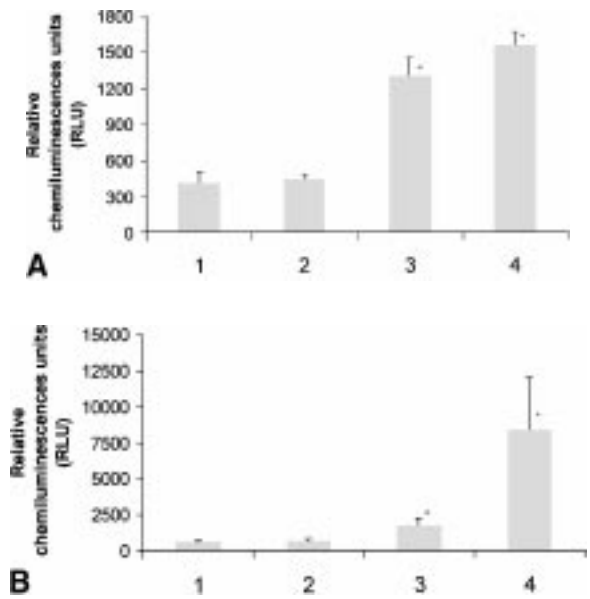


Fig. 4. **A:** Alkaline phosphatase activity was measured in W20-17 cell 3 days after; culturing with no additions (bar 1), infection with 5,000 particles/cell Ad5GFP (bar 2), culturing with 10 ng recombinant BMP2 (bar 3), or infection with Ad5BMP2 (bar 4). Alkaline phosphatase activity is depicted as the average relative chemiluminescences units (RLU) where n = 3. Error bars represent means ± SD for n = 3. A Student's *t*-test was applied to demonstrate significance (*$P < 0.05$). **B:** Alkaline phosphatase activity was measured in W20-17 cell 6 days after; culturing with no additions (bar 1), infection with 5,000 particles/cell Ad5GFP (bar 2), culturing with 10 ng recombinant BMP2 (bar 3), or infection with Ad5BMP2 (bar 4). Alkaline phosphatase activity is depicted as the average relative chemiluminescences units (RLU) where n = 3. Error bars represent means ± SD for n = 3. A Student's *t*-test was applied to demonstrate significance (*$P<0.05$).

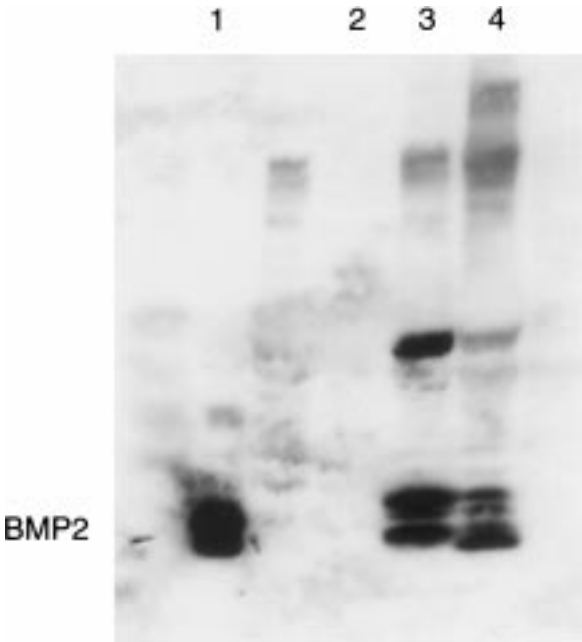


Fig. 5. Western blot analysis for BMP2 in Ad5BMP2 infected human bone marrow stromal cell cultures or Ad5BMP2 infection of bone marrow cultures that were induced to differentiate. Recombinant BMP2 (50 ng) was used as a standard (**lane 1**). Bone marrow stromal cells were cultured with; no additions (**lane 2**), 1 μM dexamethasone for three days (**lane 3**), or 1 μM dexamethasone for three days followed by two weeks with 50 μg/ml ascorbic acid (**lane 4**) and then infected with Ad5BMP2 (5,000 particles/cell). The samples were normalized for total cellular protein prior to electrophoresis.

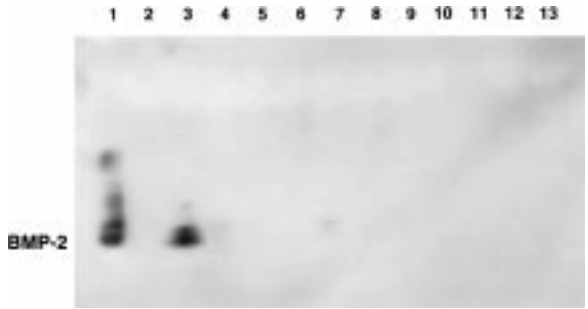


Fig. 6. Western blot analysis for BMP2 in human bone marrow stromal cells subjected to culturing in the various conditions for two days. One μM dexamethasone (**lane 2**), 1 μM dexamethasone followed by infection with Ad5BMP2 (**lane 3**), infection with Ad5BMP2 followed by addition of 1 μM dexamethasone for two days (**lane 4**), or infection with Ad5BMP2 alone (**lane 5**) and human bone marrow stromal cells subjected to culturing in the various conditions for four days; 1 μM dexamethasone (**lane 6**), 1 μM dexamethasone followed by infection with Ad5BMP2 (**lane 7**), infection with Ad5BMP2 followed by addition of 1 μM dexamethasone for four days (**lane 8**), or infection with Ad5BMP2 alone (**lane 9**) and human stromal cells subjected to culturing in the various conditions for six days; 1 μM dexamethasone (**lane 10**) 1 μM dexamethasone followed by infection with Ad5BMP2 (**lane 11**), infection with Ad5BMP2 followed by addition of 1 μM dexamethasone for 6 days (**lane 12**), or infection with Ad5BMP2 alone (**lane 13**). Recombinant BMP2 (50 ng) was used as a standard (**lane 1**). Equivalent numbers of cells were plated for all samples and Western blot analysis for BMP2 in duplicate samples yielded similar results.

cell) before or after treatment with 1 μM dexamethasone. The results (Fig. 6) show that stromal cell extracts, which are pretreated with 1 μM dexamethasone for 2 days prior to infection with Ad5BMP2, produced approximately 20 fold more BMP2 than cells preinfected with Ad5BMP2 prior to being differentiated with dexamethasone.

A small but detectable amount of BMP2 was identified in Ad5BMP2 transduced untreated stromal cells (Fig. 6; lane 5), suggesting that these cells are capable of synthesizing mature BMP2 at a level that is greatly reduced when compared to the dexamethasone-treated cells. Interestingly, as the culturing times in dexamethasone increased, the amount of BMP2 produced appears to decrease (Fig. 6; lanes 6–13) which is not seen when the cells are further differentiated into osteoblasts by culturing with 50 μg/ml ascorbic acid (Fig. 5; lane 4). Also, when the stromal cells were maintained at confluent levels for several days prior to the start of this experiment, we were unable to detect BMP2 in any of the samples by Western blot analysis.

DISCUSSION

Recent studies have demonstrated the ability to induce bone formation, hetero or orthotopically by the delivery of osteoprogenitor cells transduced with viral vectors to express BMP2 [Lieberman et al., 1998, 1999; Lou et al., 1999; Musgrave et al., 1999]. Much attention is now being focused on the development of these gene therapy techniques for eventual use in the clinic. With the essential need to provide safe and effective gene delivery systems, we have chosen to focus on optimizing a cell based adenovirus delivery system which can produce high levels of active BMP2.

The first set of experiments demonstrated the ability of Ad5BMP2 to produce and secrete BMP2 in A549 cells. The concentration of BMP2 in media increased when cells were infected with escalating doses of Ad5BMP2.

Further studies using a cell based assay to measure BMP2 responsiveness confirmed that the virally synthesized protein was functionally active. Surprisingly, BMP2 produced by viral infection appeared to be consistently three fold more active than an equivalent concentration of recombinant BMP2. The difference in activity is not due to protein instability of the recombinant protein versus the crude media extracts of the BMP2 produced by virally infected cells, since purified hrBMP2 diluted and stored in culture supernatants from A549 cells was found to yield consistent results upon long term storage and freeze-thawing. A more likely explanation is that the purification of the recombinant protein may lead to a slight loss in activity.

With confirmation that the construct could produce functional BMP2 in cells, we next measured its expression after transduction of bone marrow stromal cells with the Ad5BMP2 construct. Since adenovirus type 5 has a limited tropism for cells in whole bone marrow we chose to focus on bone marrow stromal cells [Frey et al., 1998; Shayakhmetov et al., 2000]. Several recent reports in the literature document the production of bone, in vivo, in response to implantation of Ad5BMP2 transduced W20-17 mouse bone marrow stromal cells [Lieberman et al., 1999; Engstrand et al., 2000]. Therefore, we chose to focus on optimization of the W20-17 cells. We used an amount of virus found optimal in preliminary dose-ranging experiments using AdGFP. Results showed a dramatic decrease in BMP2 production in either adenovirus transduced human bone marrow stromal cells or the mouse stromal cell line W20-17 to levels undetectable by Western blot analysis as compared to the A549 cell line. We have also demonstrated the ability to dramatically increase the level of expression of mature BMP2 in the human bone marrow stromal cells by dexamethasone treatment, which is known to induce the osteoblast phenotype.

Other researchers have demonstrated the expression of BMP2 from bone marrow stromal cells. Lieberman et al., 1998, detected BMP2 expression from both the W20-17 cells as well as primary mouse bone marrow stromal. In these experiments the authors start by infecting 80 times more W20-17 cells than in our experiments, and then purifying the BMP2 from 20 ml of culture media using heparin-Sepharose beads, prior to detection by immunoblotting [Lieberman et al., 1998]. Interestingly, the authors report that the expression of BMP2 decreases with time. By contrast we see a significant increase in BMP2 production with A549 cells over time (data not shown) and have found the BMP2 in culture media to be stable. Further our results comparing alkaline phosphatase induction in W20-17 cells shows a significant increase in cells that were cultured for 6 days vs. 3 days post-infection. We propose that this increase in alkaline phosphatase activity may be due to BMP2 induced differentiation of W20-17 cells, and that the further differentiation of these cells may result in a greater ability of the cell to produce BMP2.

Recently, Engstrand et al., 2000 generated, through retroviral insertion and transduction, a W20-17 cell line that constitutively expressed high levels of BMP2. To generate this cell line the authors selected for cells that had incorporated the retrovirus into their genome over several weeks time. Due to the long term exposure to BMP2 during this process, the differentiation state of the W20-17 cells may have been altered. The W20-17 cells have been shown to become osteoblast-like cells after exposure to BMP2 in culture [Thies et al., 1992].

The exact mechanism of attenuation of BMP2 expression in osteoprogenitor cells remains unclear. One potential step for regulation may involve processing of the precursor BMP2 molecule. Expression of the protease that cleaves the BMP2 precursor may be highly regulated during cell differentiation, this would then lead to regulation of the amount of active BMP2 that the cells could secrete.

Musgrave et al., 1999, demonstrated the production of ectopic bone in the mouse muscle after direct injection of Ad5BMP2. Interestingly, the authors admit that they were unable to detect expression of the BMP2 protein in the muscle, but suggested rapid protein diffusion and clearance of the virus as possible explanations. Another possible explanation may be that myoblasts like bone marrow stromal cells are deficient in adenovirus-mediated BMP2 synthesis. Interestingly, Baltzer et al., 2000, have achieved significant bone formation with direct injection of Ad5BMP2. However, in these experiments the Ad5BMP2 vector was delivered to a fracture site which may not only possess a large number of progenitor cells but also differentiated osteoblasts that can most likely synthesize BMP2 upon adenovirus transduction.

Our results demonstrate that infection of bone marrow stromal cells with Ad5BMP2 prior to induction of differentiation greatly decreases the production of BMP2 relative to cells that have been infected after they were induced to differentiate. One possible explanation may be that differentiation of the cells up-regulates the receptor for adenovirus. Recently Rebel et al., 2000, reported the up-regulation of CAR during differentiation of CD34+ cells to more committed progenitor cells such as bone marrow stromal cells. Another explanation may be that the high levels of adenovirus infection block cellular differentiation and in turn prevent synthesis of BMP2. MacKenzie et al., 2000, reports that with high multiplicity of infection, similar to the levels used in our experiments, approximately 79% of CD34+ cells were transduced with adenovirus type 5 even in the absence of CAR, yet less than 50% of these cells were viable to continue differentiation. Others have shown a decrease in cell growth with escalating doses of adenovirus type 5 [Teramoto et al., 1999] and the phenomenom of multiplicity dependent reactivation i.e., the replication of the virus in the absence of one or more gene products is well known with adenovirus as is non-specific cytolysis at high viral doses.

In conclusion, results of the study show that there is a deficiency in Ad5BMP2-mediated synthesis of BMP2 in bone marrow stromal cells. Although this paper does not address the mechanisms involved, it points out some of the possibilities that may account for this phenomenon. Indeed, more than one of these mechanisms may be operative. It is conceivable that improved adenovirus-mediated BMP2 synthesis upon transduction of bone marrow stromal cells, either in vitro or in vivo, may improve the safety and efficacy of this potential treatment modality. We believe that this work provides crucial information for the eventual clinical development of a safe and efficacious system for the production of bone.

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