

# Effects of osteogenic inducers on cultures of canine mesenchymal stem cells

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**Objective**—To examine age-related efficacy of bone morphogenetic protein (BMP)-2, ascorbate, and dexamethasone as osteogenic inducers in canine marrow-derived stromal cells (MSCs).

**Sample Population**—Samples of femoral bone marrow obtained from 15 skeletally immature (< 1 year old) and 4 skeletally mature (> 1.5 years old) dogs.

**Procedure**—First-passage canine MSC cultures were treated with 100 µg of ascorbate phosphate/mL, 10<sup>-7</sup>M dexamethasone, 100 ng of BMP-2/mL, or a combination of these osteoinducers. On day 6, cultures were harvested for quantitation of alkaline phosphatase (ALP) activity and isolation of RNA to prepare cDNA for real-time polymerase chain reaction analyses of osteoblast markers.

**Results**—Early markers of osteogenesis were induced in canine MSCs by BMP-2 but not dexamethasone. In young dogs, the combination of BMP-2 and ascorbate yielded the highest ALP mRNA concentrations and activity. This combination also induced significant increases in mRNA for osteopontin and runt-domain transcription factor 2. In comparison to MSCs from immature dogs, those from mature dogs had diminished ALP activity in response to BMP and ascorbate. Results for cultures treated with 3,4-dihydroxyproline suggested that ascorbate-induced production of extracellular matrix was important for maximal BMP-2 response in canine MSCs.

**Conclusions and Clinical Relevance**—BMP-2 was capable of inducing markers of osteogenesis in short-term cultures of canine MSCs. In MSCs obtained from skeletally immature dogs, ascorbate was required for maximal effects of BMP. These results define optimal conditions for stem cell osteogenesis in dogs and will facilitate development of stem cell-based treatments for dogs with fractures. (*Am J Vet Res* 2005;66:1729–1737)

Development of a multicellular organism is characterized by the progression from cellular multipotency to the differentiated phenotypes of specific tissues necessary for specialized life-sustaining functions. In postnatal life, repair of injured tissues is not possible

without stem cells capable of replication and differentiation at sites of healing.<sup>1</sup> The osteogenic differentiation of inducible multipotential stem cells is a key component of the processes of fracture repair and bone remodeling required to maintain skeletal integrity.<sup>2</sup> These inducible stem cells are of major interest for their promise as cell-based treatments to augment fracture repair or attenuate the attrition of bone in diseases such as osteoporosis.<sup>3-7</sup>

Bone marrow is recognized as a ready source of multipotential mesenchymal cells capable of differentiating into osteoblasts, chondrocytes, adipocytes, fibroblasts, and hematopoietic supporting cells.<sup>8,9</sup> These marrow-derived stromal cells (MSCs) display inducible osteogenesis during in vitro and in vivo studies.<sup>9-12</sup> Cultured in medium containing the appropriate inducers, these cells express markers collectively associated with the osteogenic phenotype (eg, type I collagen, osteopontin, alkaline phosphatase (ALP), and bone sialoprotein). During in vivo studies,<sup>3,4,10,13,14</sup> MSCs implanted alone or on matrices accelerate repair of craniofacial and critical-size long-bone defects in small and large animals. Impressive osteogenesis has been achieved by use of MSCs engineered to produce growth factors.<sup>7,10</sup> Because of their proliferative capacity and potential for multiple-lineage differentiation, MSCs can provide an autologous cell source for therapeutic strategies for tissue repair and engineering in postnatal life.

For in vitro studies, the most common inducers used to initiate osteogenesis are bone morphogenetic proteins (BMPs) and glucocorticoids, such as dexamethasone.<sup>15-22</sup> Although inducible osteogenesis is a property of MSCs isolated from all species investigated, the efficacy of specific osteogenic inducers in MSCs varies in a species-dependent manner.<sup>22,23</sup> Although BMP-2 or dexamethasone will promote osteoblast formation from rat MSCs, BMP is far less effective than dexamethasone in human MSCs, and the converse is true for mouse MSCs.

A large number of studies<sup>24-34</sup> in which dogs have been subjected to orthopedic techniques have revealed that BMPs can accelerate bone healing during craniofacial reconstruction, fracture and gap-defect repair, and spinal fusion in dogs. Treatment with mesenchymal stem cells has also received attention for its ability to enhance fracture and gap-defect repair in dogs.<sup>3,4</sup> Although canine MSCs can be expanded in vitro and osteogenesis induced by use of a combination of ascorbate and dexamethasone in long-term cultures,<sup>11</sup> the effect of specific inducers on osteogenesis of canine MSCs has not been defined.

Therefore, the purpose of the study reported here was to compare the efficacy of BMPs and dexametha-

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son, with and without ascorbate, on the induction of osteogenesis in canine MSCs in vitro. Efficacy was assessed by examining expression of several early markers of osteogenesis, including ALP, osteopontin, and runt-domain transcription factor (RUNX)2 (ie, core binding factor 1), by use of real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and ALP activity assays. In addition, we investigated the mechanism of ascorbate enhancement of BMP-induced osteogenesis in canine MSCs.

## Materials and Methods

**Sample population**—Marrow stromal cells were harvested from skeletally immature and mature dogs. The skeletally immature population comprised 15 purpose-bred mixed-breed dogs (minimum age, 6 days; maximum age, 31 weeks; mean  $\pm$  SD, 6.1  $\pm$  2.1 weeks). All research dogs were raised and cared for in accordance with National Institutes of Health and USDA guidelines for the care and use of animals in research. Research dogs used for this study were the subjects of unrelated research projects, and no additional research animals were used solely for this study.

Marrow stromal cells were also obtained from 4 skeletally mature dogs (range, 2 to 8.5 years). Three of these dogs were client-owned dogs undergoing surgery for total hip replacement. All owners of client-owned dogs provided informed consent for participation in this study. The remaining dog was from the previously described research population. The protocol for procuring bone marrow and care and use of all dogs was approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania.

**Isolation and culture of MSCs**—Marrow stromal cells were obtained from the femoral medullary cavities of skeletally mature dogs by aspiration of the femoral canal of dogs during surgery for total hip replacement. Marrow stromal cells were obtained from skeletally immature dogs and the single mature dog by flushing the medullary canal of femurs harvested immediately after the dogs were euthanized as part of other research projects.

Marrow samples were kept on ice for transport. All subsequent processing was performed at 25°C unless otherwise indicated. Each marrow sample was suspended in  $\alpha$  minimal essential medium ( $\alpha$ MEM) and centrifuged to remove the fatty fraction. Marrow cells were layered on a hydrophilic polysaccharide gradient<sup>a</sup> and centrifuged for 30 minutes at 1,900  $\times$  g to concentrate nucleated cells at the interface. The mononuclear cell fraction was collected and washed once with  $\alpha$ MEM, and the cell number was determined by use of a hemacytometer. Primary marrow cultures were established (day 0) at a density of 5  $\times$  10<sup>5</sup> cells/cm<sup>2</sup> in  $\alpha$ MEM with 10% fetal calf serum and antimicrobials with or without dexamethasone. An initial change of medium was performed on day 4 and then at intervals of 2 or 3 days. Immediately before cells reached confluence (approx day 7), adherent cells were transferred to another culture at a concentration of 1  $\times$  10<sup>4</sup> cells/cm<sup>2</sup>, and these first-passage cultures were used for all experiments. Because the study focused on use of first-passage cells for each experiment, limited cell numbers precluded all assays being performed on samples from each dog. Priority was given to cells used for measurement of ALP activity and mRNA analyses.

Twenty-four hours after first-passage cultures were established, designated cultures were supplemented with 10<sup>-7</sup>M dexamethasone,<sup>b</sup> ascorbate-2-phosphate<sup>c</sup> (100  $\mu$ g/mL), BMP-2<sup>d</sup> (100 ng/mL, unless otherwise indicated), or combinations of these osteogenic inducers. Medium was exchanged for medium containing fresh osteogenic inducers at intervals of 2 to 3 days. Cultures were incubated for 6 days

and then harvested for use in isolation of total RNA or measurement of ALP activity. For assays focusing on ascorbate mechanism, N-acetylcysteine<sup>e</sup> (NAC; 1.5mM) and 3,4-dehydroproline<sup>f</sup> (3,4-DHP; 0.5mM) were added to first-passage cultures at day 1 and at each change of medium.

**Assay of ALP activity**—Immediately before cells were harvested for assay of ALP activity, the number of viable cells was determined by use of a tetrazolium (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium inner salt [MTS])-based assay<sup>g</sup> for use in adjusting ALP activity. Cell layers were then washed twice with Hank's buffered saline solution followed by determination of ALP activity as described elsewhere.<sup>16</sup>

**Isolation of RNA and reverse transcriptase reactions**—Total RNA was isolated by use of reagent<sup>h</sup> in accordance with the manufacturer's protocol. Briefly, separation of the aqueous and organic phases was achieved by the addition of 1-bromo-3-chloropropane. The RNA was precipitated from the aqueous phase by use of isopropanol, and the precipitated RNA pellet was washed in ice-cold 75% ethanol. Total RNA quality and quantity were determined spectrophotometrically, and samples were stored at -80°C. Complementary DNA was prepared from 2  $\mu$ g of total RNA by use of oligo(dT) and a commercially available RT-PCR kit.<sup>i</sup>

**PCR assay**—Primer sets for determining mRNA concentrations by use of real-time RT-PCR assays were prepared from primer sequences selected from known canine gene sequences or those that were homologous between humans and dogs.<sup>35</sup> Primer sets used were as follows: canine ALP (forward primer, 5'-TCA AAC CGA GAC ACA AGC AC-3'; reverse primer, 3'-TCC TTG TTG CAC TGA CTG GG-5'), osteopontin (forward primer, 5'-GAT GGC CGA GGT GAT AGT GT-3'; reverse primer, 3'-AGG ATG CAA AGG GTC CTG TC-5'), RUNX2 (forward primer, 5'-GAT GAT GAC ACT GCC ACC TCT G-3'; reverse primer, 3'-CGT CAA GGG TTC GTA AAG TAG GG-5'), and hypoxanthine phosphoribosyl transferase (HPRT; forward primer, 5'-GGT GGA GAT GAC CTC TCA AC-3'; reverse primer, 3'-TCG GAT TCT ACT CTC AAG-5').

Samples of cDNA were amplified and measured by use of real-time PCR assays<sup>j</sup> in accordance with the manufacturers' protocols. Amplifications consisted of an initial incubation at 95°C for 10 minutes followed by 45 cycles of denaturation at 95°C for 15 seconds, annealing at primer-specific annealing temperatures for 30 seconds, and extension for 30 seconds at 72°C. The final melt-curve step was from 60° to 96°C at a rate of 0.2°C/s. The annealing temperature was calculated for each primer pair (ALP and RUNX2 at 60°C, osteopontin at 61°C, and HPRT at 59°C). Results from real-time RT-PCR assays were converted to arbitrary units as described elsewhere.<sup>23</sup>

**Statistical analyses**—Statistical analyses for significant differences in enzyme activity and mRNA concentrations were performed by use of paired or unpaired *t* tests, when appropriate, in a statistical program.<sup>m</sup> Values of *P* < 0.05 were considered significant.

## Results

**Induction of ALP activity in MSCs from skeletal immature and mature dogs**—For an initial assessment of the separate and collective effects of BMP-2, ascorbate, and dexamethasone on osteogenesis of canine MSCs, extracts from cultures of MSCs on day 6 were assayed for ALP activity. The influence of age of marrow donor on osteoinduction was analyzed by examining 2 populations of dogs. Responses of MSCs obtained from the femoral cavity of immature dogs

were compared with responses of MSCs obtained from skeletally mature dogs (Figure 1).

The addition of BMP alone or in combination with ascorbate consistently resulted in increased ALP activity. Although ascorbate alone caused a significant 2.5-fold increase in mean ALP activity, BMP alone induced a significant 25-fold increase in mean ALP activity. A combination of ascorbate and BMP resulted in a synergistic effect, yielding ALP activity that was nearly 85-fold higher than control values ( $P < 0.001$ ). Specific dogs had varied responses to BMP, with induction of ALP activity ranging from approximately a 2- to 240-fold increase (mean  $\pm$  SD fold increase,  $27.2 \pm 61.6$ ), relative to control values, whereas a combination of BMP and ascorbate induced responses ranging between a 20- and 200-fold increase (mean  $\pm$  SD fold increase,  $101 \pm 66.9$ ).

In contrast to its effect on human MSCs,<sup>22</sup> dexamethasone alone or in combination with ascorbate did not increase ALP activity above control values (Figure 1). In cultures treated with BMP, the addition of dexamethasone consistently decreased ALP activity. Thus, this glucocorticoid did not act as an inducer of ALP activity in canine MSCs and had a modest inhibitory effect. Addition of dexamethasone to primary cultures also did not significantly affect BMP potency in the subsequent first-passage cultures. Mean  $\pm$  SEM ALP

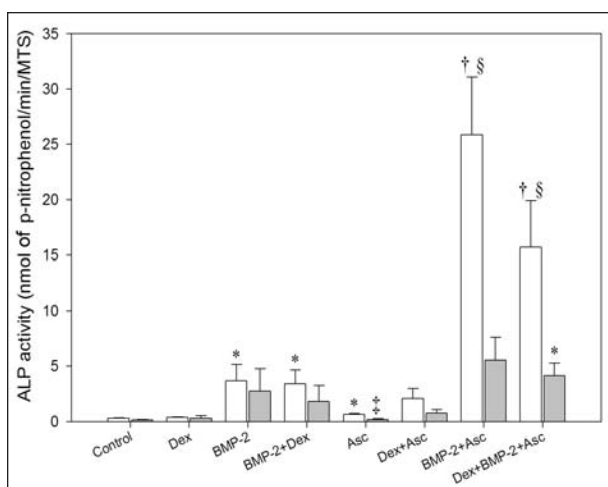


Figure 1—Mean  $\pm$  SEM adjusted alkaline phosphatase (ALP) activity as a result of culture of first-passage marrow-derived stromal cells (MSCs) for 6 days with each osteogenic inducer alone or a combination of osteogenic inducers. The MSCs were obtained from 15 skeletally immature (< 1 year old; white bars) and 4 skeletally mature (> 1.5 years old; gray bars) dogs. The ALP activity was adjusted on the basis of cell numbers determined by use of a tetrazolium-based assay. Notice that bone morphogenetic protein (BMP)-2 was the most reliable inducer of ALP activity, especially in cultures of MSCs obtained from young dogs. A combination of BMP-2 and ascorbate (Asc) in cultures of MSCs obtained from young dogs markedly increased ALP activity, compared with values for cultures treated with BMP-2 alone. Dexamethasone (Dex) alone or in combination with other osteogenic inducers had no apparent influence on ALP activity after culture for 6 days. \*†Within an age group, value differs significantly ( $*P < 0.05$ ;  $†P \leq 0.001$ ) from the value for the control group. ‡Within a treatment group, value differs significantly ( $P < 0.05$ ) from the value for the MSCs obtained from the young dogs. §Within the MSCs obtained from the young dogs, value differs significantly ( $P < 0.01$ ) from the value with and without Asc. MTS = 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt.

activity in 9 BMP-treated first-passage MSC cultures was  $6.34 \pm 2.51$  and  $8.75 \pm 4.04$  nmol of p-nitrophenol/min/MTS for cells cultured with and without dexamethasone in primary cultures, respectively.

Age of donor had little effect on ALP activity when cells were cultured without ascorbate (Figure 1). However, ascorbate-treated cultures from immature and mature donors differed significantly in ALP activity, with cultures from immature dogs having a greater response to ascorbate, compared with the response for cultures from mature dogs. The decreased impact of ascorbate in mature dogs was apparent in control and dexamethasone-treated cultures but was most dramatic in BMP-treated MSCs; adding ascorbate to BMP-containing cultures caused a 7-fold increase in MSCs from young dogs but only a 2-fold increase in MSCs from mature dogs. Analysis of these results indicated that the ability of BMP alone to promote expression of ALP in canine MSCs is dependent on age, with MSCs from younger dogs requiring ascorbate and BMP for maximal stimulation.

**Analysis of mRNA concentrations of early markers of osteogenesis**—To complement our data on ALP activity, mRNA concentrations of ALP and 2 additional early markers of osteogenesis (ie, osteopontin and RUNX2) were analyzed. Results from real-time RT-PCR analysis of ALP mRNA concentrations in MSCs from young dogs, adjusted on the basis of concentrations of HPRT mRNA, were summarized (Figure 2). The adjusted ALP mRNA results were similar to the induction pattern for ALP activity. Treatment with BMP was required to obtain a significant increase in ALP mRNA content, and dexamethasone was not an inducer of ALP expression. The combination of BMP-2 and ascorbate was much more effective (increasing ALP mRNA

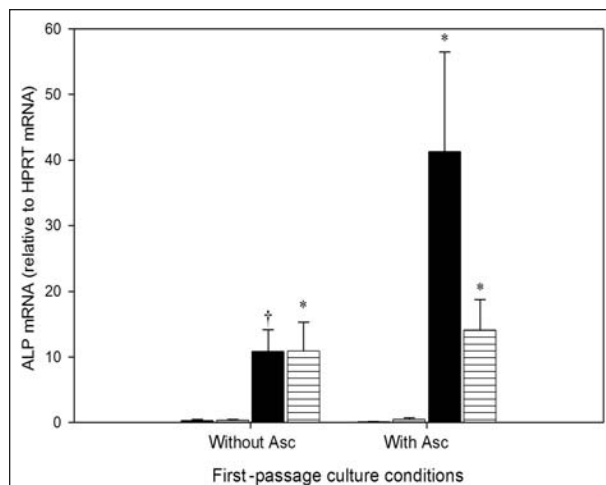


Figure 2—Mean  $\pm$  SEM ALP mRNA concentrations as a result of culture of first-passage MSCs for 6 days with or without Asc and no osteogenic inducers (control treatment; white bars), BMP-2 (black bars), Dex (gray bars), or a combination of Dex and BMP-2 (horizontal striped bars). The MSCs were obtained from 9 skeletally immature dogs. The ALP results were determined by use of real-time reverse transcriptase-polymerase chain reaction assays and adjusted on the basis of hypoxanthine phosphoribosyl transferase (HPRT) mRNA concentrations assayed in the same samples. \*†Within a treatment group, value differs significantly ( $*P < 0.05$ ;  $†P = 0.01$ ) from the value for the control group.

concentrations approx 150-fold relative to control values) than BMP alone.

The combination of BMP and ascorbate also significantly increased mRNA concentrations of osteopontin and RUNX2 (Table 1). Bone morphogenetic protein was also capable of inducing osteopontin mRNA and did not appear to require ascorbate for this effect. Bone morphogenetic protein was an inducer of osteopontin expression, and combining BMP-2 with ascorbate significantly ( $P = 0.01$ ) increased osteopontin concentrations relative to control values. Dexamethasone attenuated the high osteopontin mRNA concentrations induced by BMP and a combination of BMP and ascorbate to values similar to those for control cultures.

We also examined the effect of the 3 osteogenic inducers on RUNX2, a transcription factor expressed early in osteogenesis and required for bone formation.<sup>36</sup> Regulation of RUNX2 is similar to that for the other markers in that BMP was an effective inducer and dexamethasone was mildly inhibitory. In addition, ascorbate alone was capable of increasing RUNX2 mRNA concentrations above those for control cultures.

The efficacy of BMP-2 and dexamethasone, with and without the addition of ascorbate, as osteogenic inducers of canine MSCs was summarized (Table 2). Species-specific differences in response to these inducers among dogs, humans, rats, and mice were highlighted. The response of canine MSCs to BMP-2 and

dexamethasone was most similar to that seen in the mouse and contrasted the weak BMP-2 and strong dexamethasone responses by human MSCs previously reported by our laboratory as well as others.<sup>15,16,22,37,38</sup>

#### BMP-2 dose response in cultured canine MSCs—

Because of the wide range of BMP-2 dosages used in vitro for numerous species, we developed an ALP activity dose-response curve of immature canine MSCs for BMP-2 with and without ascorbate (Figure 3). Without ascorbate, BMP concentrations in the range of 20 to 100 ng/mL significantly increased ALP activity, compared with values for cultures without BMP, but ALP activity was relatively low at all BMP concentrations. When BMP was used in combination with ascorbate, a significant ( $P < 0.005$ ) increase in ALP activity was evident for each increase of 20 ng/mL in BMP-2 concentration up to 80 ng/mL. The dose-response curve highlighted the ability of ascorbate to augment BMP-2 induction of ALP activity in these cells; for all concentrations of BMP-2 tested, the addition of ascorbate dramatically increased ALP activity.

**Effects of ascorbate and required formation of the extracellular matrix on induction of osteogenic markers—**To investigate the mechanism of ascorbate augmentation of BMP-2-induced osteogenesis in canine MSCs, cultures were prepared that contained 3,4-DHP or NAC. Addition of 3,4-DHP inhibits proline hydroxylation and prevents secretion of collagen fibrils

Table 1—Mean  $\pm$  SEM mRNA concentrations of osteogenic markers as a result of culture of first-passage marrow-derived stromal cells for 6 days with various osteogenic inducers.

Osteogenic inducer	Alkaline phosphatase (9)	Osteopontin (8)	RUNX2 (7)
Control	0.28 $\pm$ 0.15	0.03 $\pm$ 0.01	1.33 $\pm$ 0.14
Ascorbate	0.09 $\pm$ 0.05	0.03 $\pm$ 0.03	3.11 $\pm$ 1.70
Dexamethasone	0.33 $\pm$ 0.13	0.02 $\pm$ 0.00	0.71 $\pm$ 0.19
Dexamethasone and ascorbate	0.44 $\pm$ 0.28	0.12 $\pm$ 0.12	2.04 $\pm$ 0.99
Dexamethasone and BMP-2	10.89 $\pm$ 4.40*	0.02 $\pm$ 0.01	2.75 $\pm$ 1.19 <sup>a</sup>
BMP-2	10.85 $\pm$ 3.30†	1.58 $\pm$ 1.50	9.26 $\pm$ 4.53 <sup>b</sup>
BMP-2 and ascorbate	41.24 $\pm$ 15.29*	1.64 $\pm$ 0.35†, <sup>c</sup>	4.57 $\pm$ 1.78*
Dexamethasone, BMP-2, and ascorbate	14.10 $\pm$ 4.69*	0.08 $\pm$ 0.06 <sup>d</sup>	2.39 $\pm$ 0.85

Results reported were determined by use of real-time reverse transcriptase-polymerase chain reaction assays and adjusted on the basis of hypoxanthine phosphoribosyl transferase mRNA concentrations assayed in the same samples. Numbers in parentheses indicate the number of skeletally immature (< 1 year old) dogs from which stem cells were obtained.

\*†Within a column, value differs significantly ( $*P < 0.05$ ;  $\dagger P = 0.01$ ) from value for the control treatment. RUNX2 = Runt-domain transcription factor 2. Ascorbate = 100  $\mu$ g of ascorbate phosphate/mL. Dexamethasone = 100nM dexamethasone. BMP-2 = 100 ng of bone morphogenetic protein-2/mL.

<sup>a-d</sup>Values with different superscript letters differ significantly (<sup>a,b</sup> $P < 0.05$ ; <sup>c,d</sup> $P < 0.005$ ).

Table 2—Efficacy of BMP-2 and dexamethasone as osteogenic inducers in cultured bone marrow stromal cells obtained from various animals.

Source	BMP-2	Dexamethasone	BMP-2 and dexamethasone	References
Humans	+/-	++++	++++	22, 23
Rats	++	++	++++	16, 22, 23
Mice	++++	+/-	+++	15, 22, 37, 38
Dogs				
Without ascorbate	+	-	+	NA
With ascorbate	++++	-	+++	NA

+/- = Equivocal response. - = No response. + = Mild response. ++ = Mild to moderate response. +++ = Moderate response. ++++ = Maximal response. NA = Not applicable; data provided in the study reported here.

See Table 1 for remainder of key.

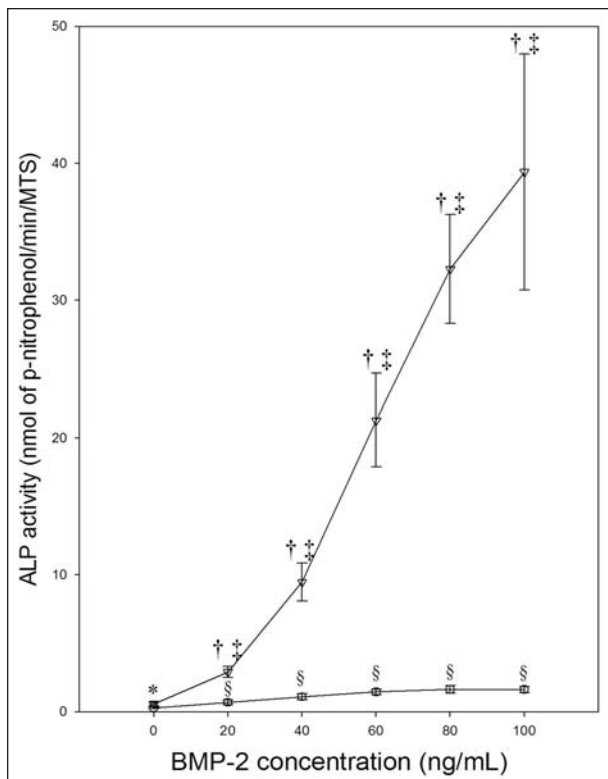


Figure 3—Mean  $\pm$  SEM adjusted ALP activity as a result of culture of first-passage MSCs for 6 days with increasing concentrations of BMP-2 without Asc (circles) or with Asc (triangles). The MSCs were obtained from 7 skeletally immature dogs. \*The ALP activity was significantly ( $P < 0.05$ ) increased in cultures with Asc, compared with those without Asc. †Within an Asc culture condition, value differs significantly ( $P < 0.005$ ) from value for 0 ng of BMP-2/mL. ‡§Within a BMP-2 concentration, value differs significantly from the value without Asc ( $\dagger P < 0.005$ ) and from the control (no inducers) value (§  $P < 0.05$ ). See Figure 1 for remainder of key.

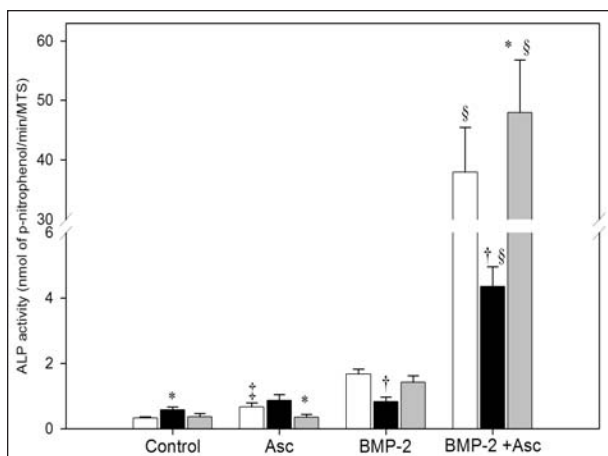


Figure 4—Mean  $\pm$  SEM adjusted ALP activity as a result of culture of first-passage MSCs for 6 days without osteogenic inducers (control group) or with Asc, BMP-2, or a combination of BMP-2 and Asc and 2 treatment groups to assess Asc mechanism of osteoinduction (no treatment [control; white bars]; the matrix inhibitor, 3,4-dihydroxyproline [black bars]; or the antioxidant, N-acetylcysteine [gray bars]). \*†Within an osteogenic inducer group, value for treatment with matrix inhibitor or antioxidant differs significantly ( $* P < 0.05$ ;  $\dagger P < 0.005$ ) from the value without treatment. ‡§Within a treatment group, value differs significantly ( $\dagger P < 0.05$ ; §  $P < 0.005$ ) from the corresponding osteogenic inducer without Asc. See Figure 1 for remainder of key.

into the extracellular matrix,<sup>39</sup> whereas NAC is a cell-permeant antioxidant that has been used to replace the antioxidant effects of ascorbate.<sup>40</sup> Preventing secretion of a collagen-rich matrix by the addition of 3,4-DHP had a significant ( $P < 0.005$ ) negative effect on induction of ALP activity in canine MSCs treated with BMP-2 alone or BMP-2 in combination with ascorbate (Figure 4). As expected, the greatest effect of 3,4-DHP on induction of ALP activity was evident in MSCs cultured with a combination of BMP and ascorbate. For those cultures, mean ALP activity after addition of 3,4-DHP was 11% of the ALP activity in cultures treated with a combination of BMP and ascorbate but without addition of 3,4-DHP.

In contrast, NAC had relatively little effect on ALP induction. Addition of NAC to BMP-treated cultures did not mimic the synergistic effect evident with ascorbate. Instead, MSCs treated with NAC in cultures treated by a combination of BMP and ascorbate had a modest but significant increase in ALP activity, compared with ALP activity in cultures treated with a combination of BMP and ascorbate but without addition of NAC.

## Discussion

Because BMPs can promote ectopic bone formation in laboratory animals, they have been viewed as promising therapeutic agents for bone repair.<sup>41-43</sup> In vitro, BMPs promote osteoblast differentiation of primary preosteoblasts and osteoblastic cell lines.<sup>41</sup> In vivo, BMPs can increase the rapidity of fracture repair and fill bone defects in numerous animals, including dogs.<sup>27,30,32,42-44</sup> Because in vivo bone repair is believed to require the differentiation of MSCs into osteoblasts, there is an expanding interest in research to examine factors that may promote osteogenesis of MSCs. A major challenge for cell-based repair treatments is to elucidate the optimal conditions for ex vivo expansion of stem cells while maintaining a differentiation capacity sufficient to maximize the therapeutic effect. Similarly, for drug-based treatments, it is essential to define those modulators that are effective in promoting a specific differentiation pathway.

Despite the fact that dogs are excellent animals for use in evaluating orthopedic repair, data on the efficacy of osteogenic inducers in canine MSCs are limited. The experiments reported here used high ALP activity and increased concentrations of mRNA for ALP, osteopontin, and RUNX2 as indicators of MSC osteogenesis. Analysis of our findings suggests that BMPs are excellent inducers of osteogenesis in canine MSCs. This is in contrast to dexamethasone, which appears to have no osteoinductive effect and inhibits BMP-stimulated osteogenesis. Furthermore, when young dogs are the source of MSCs, it is necessary to supplement BMP-containing medium with ascorbate, which functions to promote formation of a collagen-rich matrix. In another report,<sup>11</sup> canine MSCs cultured for at least 3 weeks with a combination of ascorbate and dexamethasone had ALP activity 10-fold higher than in control cultures. Because our primary interest was to define conditions that would rapidly promote osteogenesis, all of our experiments were limited to a culture period of 6 days. By use of this shorter time period, we found that

a combination of BMP and ascorbate caused a > 75-fold increase in ALP activity and ALP mRNA content, whereas the combination of dexamethasone and ascorbate was ineffective in inducing osteogenesis.

Although a combination of ascorbate and  $\beta$ -glycerophosphate is often sufficient to induce mineralizing osteoblasts from cells already committed to the osteoblast lineage, only BMPs and dexamethasone reportedly are efficient osteogenic inducers for multipotential MSCs. Evaluation of data on the efficacy of BMPs and dexamethasone with MSCs from various sources indicates that although inducible osteogenesis is a property of MSCs from all mammalian species studied to date, there is noticeable interspecies variation in response to these osteogenic inducers, suggesting that a uniform mechanism for inducing osteogenesis in MSCs does not exist among species.

Results of *in vivo* studies tend to support the differences detected with cultured MSCs. Treatment with BMP increases bone formation in numerous animals, and studies<sup>25-32,43</sup> in dogs reveal efficacy of BMP at alveolar and long-bone sites. However, BMP has not had equivalent potency in clinical trials in humans.<sup>45-47</sup>

The amount of BMP-induced osteogenesis in canine MSCs appeared to be age related. The MSCs from 15 immature dogs (< 1 year old) had a pattern of osteogenic induction similar to that for MSCs from the 4 skeletally mature dogs, but mean ALP activities were decreased in the mature population. The effect of ascorbate on osteogenesis was also an age-related phenomenon, with BMP-treated MSCs from young dogs having a 5- to 7-fold increase in ALP activity when cultured with ascorbate, whereas addition of ascorbate to BMP-treated MSCs from mature dogs yielded a marginal increase that was not significantly different from ALP activity in untreated control cultures (Figure 1). The major effects of age reported for MSCs are a decrease in mononuclear cells at the time of marrow harvest and a decrease in colony-forming efficiency with older patients, both of which lead to reduced numbers of ALP-positive colonies in MSCs from older donors.<sup>48-51</sup> However, the ability of cultured human and rat MSCs to undergo osteogenesis does not seem to decrease with age of donor.<sup>52,53</sup> Studies<sup>54-56</sup> focusing on age-related changes in rats revealed that although BMP-induced osteogenesis decreased proportionately as the recipient aged, BMP still effectively promoted osteogenic induction. The results reported here used passaged cells seeded at a uniform density; therefore, age-related differences cannot be ascribed to a change in proportion of MSCs in the bone marrow population. It is also likely that with a larger sample size for mature dogs, the MSCs would have yielded a significant difference between osteogenesis with and without addition of BMP. Nonetheless, the results reported here clearly documented that MSCs from older, skeletally mature dogs have a decreased osteogenic response to BMP and to ascorbate.

Evidence provided by use of real-time RT-PCR assays indicated that the results for measurement of ALP activity reflected differences in the amount of ALP gene expression (Figure 2; Table 1). By use of MSCs from skeletally immature dogs, real-time RT-PCR

analyses for osteopontin and RUNX2 yielded results similar to those seen for ALP expression, indicating that BMP is capable of marked induction of mRNA concentrations. The addition of dexamethasone to cultures prevented induction of osteopontin mRNA by BMP with or without ascorbate. The osteogenic marker RUNX2 is an osteoblast-specific transcription factor and regulator of osteoblast differentiation.<sup>57</sup> Similar to results for the canine MSCs reported here, BMP is capable of upregulating the transcription of RUNX2 in mouse C2C12 cells.<sup>57,58</sup> We also detected a modest increase in RUNX2 concentrations with ascorbate alone, which is an effect also evident in mouse cells.<sup>59</sup> Dexamethasone-induced downregulation of RUNX2 mRNA reflects the overall lack of effect that dexamethasone has on osteoinduction in canine MSCs. In contrast to its effects in dogs, dexamethasone is an osteogenic inducer in human and rat MSCs. Dexamethasone stimulates RUNX2 mRNA in cultures of osteoblasts obtained from humans and rats.<sup>60,61</sup>

The combination of BMP and ascorbate is effective at inducing chondrocyte hypertrophy and osteogenesis.<sup>62-64</sup> The most commonly documented effect of ascorbate is on collagen hydroxylation, a necessary step in the formation of stable, secreted triple-helical collagen. Ascorbate can stimulate differentiation of a murine osteoprogenitor cell line by increasing matrix-integrin interactions.<sup>59,65</sup> Our results documented that 3,4-DHP, an inhibitor of collagen hydroxylation, inhibits ALP activity in canine MSCs cultured with BMP alone or with a combination of BMP and ascorbate. This is consistent with the assumption that the mechanism of action for ascorbate in canine MSCs is identical to that reported for ascorbate-stimulated differentiation of osteoprogenitors in other species (ie, it is required for maximal ALP expression in canine MSCs because of its ability to increase collagen secretion). Evidence from other studies<sup>59,66</sup> indicates that a triple-helical region of type I collagen containing an aspartic acid-glycine-glutamic acid-alanine peptide sequence binds to  $\alpha$ 1- or  $\alpha$ 2-containing integrins to promote osteoblast differentiation. The fact that canine MSCs appear to have a more stringent ascorbate requirement than MSCs from other animals suggests that cell-matrix interactions may be particularly important for bone repair in dogs and that definition of matrices conferring maximal induction of osteogenesis in canine MSCs may be necessary to enhance the success of cell-based orthopedic applications in animals.

Because the addition of 3,4-DHP did not completely abolish the effect of ascorbate on BMP-2-treated cells, we examined the alternative hypothesis that ascorbate may also function as an antioxidant to protect osteoprogenitors from toxic hydroxyl radicals and peroxide or modulate intracellular signaling.<sup>40</sup> Analysis of our results for NAC indicates that this antioxidant caused a significant increase in ALP activity when added in addition to BMP or ascorbate. The fact that this was a minor contribution, compared with the effect of ascorbate, and that NAC had no effect without ascorbate suggests that NAC maintained the added ascorbate in a reduced state so that it could sustain collagen secretion.

In an *in vivo* study,<sup>67</sup> BMP-induced osteogenesis

in dogs provided successful treatment of nonunion fractures. In addition, BMP-2 and -7 can augment repair of craniofacial<sup>24,28,31,68</sup> and long-bone gap defects,<sup>27,30,32,34,69</sup> promote healing of arthrodeses<sup>67</sup> and spinal fusions,<sup>25,69-71</sup> improve incorporation of allografts,<sup>30,34</sup> and enhance bone formation within implants.<sup>26,32</sup> Most of these studies were performed in dogs as part of preclinical studies for use of BMPs in humans. However, it should be mentioned that the combination of BMP-2 and ascorbate that was most effective for inducing ALP expression in canine MSCs in the study reported here is incapable of inducing ALP activity in human MSCs.<sup>22</sup> Similarly, it is likely that each species has differing requirements for the BMP dose that will promote effective osteogenesis in vivo, with dogs being intermediate in responsiveness between small animals (eg, rodents and rabbits) and humans.<sup>72</sup> The minimal effective dose of 50 µg of BMP-2/mL in an absorbable collagen sponge was established during an in vivo study<sup>69</sup> in dogs with a diaphyseal segmental defect. In that study, deleterious effects, such as cyst-like void formation, were evident at higher doses of BMP-2. In our study, we documented that the in vitro response of canine MSCs to BMP-2 alone appeared to plateau at a concentration of 60 to 100 ng/mL (Figure 3). However, concentrations > 100 ng/mL were not tested.

Allogeneic MSCs enhance repair of critical-sized bone defects in dogs without inducing adverse immune responses.<sup>3,13</sup> This establishes the practicality of ex vivo expansion of canine MSCs to provide a ready source of cells for tissue engineering. Although MSCs can improve fracture and gap-defect repair in dogs,<sup>4,5</sup> it is unclear which inducers best direct canine MSCs to differentiate into osteoblastic cells. In the study reported here, we documented that the combination of BMP-2 and ascorbate was more potent for inducing osteogenesis in short-term cultures of canine MSCs than was the combination of dexamethasone and ascorbate. Because the efficacy of BMP-2-induced augmentation of fracture and gap-defect repair has been established in dogs, it is possible that culturing MSCs with BMP (alone or in combination with ascorbate) may create an improved therapeutic strategy for challenging orthopedic procedures in dogs.

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- a. Ficoll-Paque, Amersham Pharmaceutical Biotech, Piscataway, NJ.
  - b. Dexamethasone, Sigma Chemical Co, St Louis, Mo.
  - c. Ascorbate-2-phosphate, Wako, Richmond, Va.
  - d. Recombinant human BMP-2, Genetics Institute, Wyeth Labs, Cambridge, Mass.
  - e. N-acetylcysteine, Sigma Chemical Co, St Louis, Mo.
  - f. 3,4-Dehydroproline, Sigma Chemical Co, St Louis, Mo.
  - g. Cell Titer 96 AQueous cell proliferation assay, Promega Corp, Madison, Wis.
  - h. TRI reagent, Molecular Research Center, Cincinnati, Ohio.
  - i. First-strand synthesis system for RT-PCR, Invitrogen Life Technologies, Carlsbad, Calif.
  - j. Cepheid Smart Cycler, Cepheid, Sunnyvale, Calif.
  - k. Roche LightCycler, Roche Molecular Biochemicals, Mannheim, Germany.
  - l. LightCycler-Fast Start DNA Master SYBG Green I kit, Roche Molecular Biochemicals, Mannheim, Germany.
  - m. SigmaPlot 8.0, Systat, Point Richmond, Calif.
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