Induction of Bone Matrix Protein Expression by Native Bone Matrix Proteins in C2C12 Culture

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Objective To study the expression of bone matrix protein (BMP) induced by bovine bone morphogenetic proteins (BMPs) *in vitro*. **Methods** Type I collagen, osteopontin (OPN), osteonectin (ON), osteocalcin (OC), and bone sialoprotein (BSP) were detected by immunohistochemistry in C2C12 cultured from day 1 to day 28. **Results** The signaling of bone matrix protein expression became weaker except for type I collagen, OC and BSP after 5 days. Fourteen days after culture, the positive signaling of type I collagen, OPN, ON, OC, and BSP was gradually declined, and could be detected significantly as compared with that of the negative control on day 28. BMP assay showed that the lkaline phosphatase (ALP) activity was higher in C2C12 culture than in the control during the 14-day culture. Also, total protein and DNA significantly increased during the 14-day culture. High levels of ALP were seen in preosteoblasts and osteoblsts *in vivo* and in differentiating osteoblasts *in vitro*. ALP was well recognized as a marker reflecting osteoblastic activity. **Conclusion** Native bovine BMP induces conversion of myoblasts into osteoblasts, produces type I collagen, and plays significantly role in osteoinduction and bone matrix mineralization of C2C12 *in vitro*.

Key words: Bone matrix proteins; Bone morphogenetic proteins; Immunohistochemistry; C2C12

INTRODUCTION

Bone morphogenetic protein (BMP) is a growth factor "stored" in the bone matrix, which enhances osteoblast activities and induces bone formation[1-2]. However, the mechanism of bone formation has not been fully elucidated. Osteoblasts play a major role in bone formation and produce type I collagen, a major organic component of the mineralized bone matrix, and also express a variety of non-collagenous proteins (NCP), including osteopontin (OPN), bone sialoprotein (BSP), osteonectin (ON) and osteocalcin (OC), which play a role in the regulation of mineralization[3-4], attachment of osteoblasts and osteoclasts to the bone matrix, and/or attraction of cells to the bone matrix[5-6]. Therefore, BMP can be considered a non-collagenous bone matrix protein. Effects of BMP on non-collagenous proteins and regulation of mineralization in osteoblast-like cells have not been fully studied. In the present study, we have examined the expression of OPN, ON, OC, BSP, and type I collagen by immunohistochemistry with an *in vitro* assay to measure the mineralization of bovine BMP using cultured C2C12 mouse myoblasts.

MATERIALS AND METHODS

Extraction of BMP

BMP was extracted with the modified Urist's method[7]. Briefly, 5 kg of fresh bovine cortical bone was frozen in liquid nitrogen and ground to approximately 1 mm^3, bone particles were defatted overnight in chloroform-methanol (1:1), dried for over 4 h, and decalcified with 0.6 mmol/L HCl for 72 h. The protein was extracted from 6 mol/L urea/0.5 mol/L $CaCl_2$, and dialyzed against distributed water for 72 h. Precipitate was collected and resolved into 6 mol/L urea/0.5 mol/L $CaCl_2$, and dialyzed against 11 volumes of 0.25 mol/L citric acid buffer (pH 3.1). Precipitate was collected and defatted in

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chloroform-methanol (1:1) for 4 h, and lyophilized as partially purified BMP.

C2C12 Culture

C2C12, a mouse myoblast cell line, can convert its differentiation pathway from myoblasts to osteoblasts in the presence of BMP[5], and is readily available from American Type Culture Collection (ATCC, VA). C2C12 cells (5×10^4) were seeded into each 2 cm × 2 cm well of a 24-well plate and incubated on a growth medium (alpha-MEM, 15% FBS, 50 ug/mL ascorbic acid, 10 mmol/L β-GP, antibiotics). Bovine BMP to be tested was placed in the wells containing 100 ug (20 uL) of the extracted BMP. C2C12 cell culture was set as a negative control. The growth medium was changed every 3 days. C2C12 cells were collected on days 1, 3, 5, 7, and 14 for von Kossa staining, alkaline phosphatase assay, protein and DNA analysis. Cultures were tested on days 1, 3, 5, 7, 14, and 28 by immunohistochemistry.

Alkaline Phosphatase Assay

Cultures were rinsed three times with ice cold Tris buffered saline (TBS, 20 mmol/L Tris, 137 mmol/L NaCl, pH 7.4). One mL of lysis buffer (TBS + 0.1% Triton X-100) was added to each well and cell layer was scrapped into the buffer. The lysate was transferred to epindorf tubes, sonciated, and centrifuged at 5 000 × g at 4 ℃ for 5 min. The supernatant was assayed for alkaline phosphatase and protein content. Alkaline phosphatase assay was performed in samples following Sigma protocol 104. Enzyme activity was detected by spectrophotometry at 405 nm after 30 min of incubation. Standard curves were plotted using p-nitrophenol standard solution in 0.08 mmol/L NaOH, and 2-N Sigma enzyme controls were used as a positive control. Enzyme activity data (nmol phosphate released/well/hour at 37 ℃) were expressed as a mean as previously described[8]. For clarity, the standard error bars are omitted in Fig. 3. Statistical significance of the data was determined using SigmaStat (SPSS).

Immunohistochemical Study

The medium was removed from 24 wells culture plate with vacuum suction, and the cells were fixed in 2% paraformaldehyde for 20 min. The cover-slips were washed twice with PBS (5 min each time). Monoclonal mouse anti-collagen type I (Sigma, C 2456), MPIIIB101 (mouse anti-rat osteopontin, Department of Biology, University of Iowa, IA), LF-32 (mouse anti-bovine osteocalcin, NIDR, NIH, MD20892), AOV-1 (mouse anti-bovine osteonectin, NIDR, NIH, MD20892), WVID1 (mouse anti-rat bone sialo proteins I and II, Department of Biology, University of Iowa, IA) and LF-83 (bone sialo protein) were used as primary antibodies as previously described[9-10]. Briefly, cover-slips were incubated with monoclonal mouse anti-collagen type I (1:1000), mouse anti MPIIIB101 (1:250), LF-32 (1:1000), AOV-1 (1:500), and WVID1 (1:250) overnight at 4 ℃ after endogenous peroxidase was blocked with 0.3% H_2O_2 methanol for 30 min. Pepsin (Sigma) enzyme (0.1%) and 0.01 mol/L HCl (pH 2.25) were incubated for 30 min at room temperature in a moist incubation chamber for digestion. Only C2C12 cell culture staining with these primary antibodies was set as a normal control. For negative controls, PBS and normal horse serum were used instead of primary antibodies as a blank control. Cover-slips were washed with PBS/0.2% Tween-20, incubated with biotinylated horse anti-mouse immunoglobulin G or/and goat anti-rabbit IgG (immunopure ultra-sensitive ABC peroxidase staining kit, Pierce, Rockford, IL) for 1 h at room temperature in a incubation chamber, and then incubated for 30 min with avitin-biotin-complex. Substrate was developed with 0.03% 3, 3'-diaminobenzidine tetrahydrochloride (DAB Sigma), and incubated for 7min. The reaction was terminated by rinsing the sections in ddH_2O for 10 min and PBS/0.2% Tween-20 for 5 min. The sections were counter stained with hematoxylin for 2 seconds.

Total DNA Content

Cultures were rinsed three times with ice cold Tris buffered saline L (TBS, 20 mmol/L Tris, 137 mmol/L NaCl, pH 7.4). Trizol (0.2 mL) was taken out from each well, 40 ug glycogen was added directly into Trizol, by passing the cell lysate several times through a pipette. The mixed culture was incubated for 5 min at room temperature, into which 60 uL of chloroform was added, and centrifuged 12 000 r/min for 15 min at 4 ℃. DNA was precipitated from the interphase and organic phase with EtOH, while the supernatant was removed for protein isolation. DNA pellet was washed twice with a 0.1 mol/L sodium citrate solution in 10% EtOH, and DNA was suspended in 75% EtOH. After air-dry for 5-10 min, DNA samples were dissolved in 8 mmol/L NaOH (100 uL). DNA content in some samples was determined using UV and visible lamps in TE at 260 and 280 nm.

Total Protein Assay

Isopropyl alcohol (1.5 mL) was added into the supernatant, and centrifuged 12 000 r/min at 4 ℃.

Protein pellet was washed 3 times in a solution of 95% EtOH containing 0.3 mol/L guanidine-HCl and dried in vaccum, dissolved in 1% SDS at 50 ℃. Insoluble materials were removed by centrifugation. Samples were assayed with the BCA™ protein assay reagent (Pierce, #) following the Bio-Rad protocol. Bovine serum albumin in 1% SDS was used to plot a standard curve at 562 nm.

RESULTS

Expression of BMP

As shown in Table 1, a wide variety of BMP could be detected in C2C12 cells cultured from day 1 to day 28. OPN, ON, OC, and BSP were found in cover-slips on days 1-5. The signaling of protein expression became weaker except for OC after 5 days. When cultured for 14 days, the positive signaling of OPN, ON, OC, and BSP was gradually declined, and became more significant than that of the negative control on day 28. During the cultures, the signaling of type I collagen, OC and BSP protein expression was stronger, especially in OC on day 28. Compared with the other three proteins, OPN expression was weaker on day 5, but increased on day 7 and could be detected significantly on day 28. In the normal control, weaker IR staining was shown (Table 1), and IR of type I collagen appeared on day 1. Blank and negative controls were consistently negative (Figs. 1-2).

TABLE 1

BMP Expression in C2C12 Culture

Days	Normal Control			BMP 100 ug/Well				
	Type I Collagen	Osteocalcin	Osteonectin	Type I Collagen	Osteopontin	Osteocalcin	Osteonectin	BSP
1	++	++	±	++++	++++	++++	++++	++++
3	+	+	-	++++	+++	++++	+++	++++
5	+	+	-	++++	++	++++	++++	++++
7	+	+	-	+++	+++	++++	+++	+++
14	+	+	-	+++	++	+++	++	++
28	+	+	-	+++	++	+++	++	++

Note. -: no immunostaining; ± faint staining; +: mild immunostaining; ++: moderate immunostaining; +++: strong immunostaining; ++++: very strong immunostaining.

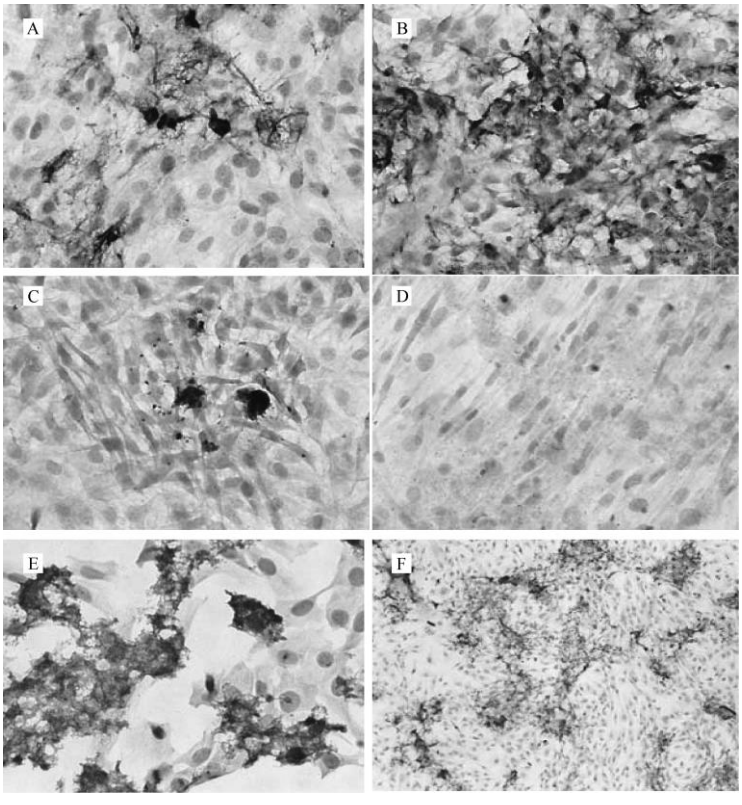

FIG. 1. Immunostaining for type 1 collagen in C2C12 culture on days 3 (A) and 6 (B) (× 400), for osteopontin on days 3 (C) and for IR on day 28 (D) (× 400), for osteocalcin on day 1 (E) and day 3 (F) (× 100), respectively.

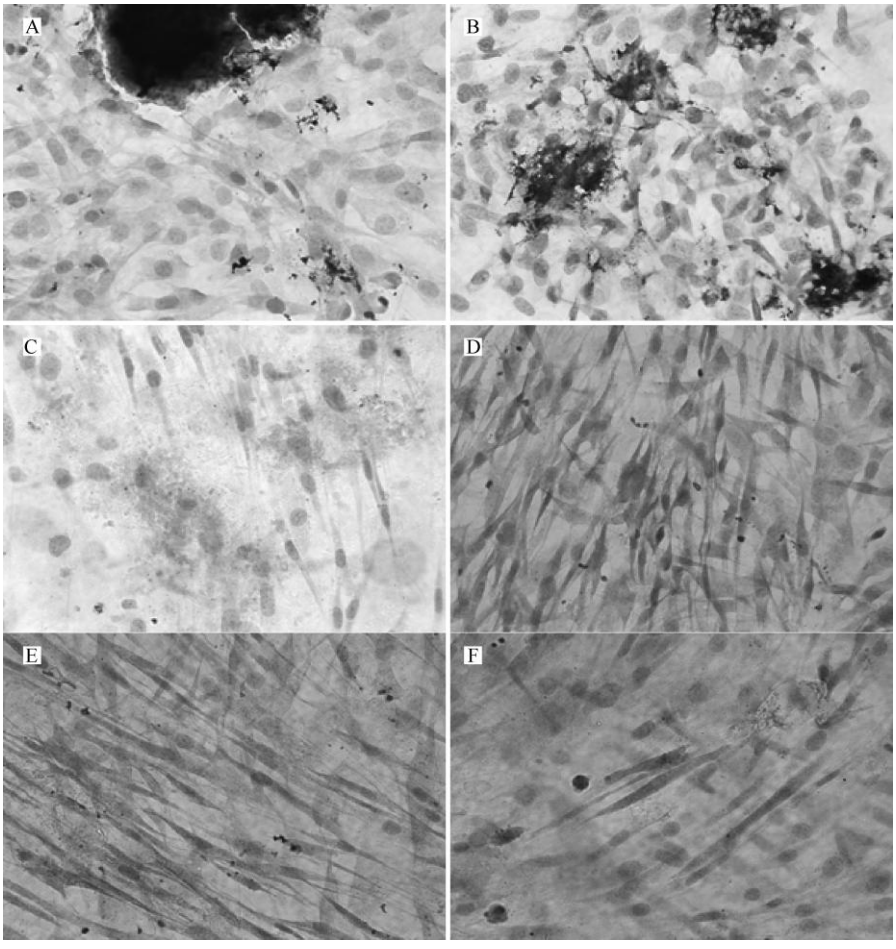

FIG. 2. Osteonectin on days 3 (A), IR in cell matric on days 5 (B) and 28 (C) (×400), BSP on days 5 (D), IR on days 14 (E) and 28 (F) in C2C12 culture (×400).

Alkaline Phosphatase Activity

The ALP activity of ALP in BMP was highly osteoinductive, and much higher than that of the control during the 14-day culture (Fig. 3). Typically, the tested BMP elevated the activity of ALP by 5-20 folds. The activity of ALP reached its peak on day 3, and declined from day 5 to day 14, compared with the control (P<0.01). The ALP in BMP almost kept a significantly high level (P<0.05-0.01) on day 14.

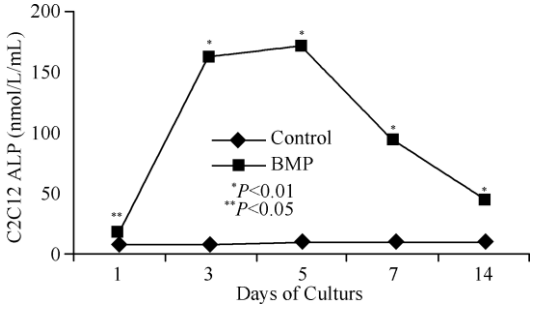

FIG. 3. Elevated ALP activity in BMP during the 14-day culture.

Total DNA Content

DNA content increased significantly on day 3 (P<0.05) and reached its peack on day 7 (P<0.001), and was higher on day 14 (P<0.01) in BMP than in the control (Fig. 4).

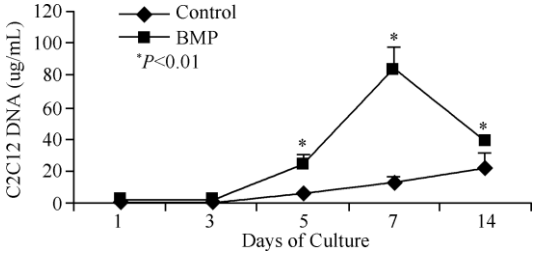

FIG. 4. Increased DNA content on days 5 (P<0.01), 7 (P<0.001), and 14 (P<0.01) in BMP.

Total Protein Assay

Sample assay showed that the protein content was increased in BMP on days 1, 3, 7, and 14 ($P<0.05$, Fig. 5).

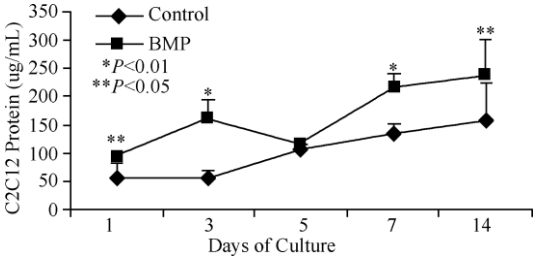

FIG. 5. Increased protein content in samples on days 1, 3, 7, and 14 ($P<0.05$).

DISCUSSION

The results of our study demonstrate that native bovine BMP had significant effects on the expression of type I collagen, OPN, ON, OC, and BSP in cultured C2C12 cells. Total DNA and protein were also obviously increased. However, different positive immuno-reactions were detected in proteins. The signaling of protein expression was weaker except for type I collagen, OC and BSP after 5 days. After cultured for 14 days, the positive signaling of type I collagen, OPN, ON, OC, and BSP was gradually declined, which was more significant than that of the negative control on day 28.

NCP is an important regulator of bone metabolism, accounting for about 10% of the total bone protein content. A variety of NCP have been identified, including osteocalcin(OC), osteopontin(OPN), osteonectin(ON), bone sialoprotein(BSP), decorin, and biglycan[3-4]. Only OC and BSP are specific for bone, whereas the other proteins are also present in non-calcifying tissues and play a limited role in bone metabolism. In our experiments, bovine BMP obviously enhanced OC and BSP signaling, thus supporting the hypothesis that BMP increases the specific NCP for bones in the regulation of mineralization.

Bone matrix mainly comprises type I collagen and a variety of non-collagenous proteins, such as proteoglycans, OC, matrix Gla protein, ON, OPN, BSP, and bone acidic glycoprotein-75[11]. Although the exact function of these non-collagenous proteins has not been conclusively determined, they play a critical role in regulating cell differentiation, bone matrix formation and mineralization[12], and are expressed during osteoblast differentiation. Type I collagen, a most abundant protein in bone matrix, is an early marker of osteoblast differentiation and a major organic component of the mineralized bone matrix. Osteoblasts play a major role in bone formation and produce type I collagen. The native bone morphogenic protein used in this experiment contains transforming growth factor 1, as well as OPN, ON, other proteoglycans and lipoproteins[13-14], which are acidic proteins with a strong affinity for hydroxyapatite and can bind to calcium ions. ON and OPN which are not specific to mineralizing tissues have been identified in a number of no-mineralizing tissues, while OC is tissue specific and does not appear to be necessary for tissue mineralization. However, osteocalcin appears to be important in bone resorption as a chemoattractant of osteoclast precursors, because its expression is strongly enhanced by 1, 25-dihydroxyvitamin D_3. In contrast, BSP produced by osteoblasts is specifically expressed in cells directly involved in the formation of mineralizing connective tissue matrices. Cells accumulate in BSP of extracellular matrix, and the distribution of BSP is restricted to bone and mineralized tissues *in vivo*. In bone, BSP in the bone matrix, a nucleator of hydroxyapatite crystal precipitation, is one of the major non-collagenous proteins, and plays a role in the maintenance of bone cells on mineralized matrix[15-17]. Mizuno *et al.*[18] found that BSP could stimulate the expression of MC3T3-E1 osteoblastic phenotypes, indicating that BSP regulates the expression of osteoblastic phenotypes. BSP, produced by osteoblasts and odontoblasts, is a marker of osteoblastic phenotypes, and can stimulate the calcification of MC3T3-E1 osteoblastic cells, suggesting that BSP accelerates the expression of osteoblastic phenotypes. Collagen matrices and other extra-cellular proteins support phenotypes and tissue-specific functions, indicating that extra-cellular matrices maintain cell functions.

In the present study, OPN expression reached its peak on days 1-3, and the signaling was weak on day 5 and increased on day 7. OPN has a high-affinity for hydroxyapatite, and plays a role in modulating the mineralization of bones, though it is not specific for bone. The deposition of OPN protein is exactly consistent with that of calcium phosphate and vise versa[19-20], indicating that OPN plays a significant role of OPN in ectopic calcification. Further study is needed to observe the role of OPN in bone metabolism.

The activity of ALP in BMP was highly osteoinductive compared with the control. ALP is widely used as a marker of osteoblast phenotypes. Osteoblasts expressing the non tissue specific form of enzymes and high levels of ALP have been observed

in preosteoblasts and osteoblsts *in vivo* and differentiating osteoblasts *in vitro*[4,21]. Osteoblasts play a major role in bone formation and produce type I collagen, which is the most abundant protein in bone matrix[15-16]. This study demonstrated that ALP activity in cultured C2C12 cells was elevated by cultured native BMP, which induces conversion of myoblasts into osteoblasts, produces type I collagen and significantly increases the signaling of type I collagen, OPN, ON, OC, and BSP in cultured C2C12 cells.

In conclusion, native bovine BMP induces conversion of myoblasts into osteoblasts, produces type I collagen, and significantly increases the role of osteoinduction and bone matrix mineralization in C2C12 cells *in vitro*.

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