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Original Article

Interaction of TGF-β1 and rhBMP-2 on Human Bone Marrow Stromal Cells Cultured in Collagen Gel Matrix

Myung Ku Kim[1], and Christopher Niyibizi[2]

[1]Department of Orthopaedic Surgery, Inha University Hospital, Incheon, Korea;
[2]Department of Orthopaedic Surgery, Musculoskeletal Research Center, University of Pittsburgh Medical Center, Pittsburgh, PA, USA.

Transforming growth factor-β1 (TGF-β1) and bone morphogenetic protein-2 (BMP-2) are abundant proteins in the bone matrix. However, their interaction in controlling osteoblast differentiation is not clearly understood. In this study, HBMSCs were cultured in collagen gel matrix with different condition of exogenous rhBMP-2 and TGF-β1 in order to determine the interaction of BMP-2 and TGF-β1 on human bone marrow stromal cells (HBMSCs) differentiation. The cultured cells were analyzed for cell proliferation, alkaline phophatase (ALP) activity and mineralization stainning with Von-Kossa. The cells treated with TGF-β1 exhibited a higher rate of cell growth than those without. However, the cells cultured in collagen gel matrix showed a lower rate of cell growth than the cells cultured in a monolayer. To investigate the effects of both cytokines on osteoblast differentiation, the cells were treated with 0, 1, 5, 10 ng/ml of TGF-β1 for 2 days. This was followed by culturing with 0, 1, 5, and 10 ng/ml of TGF-β1 and 100 ng/ml of rhBMP-2 together for 3 days with the alkaline phosphatase (ALP) activity measured. The cells treated with 1 ng/ml of TGF-β1 responded efficiently to rhBMP-2 and expressed ALP activity with a level equivalent to that exhibited by cells that were not treated with TGF-β1. The cells treated with 5 and 10 ng/ml of TGF-β1 showed a dramatic decrease in ALP activity. The cells treated with 10ng/ml of TGF-β1 followed by rhBMP-2 alone exhibited an intermediate ALP activity. The cells treated with 100 ng/ml of rhBMP-2 demonstrated Von-Kossa positive solid deposits after 3 weeks, while there were few Von-Kossa positive solid deposits when the cells treated with 10 ng/ml of TGF-β1. These results show that TGF-β1 inhibits the effects of rhBMP-2 on the osteoblast differentiation of HBMSCs in a dose dependant manner. Furthermore, the effects of TGF-β1 on HBMSCs are reversible. This suggest that TGF-β1 and rhBMP-2 are coordinately controlled during the osteoblast differentiation of HMBSCs.

Key Words: Human bone marrow stromal cell, rhBMP-2, TGF-β1, osteoblast differentiation

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INTRODUCTION

The bone marrow stroma is a complex network of cells including fibroblasts, adipocytes, reticular cells, endothelial cells and pluripotential stem cells.[1-3] Regulation of osteoblast differentiation must occur in order to maintain a continuous supply of mature osteoblasts needed for bone growth, remodeling and fracture repair. Osteoblast precursors as well as precursors of the other mesenchymal lineages-fibroblast, myoblast, adipocytes and chondrocytes-are believed to be derived from the multipotential stem cells of the bone marrow stroma.[1,4] Evidence for the presence of osteoprogenitor cells in bone marrow is based on data showing that transplantation of undifferentiated human bone marrow cells or fibroblastic-like stromal cells into immuno-compromized animals results in bone formation *in vivo*.[5-7]

During osteoprogenitor cell differentiation in the bone marrow stroma, local factors that are produced by bone cells, act in a cooperative fashion to control cell proliferation and differentiation of these cells toward a different lineage. Among them, TGF-β1 and BMP-2 are believed to play a major role in controlling bone formation.[8-15] However, there is much controversy in literature regarding the effects of TGF-β1 and BMP-2 on bone marrow stromal cell differentiation toward the osteoblast lineage, which depend on the culture condition and cell type. Alkaline phosphatase activity and osteocalcin production as osteoblastic makers are typical examples.[16-21]

The collagen gel matrix is a good culture scaffold, but there are few reports of human mesen-

chymal stem cells being cultured in a collagen gel matrix. There was a report showing that a collagen matrix induced the osteoblastic differentiation itself[22] and TGF-β1 accelerated the osteogenic differentiation of bone marrow cells induced by the collagen matrix.[23] However, there is still controversy on cell proliferation and the effect of TGF-β1 and BMP-2 on human bone marrow stromal cells (HBMSCs) in a collage gel matrix culture.

To gain more insight into the effect of both TGF-β1 and BMP-2 on the induction of human bone marrow stromal cells in a collagen gel matrix as a culture scaffold, HBMSCs were cultured in a collagen gel with different concentrations of exogenous TGF-β1 and rhBMP-2. These were assessed for alkaline phosphatase (ALP) activity and mineralization staining with Von-Kossa stain as an index for HBMSCs differentiation. In addition, the number of cells were measured to determine the effect of TGF-β1 on cell proliferation in collagen gel matrix.

MATERIALS AND METHODS

All tissue culture reagents were purchased from GIBCO BRL (Grand Island, NY, USA) and all other reagents were purchased from Sigma (Sigma Chemical Co, St. Lous, MO, USA).

Human bone marrow stromal cell isolation

Human bone marrow was obtained from a pelvic bone during a hip operation and was washed 4 times by centrifugation in low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin (P/S). The pellet cells were plated into T-25 flasks at a low density in DMEM supplemented with 10% FBS, 1% P/S, 50 μg/ml of ascorbic acid and 10^{-7} M dexamethason. After 4 days incubation, all non-adherent cells were removed and the adherent cells were maintained in culture with change of medium every 3 days.

Culture of the HBMSCs in collagen gel

Type I collagen matrix gels (Cow skin, Vitrogen 100, Collagen Co, Palo Alto, CA, USA) were formed by a slight modification of a method described previously.[23] Briefly, 1ml of type I collagen gel was mixed with 125 μl of HEPES buffer and 125 μl of tenfold concentrated high glucose DMEM to neutralized the pH of the mixed solution. After the subculture was carried out once to expand the cell numbers, the cells were trypsinized and mixed with the type I collagen complex solution, which was previously prepared, at a concentration of 1×10^6 cells/ml. Subsequently, 100 μl of the type I collagen gel- cell complex solution was again plated into a 12 well plate and incubated at 37℃ for 15 min in order to harden the collagen gel matrix. This was followed by culturing the HBMSCs with a 1% low glucose DMEM overnight.

Measurement of cell proliferation

To determine the effect of exogenous TGF-β1 protein on cell proliferation, the cells mixed with the type I collagen gel were maintained in a serum free medium (DMEM) supplement with 1% ITS (insulin, transferin and selenium), 50 μg/ml ascorbic acid with or without 10 ng/ml of TGF-β1. After 24 h, the medium was removed and 400 units/ml of a collagenase solution was added. The plates were incubated at 35℃ to isolate the cells from the type I collagen gel matrix and then the cells were counted. In another set of experiments, the cells were treated as above, but were maintained in culture for 72 h. The cells then were counted as above. To measure the effect of the collagen gel matrix on cell proliferation, HBMSCs were cultured as a monolayer as above for control. The experiment was performed five times.

Measurement of ALP activity

ALP activity was determined as described previously.[21,24] Briefly, the HBMSCs plated in 24 well plates were cultured in serum free DMEM supplemented with 1% ITS, and 50 μg/ml ascorbic acid. The cells were incubated at 37℃ in 5% CO_2 for 24 hr. Subsequently, the cells were cultured in 1% DMEM with 0, 1, 5, 10 ng/ml of TGF-β1 with a media change every day for 2 days. This was followed by incubation in 100 ng/ml of BMP-2

with 0, 1, 5, 10 ng/ml of TGF-β1 for further 3 days. On the 7th day, the cells were washed with Gey's balanced salt solution (GBSS) twice and lysed by two freeze thaw cycles. 5 μl aliquots of the lysates were used to determine ALP activity using a Sigma ALP kit. The experiment was performed five times.

Mineralization of collagen matrix gel (Von-Kossa staining)

To determine the mineralization of collagen matrix gel, the cells embedded in the collagen gel matrix were cultured with exogenous 100 ng/ml rhBMP-2 or 10 ng/ml TGF-β1 protein in serum free DMEM with 1 % ITS, 1% P/S and 10 mM/L b-glycerophosphate for 21 days with a change of medium every 3 days. The collagen gels matrixes were stained with Von-Kossa as described previously.[21] Briefly, the collagen gel matrix with HBMSCs were washed twice with GBSS and fixed with 2 % paraformaldehyde in a 0.2 M/L cacodylic buffer for 10 min at 4℃. The collagen gel matrix was subsequently washed extensively with 0.1 M/L cacodylic buffer at pH 7.4, then a filtered Von-Kossa (3% silver nitrate) was added to each well. The wells were exposed to ultraviolet light for 1 hr and the residual silver nitrate solution was rinsed with distilled water.

RESULTS

Cell proliferation

Initially, 10 $\times$ 10^3 cells were plated on each well. 2 days after plating, the cells cultured in the collagen gel matrix with 10 ng/ml of TGF-β1 increased in number to 10.3 $\times$ 10^3 $\pm$ 0.4 $\times$ 10^3 and, after 4 days, the cells increased in number to 26.2 $\times$ 10^3 $\pm$ 4.4 $\times$ 10^3, approximately 2.6 fold. In contrast, the cells cultured in the collage gel matrix without TGF-β1 did not increase in number 2days after plating and increased approximately 1.4 fold (14.4 $\times$ 10^3 $\pm$ 0.1 $\times$ 10^3) after 4 days (Fig. 1A). The cells cultured in the monolayer with 10 ng/ml of TGF-β1 increased in number approximately 5.7 fold (57.7 $\times$ 10^3 $\pm$ 11.4 $\times$ 10^3) after 4days (Fig. 1B). These results showed that the cells treated with TGF-β1 increased in number two times those that were not. However, the increase in number of cell was not as great when compared to the number of the cells cultured in the monolayer with TGF-β1.

ALP activity

ALP activity was used in the present study as an index for HBMSC differentiation. The HBMSCs were treated with TGF-β1 concentrations of 0, 1,

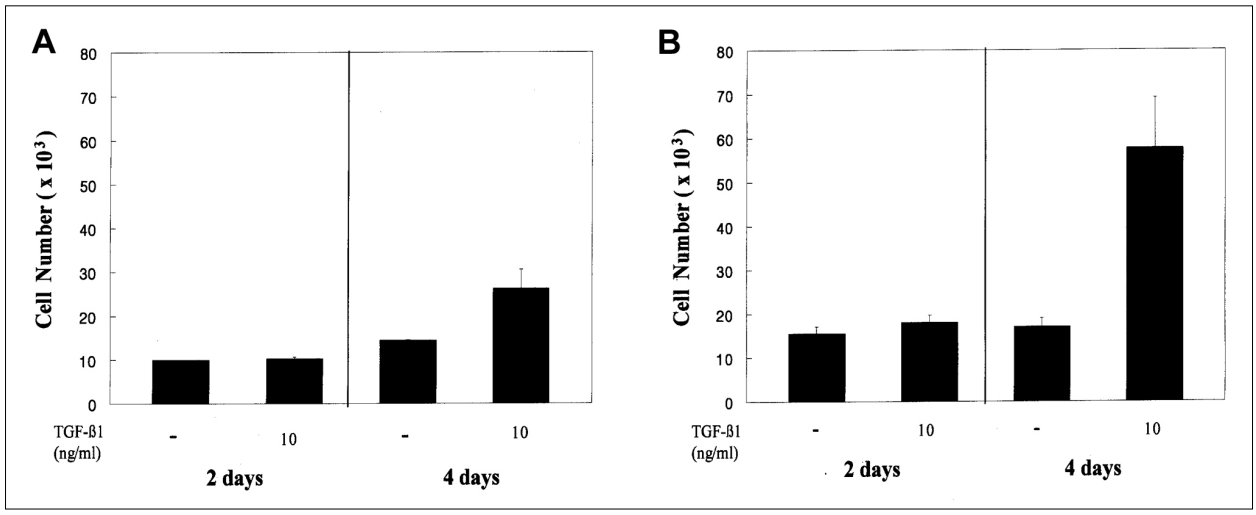

Fig. 1. Proliferation of the cells. An equal number of the cells were plated. 2 and 4 days after plating, the cells were counted. The cells cultured in collagen gel matrix with 10 ng/ml of TGF-β1 increased 1.3 and 2.6 fold after 2 and 4 days respectively, while the cells without TGF-β1 increased 1.4 fold after 4 days under similar conditions (A). The cells cultured in monolayer with TGF-β1 increased 5.7 fold after 4 days (B).

5, and 10 ng/ml for first 2 days and followed by an additional 100 ng/ml of BMP-2 for a further 3 days. The cells treated with 5 and 10 ng/ml of TGF-β1 expressed a very low ALP activity (7.253 ± 0.722 and 6.822 ± 0.853 U/L) when compared to cells not treated with TGF-β1 (18.477 ± 3.111 U/L). However, the cells treated with 1 ng/ml of TGF-β1 responded efficiently to rhBMP-2 and expressed a high ALP activity (18.879 ± 3.627 U/L) with a level equivalent to that exhibited by cells that were not treated with TGF-β1. The cells treated with 10 ng/ml of TGF-β1 and 100 ng/ml of rhBMP-2 together expressed intermediate ALP activity (8.234 ± 2.440 U/L). However the cells treated with 10 ng/ml of TGF-β1 alone for 2 days followed by 100 ng/ml of BMP-2 alone for another 3 days still expressed a high ALP activity (12.534 ± 2.981 U/L)(Fig. 2). These results show that TGF-β1 inhibits the effect of rhBMP-2 on osteoblastic differentiation of HBMSCs in a dose dependent manner.

Von-Kossa staining

The HBMSCs cultured in the collagen matrix gel treated with 100 ng/ml of rhBMP-2 in the presence of β-gylycerophosphate and ascorbic acid demonstrated Von-Kossa positive solid deposits after 3 weeks of culture (Fig. 3A). In contrast, the cells treated with 10 ng/ml of TGF-β1 showed only a few Von-Kossa positive solid deposits (Fig. 3B). The mineralization nodules were patchy but were spread randomly over the entire collagen matrix gel.

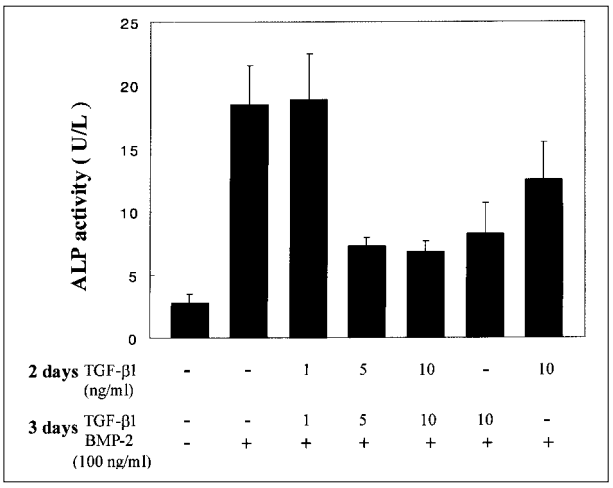

Fig. 2. ALP activity of HBMSCs treated with varying amounts of TGF-β1. The cells with 5 and 10 ng/ml TGF-β1 exhibited low levels of ALP activity when treated with rhBMP-2, while the cells with 1 ng/ml of TGF-β1 exhibited high levels of ALP activity. The cells treated with 10ng/ml of TGF-β1 first, followed by rhBMP-2 alone also exhibited high levels of ALP activity. The data shows that the presence of TGF-β1 inhibits the cell response to BMP-2 stimulation in a dose dependent manner.

DISCUSSION

The TGF-β1 protein is multifunctional peptide,

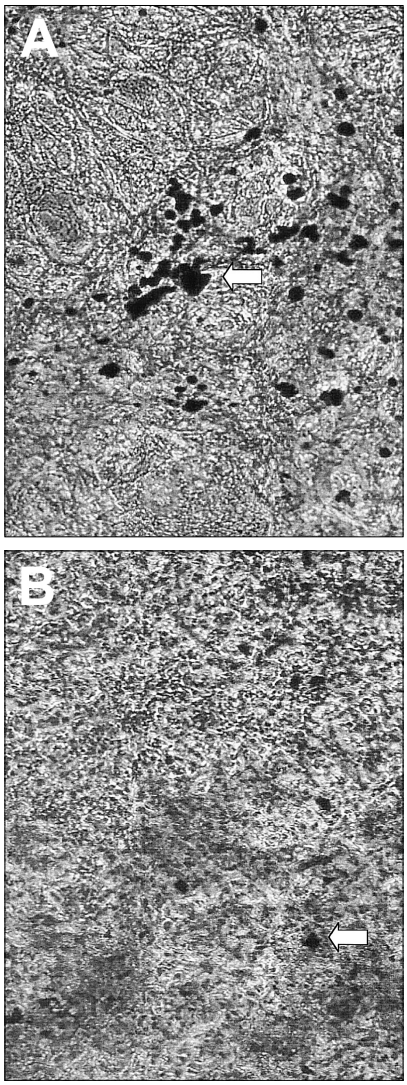

Fig. 3. Mineralization of collagen gel matrix (× 100). The cells cultured in collagen gel matrix treated with 100 ng/ml of rhBMP-2 demonstrated Von-Kossa positive solid deposits (white arrow) after 3 weeks of culture(A), while the cells treated with 10 ng/ml of TGF-β1 showed few Von-Kossa positive solid deposits (B).

stored within the bone matrix, and has a broad range of cellular activities including the control of cell proliferation and expression of the differentiated phenotypes of various cell types.[8,12,25,26] By isolating TGF-β1 from the bone matrix, numerous *in vitro* studies have demonstrated a key role of this growth factor in osteoblastic differentiation, matrix synthesis and bone remodeling.[12,15,25,27] BMP-2 is a subfamily of BMPs among members of the TGF-β superfamily and is able to induce bone formation *in vivo* and promote osteoblastic differentiation.[28-32] Both TGF-βs and BMPs may act in a sequential manner at different stages to promote mesenchymal stem cell differentiation towards the osteoblastic phenotype.[33] Although TGF-β1 has been shown to play a major role in osteoblast differentiation and bone remodeling, there are several *in vitro* and *in vivo* studies that indicate the divergent effects of this growth factor on the expression of the differentiated osteoblast phenotype.[34,35] Some studies have been shown that treating mesenchymal stem cells with TGF-β1 inhibits ALP activity and osteocalcin production.[16,17] There is also some *in vivo* data indicating that the application of TGF-β1 to fracture sites does not induce bone formation, but instead results in a tissue reaction at the injection site.[36] This contradicts the role ascribed to TGF-β1 induction of mesenchymal stem cell differentiation toward the differentiated cell phenotypes of bone and cartilage.[18,25,37] The complete induction of osteogenesis requires the effect of a multiple of local factors acting in a coordinated fashion to control cell proliferation and generate cell differentiation via a multi-step process.[33]

The mechanism by which TGF-β1 inhibits BMP-2 induction of ALP activity in human bone marrow stromal cells is not clear. BMPs induce mesenchymal stem cell differentiation into the osteogenic cells and enhance both the differentiation and function of osteoblasts.[38] BMP-2 induces osteoblast commitment by binding to the type I receptor on mensenchymal stem cells resulting in a cascade that involves phosphorylation of specific tansactivators, smad 1, 5 and 8, which associate with smad 4 then translocate to the nucleus.[39] TGF-β1 does not bind to the BMP-2 receptors although they both use smads as mediators of their signaling mechanism.[40] However, it is possible that the number of receptors occupied by TGF-β1 may influence BMP-2 binding to its receptor. A second possibility by which TGF-β1 could inhibit BMP-2 action, is that TGF-β1 in high concentrations may induce protein synthesis by the mesenchymal stem cells that block BMP-2 activity. Two proteins, noggin and chordin, have been shown to block BMP-2 and BMP-4 activity by preventing receptor binding.[38,41-43] It is therefore possible that TGF-β1 in large amounts may induce the synthesis of noggin or chordin, which in turn inhibit BMP-2 activity. Indeed, it has been shown that TGF-β1 upregulates noggin synthesis in mesenchymal stem cells.[38] These possibilities will need to be tested in order to verify these hypotheses.

It has also been reported that the collagen matrix induced the osteoblastic differentiation itself[22] and TGF-β1 accelerated the osteogenic differentiation of bone marrow stem cells induced by the collagen matrix.[23] Lynch et al. speculated that this induction might be due to the interaction of the collagen matrix with the intergrin receptors in the cells.[44] It has been previously shown that bone marrow stem cells express the osteoblast phenotype when treated with BMP-2.[21,24] The expression of ALP activity in the presence of BMP-2 was therefore used as an index to determine the effect of TGF-β1 on bone marrow stem cell differentiation toward the osteoblast lineage.

The present findings demonstrate that the cells cultured in a collagen gel matrix treated with TGF-β1 for 2 days increased in number approximately 2.6 fold. However, the cells cultured in the collagen gel matrix did not increase to such a high number when compared with the cells cultured in the monolayer. The cells treated with TGF-β1 followed by TGF-β1 and rhBMP-2 showed a reduction in the expression of the osteoblast phenotype, indicating that presence of TGF-β1 inhibited the cell response to BMP-2. The cells treated with TGF-β1 protein first followed by rhBMP-2 alone expressed ALP activity. Therefore, it is proposed that the effect of TGF-β1 on the HBMSCs was reversible and dose dependent. With Von-Kossa staining, the collagen gel matrix treated with rhBMP-2 showed mineralization, but those with TGF-β1 showed a much

lower degree of mineralization. This suggests that the collagen gel matrix cannot in itself induce osteoblastic differentiation of HMBSCs. These results were slightly different from the results from a previous study. HBMSCs were cultured in DMEM with 1% FBS to reduce the effects of other growth factors that may be present in the serum. The results indicate that TGF-β1 and BMP-2 are coordinately controlled during osteoblast differentiation of the osteoprecursor cells from the HBMSCs even in a collagen gel matrix as reported previously in monolayer culture, so the collagen gel matrix is a good scaffold to culture HBMSCs. However, the main problem with this is that cells cultured in a collagen gel matrix showed a lower rate of cell growth, when compare to the cells cultured in a monolayer.

Understanding the effects of TGF-β1 on BMP-2 in controlling mesenchymal stem cell differentiation when cultured in a collagen gel matrix, may be helpful in developing uses of these growth factors and a collagen gel in skeletal repair. This may be useful in accelerating the fracture healing and the treatment of non-union.

Myung Ku Kim, M.D., Ph.D.
Department of Orthopaedic Surgery,
Inha University Hospital,
7-203, 3Ga Shinheung-Dong, Jung-Gu,
Incheon 400-103, Korea.
Tel: 82-32-890-3662, Fax: 82-32-890-3099,
E-mail: m9kim@inha.ac.kr

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