

## Original

# Effects of Immobilized rhBMP-2/atelocollagen in vivo and in vitro

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**Abstract:** The use of recombinant human bone morphogenetic protein - 2 (rhBMP-2) to induce ectopic bone formation requires a carrier. Atelocollagen, a biomaterial with a porous structure, excellent operational features and biocompatibility, is known to be an effective carrier for rhBMP-2. However, conventionally used lyophilized rhBMP-2/atelocollagen mixture does not necessarily give adequate bone induction effect. In the present study, we evaluated the effect of immobilizing rhBMP-2 to succinylated type I atelocollagen on the cellular activity of ST2 cells and immobilized rhBMP-2/ atelocollagen and non immobilized rhBMP-2/atelocollagen implanted in subcutaneous pockets of Wister rats. Our results revealed that 1) Alkaline phosphatase activity confirmed the effectiveness of rhBMP-2/ succinylated type I atelocollagen immobilization in augmenting cellular activity. 2) Intracellular signaling continued for prolonged period when rhBMP-2 was immobilized to succinylated type I atelocollagen. 3) In rhBMP-2/atelocollagen implants were completely fully with new bone formation and cell proliferation. Whereas, in non immobilized rhBMP-2/atelocollagen implants showed new hard tissue in the periphery of the carrier with only collagen membrane in its center. This study indicated that immobilizing rhBMP-2 is an efficient method to increase bone induction.

**Keywords:** Immobilization, rhBMP-2, Collagen, Smad family, Alkaline phosphatase.

## Introduction

About twenty types of bone morphogenetic proteins (BMP) have been reported within the BMP family.<sup>1-3</sup> Among them, BMP-2 possesses the strongest bone inducing activity, and basic research has been conducted aiming at clinical application of this protein.<sup>4-7</sup> A carrier is indispensable when using rhBMP-2 to induce ectopic bone formation. Basic research has indicated that the atelocollagen, a biomaterial with a porous structure and excellent operational features and biocompatibility is highly effective as a carrier for rhBMP-2.<sup>8-10</sup> However, these studies used a lyophilized rhBMP-2/atelocollagen mixture, which may not possess an adequate bone induction effect. The mechanism of action of immobilized rhBMP-2 on cells is based on the theories of biomaterial science with respect to chemically bonding the ligand rhBMP-2 to a carrier. The ligand-carrier composite will react with the receptors on target cells and initiate the cellular function control mechanism. When growth factors such as insulin like

growth factor and epithelial cell growth factor are immobilized to a carrier, the immobilized growth factor binds with the receptor on the cell surface forming a complex.<sup>11-16</sup> Without being internalized into the cell, the growth factor-receptor complex is able to transduce signals into the cell over a long period of time, achieving high efficiency in promoting cell proliferation. BMP family is considered to be differentiation factors.<sup>17</sup> However, the effects of immobilized rhBMP-2 on cell differentiation and augmentation of cellular activity remain unknown. The purpose of this study was to test the hypothesis that chemically bonding (immobilizing) rhBMP-2 to atelocollagen may improve cell activity in vitro and increase the bone induction effect in vivo.

## Materials and Methods

### Cell culture

Mouse ST2 cell line (Cell No. RCB0224, from the cell bank of the Institute of Physical and Chemical Research, Japan.), a stromal cell line derived from mouse bone marrow, was used in this study. The cells were cultured in  $\alpha$ -minimum essential medium ( $\alpha$ -MEM, Gibco BRL, Inc., USA) supplemented with X1 antimicrobial agents (Antibiotic-Antimycotic, Life Technologies, Inc., USA).

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The cells were seeded in 10 cm petridishes (Falcon, Inc., USA) at a cell density of  $2 \times 10^5$  cells/cm<sup>2</sup>, and incubated at 37 °C with 5% CO<sub>2</sub>. The growth medium was changed every 3 days.

*Preparation of rhBMP-2/ succinylated type I atelocollagen collagen samples*

rhBMP-2 was kindly provided by Yamanouchi, Inc., Japan. Succinylated type I atelocollagen (Koken, Inc., Japan) was used as carrier. For immobilization, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (water soluble carbodiimide [WSC], Katayama Chemical, Inc., Japan) was used. The principle of rhBMP-2/ succinylated type I atelocollagen immobilization using WSC is illustrated in (Fig. 1).<sup>18</sup>

The test samples were prepared by the following methods: A. Immobilized group: succinylated type I atelocollagen 3 mg/ml was dissolved in dilute hydrochloric acid with pH 3.0. As a pre-treatment, WSC was added to the succinylated type I atelocollagen solution to obtain a final concentration of 1 mg/ml and incubated at 4 °C for 2 h. Then, rhBMP-2 4 μg/ml was added and immobilized at 4 °C for 24 h. After completion of immobilization, the samples were dialyzed for 24 h against dilute hydrochloric acid pH 3.0 to remove the WSC in the reaction mixture.

B. Non-immobilized group: succinylated type I atelocollagen was dissolved in dilute hydrochloric acid pH 3.0 and pretreated at 4 °C for 2 h without the addition of WSC. Then, rhBMP-2 was added. After reacting at 4 °C for 24 h, the mixture was dialyzed for 24 h against dilute hydrochloric acid pH 3.0.

*Effect of immobilized rhBMP-2/succinylated type I atelocollagen on ST2 cells*

ST2 cells were seeded in a 10 cm petridish (Falcon, Inc., USA)

and cultured. The medium was changed completely after a confluent growth was obtained. A sample of succinylated type I atelocollagen alone was included as control. The samples contain 150 ìg/ml of succinylated type I atelocollagen and 200 ng/ml of rhBMP-2. The cells were collected on days 1, 3, 5 and 7 to measure alkaline phosphatase (ALP) activity. At the end of the culture period, the medium was completely removed. The plates were washed twice in TBS solution (10 mM Tris HCl, 150 mM NaCl, pH 7.4) and kept them at 4 °C. Then, 500 μl of TBS solution containing 1% Triton X-100 was added, and the cells were collected and homogenized in a sonicator (Astrason, Misonix, Inc., USA) twice for 30 seconds each. The homogenate was centrifuged for 5 min at 12,000 rpm and at 4 °C. ALP in the supernatant was measured based on the method of Kind-King<sup>19</sup> using an ALP reagent kit (Wako, Inc., Japan). The DNA in the supernatant was measured quantitatively using a DNA reagent kit (Pico Green, Molecular Probe, Inc., USA). ALP activity per unit quantity of DNA was expressed as the molar quantity of PNP produced in 1 min per μg of DNA.

*Gene expression analysis by reverse transcription polymerase chain reaction*

ST2 cells were seeded in a 10 cm petridish (Falcon, Inc., USA) and cultured until confluency. The samples were added to growth medium with a final succinylated type I atelocollagen concentration of 150μg/ml and rhBMP-2 concentration of 200ng/ml. The cells were collected after incubation for 1, 3, 6, 12, 24 and 48 h. After RNA extraction, gene expression was analyzed by quantitative reverse transcription polymerase chain reaction (RT-PCR).

Total RNA was extracted from the cultured cells according to the AGPC method<sup>20</sup> using TRIzol (Life Technologies, Inc., USA).

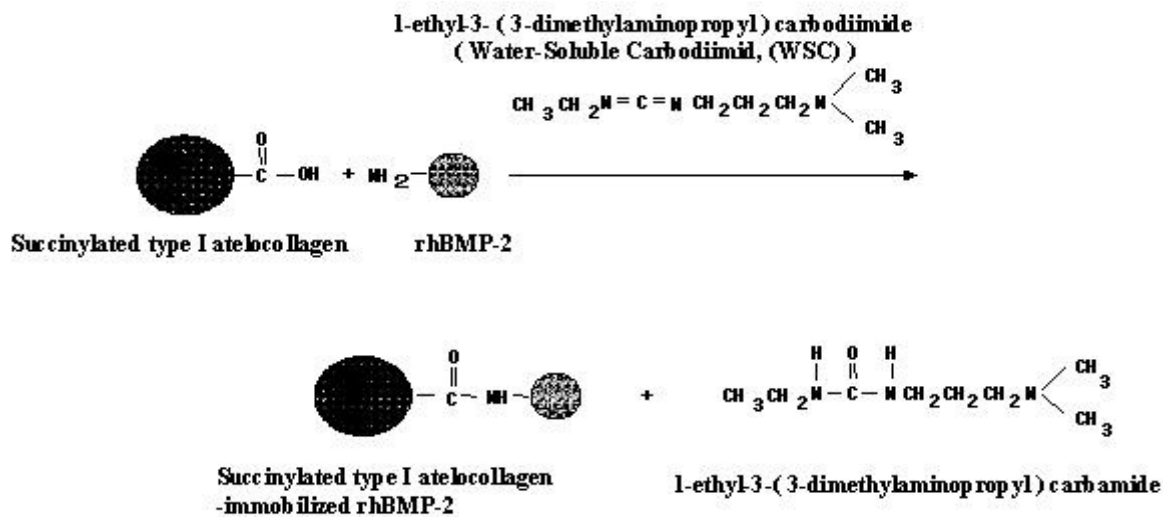


Fig. 1. Principle of immobilization of rhBMP-2 to succinylated type I atelocollagen. 1-ethyl-2-(3-dimethylaminopropyl) carbodiimide (water soluble carbodiimide:WSC) is a reagent that promotes the peptide bonding reaction (elimination of H<sub>2</sub>O) between a carboxyl residue and an amino residue. When collagen and rhBMP-2 react in the presence of WSC, the carboxyl residue of collagen and the amino residue of rhBMP are chemically bonded, and the two are immobilized through peptide bonds.

Total RNA was dissolved in DEPC-treated water and the absorption at 260 nm and 280 nm was measured by a spectrophotometer (DU-640, Beckman Instruments Inc., USA) to calculate the quantity and purity. Complementary DNA was synthesized using the AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Life Science, Inc., USA).

Quantitative RT-PCR was performed using a thermocycler (PC707, Astec, Inc., Japan). The thermal cycling condition for DNA amplification of BMPR-IA, BMPR-IB, Smad 1, 5, 8, BMP-2, BMP-4, and GAPDH was set at 94 °C for 30 sec, 58 °C for 30 sec and 72 °C for 30 sec. For Smad 6 and 7, GC buffer II (Takara, Inc., Japan) was added to the PCR reaction mixture and thermal cycling condition was set at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. The base sequences of the primers were used

- BMP-4 (F)5'-ACTCACCTCCACCAGACACG-3'  
 (R)5'-CCTCTACCACCATCTCCTGA-3', BMPR-IA  
 (F) 5'-TAGCACCAGAGGATACCTTGC-3'  
 (R) 5'-AATGCTTCATCCTGTTCCAAA-3', Smad1  
 (F) 5'-GTCTGTCTGAGGAGCGTGTAGA-3'  
 (R) 5'-GCATTCCAGAGGCTTCAGTTCATGG-3',  
 Smad5 (F) 5'-TGAACCACAGAGGGCAGGGAGTAG-3'  
 (R) 5'-ACGGAAGCTGGGTATGATGGCGGA-3',  
 Smad8 (F) 5'-TACCATTACCGCAGAGTGGGGACC-3'  
 (R) 5'-TCCTCGTAGCAAAGTGGCCGGAAG-3',  
 Smad6 (F) 5'-GAAGTCGTGTGGTCCCTGATCG-3'  
 (R) 5'-ACTCCACAGCCTCCAACAGCGT-3',  
 Smad7 (F) 5'-TCAGGACCAAACGATCTGCGCTC-3'  
 (R) 5'-GAGATCCGGCCACCTGAACACTTT-3',  
 GAPDH(F) 5'-GGGTGGAGCCAAACGGGTC-3'  
 (R) 5'-GGA GTTGCTGTTC AAGTCGCT-3'

**Subcutaneous implantation and explantation**

Wister Rats were subjected to intramuscular anesthesia with Ketamine (Fuji Chemical Industry Co., Ltd. Japan) and Dormitol (Meiji Seika Kaisha LTD. Japan). The samples contained 10mg of type I atelocollagen and 10µg of rhBMP-2. On the subcutaneous pockets of the back immobilized rhBMP-2/atelocollagen and non immobilized rhBMP-2/atelocollagen were implanted. The animals were sacrificed with an overdose of ether at 2 weeks after implantation. All specimens and surrounding tissue were removed and fixed by 4% paraformaldehyde, decalcified with 10% EDTA, embedded in paraffin, sectioned at 4 µm in thickness and stained by hematoxylin-eosin (H&E).

**Results**

Effect of immobilized rhBMP/succinylated type I atelocollagen on ST2 cells

**ALP activity with time course**

Compared to the collagen control group, both the immobilized and non-immobilized groups showed increased ALP activity along

of the period of time. The immobilized and non-immobilized groups showed no differences in ALP activity on days 1 and 3, but significantly higher ALP activity was observed in the immobilized group compared to the non-immobilized group on days 5 and 7 (Fig. 2).

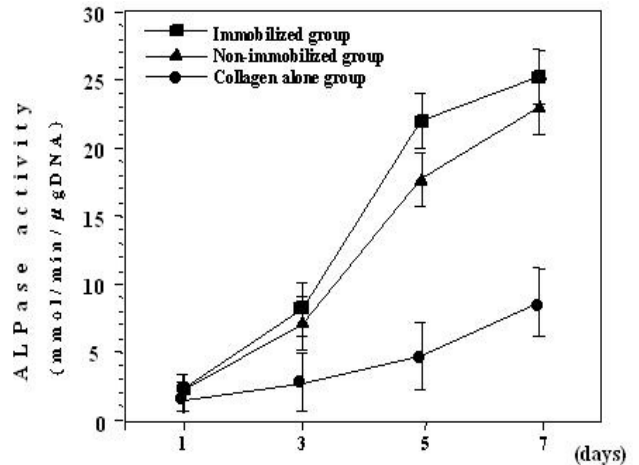


Fig.2. Changes of ALP activity in ST2 cells with time. Both the immobilized and non-immobilized groups show increased ALP activity with time. The immobilized group shows higher ALP activity than the non-immobilized group on days 5 and 7 (p < 0.02).

**RT-PCR**

The changes in gene expression detected by quantitative RT-PCR at various times after the addition of immobilized or non-immobilized rhBMP-2 are shown in fig. 3.

**Expression of BMPR-1A gene**

In the immobilized group, the expression remained unchanged after the addition of immobilized rhBMP-2 for up to 24 h, and then decreased at 48 h. In the non-immobilized group, the expression decreased sharply after the addition of non-immobilized rhBMP-2, but started to increase with time after 3 h. Expression of specific Smad 1, 5 and 8 genes

In the immobilized group, the expression remained almost unchanged after the addition of immobilized rhBMP-2 but decreased after 48 h. In the non-immobilized group, an early, sharp decrease in expression was observed after the addition of non-immobilized rhBMP-2, and thereafter gene expression started to increase with time.

The pattern of expression for Smad 8 gene differed from those of Smad 1 and 5. In the immobilized group, the expression increased along of period of time, reaching a peak at 12 h and declined thereafter. In the non-immobilized group, the expression continued to increase with time.

**Expression of inhibitory Smad 6 and 7 genes**

In the immobilized group, there was a sharp increase with time,

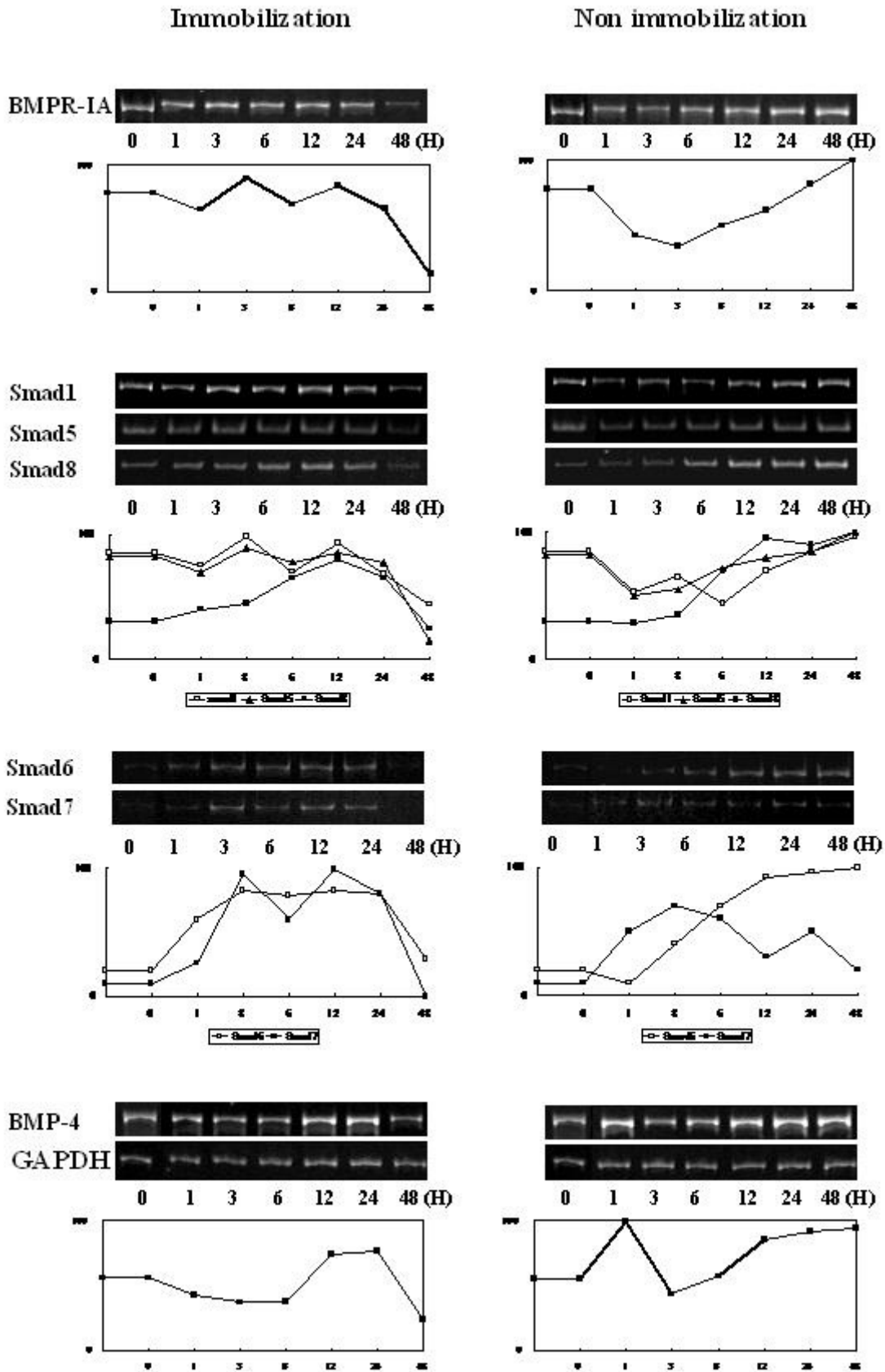


Figure 3. Effects of immobilized and non-immobilized rhBMP-2 on the expression of various genes. Immobilized or non-immobilized rhBMP-2 was added to ST2 cells and the cells were sampled after 1, 3, 6, 12, 24 and 48 h and gene expression was analyzed by quantitative RT-PCR method. RT-PCR was conducted under conditions in which the amplification reaction was not saturated.

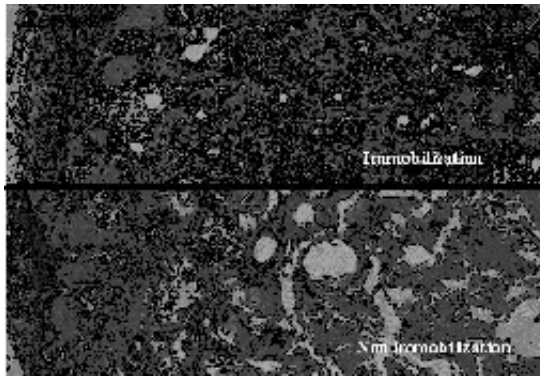


Fig. 4 In rhBMP-2/atelocollagen implants were completely fully with new bone formation and cell proliferation. Whereas, in non immobilized rhBMP-2/atelocollagen implants showed new hard tissue in the periphery of the carrier with only collagen membrane in its center.

and the high level of expression was maintained, followed by a decrease after 48 h. In the non-immobilized group, the Smad 6 gene expression showed a tendency to increase with time continuously, but Smad 7 gene expression reached a maximum at 3 h followed by a decline in its expression.

#### Expression of BMP-4 gene

In the immobilized group, there was no remarkable change up to 6 h after the addition of immobilized rhBMP-2, but the expression increased at 12 h and then declined rapidly at 48 h. In the non-immobilized group, BMP-4 gene expression increased sharply at 1 h, declined at 3 h, and then increased with time. No BMP-2 and BMP-IB gene expression was observed.

#### Histological Analysis

Immobilized rhBMP-2/atelocollagen showed the carriers fully filled by immature new bone with high cellularity. The new hard tissue was observed at the periphery and the center area of the carrier. Cellular proliferation was found in its center.

Non-immobilized rhBMP-2/atelocollagen revealed immature bone formation in the periphery of the carrier. In the center of carrier, only collagen membranes were observed. (Fig. 4).

#### Discussion

ST2 cells are stromal cells derived from mouse bone marrow. These cells have been reported to express characteristics of osteoblasts when stimulated by rhBMP-2, 4, or 6.<sup>21,22</sup> Stimulation of ST2 cells by rhBMP results in the increase of osteocalcin gene expression and ALP activity, which is an indicator of differentiation to osteoblast. It was also demonstrated that the immobilized sample showed higher ALP activity than the non-immobilized sample, indicating the beneficial effect of immobilization of rhBMP-2 on ST2 cells. Combining our findings in the present study with those reported by Ito et al<sup>12</sup>, we propose a mechanism by which immobilized rhBMP-2 acts on ST2 cells

to promote cell activity. After the collagen-immobilized rhBMP-2 forms a complex with the receptor located on the cell membrane, the large complex is not internalized by the cell but continues to transduce signals into the cell through the receptor inducing an increase of ALP activity.

The present study also attempted to examine the gene expression of intracellular signaling molecules induced by rhBMP-2 as well as the effect of immobilizing rhBMP-2 on the gene expression. BMP-2 binds with receptors on the cell membrane to mediate intracellular signal transduction<sup>26</sup>. The Smad protein family comprising the common Smad: Smad 4, the specific Smads: Smad 1, 5 and 8, and the inhibitory Smads: Smad 6 and 7, is known to be involved in the intracellular signaling pathway of BMP<sup>23-28</sup>. Once the receptor binds with BMP-2, Smad 1, 5, and 8 will bind to phosphorylated BMPR-IA, thereby becoming phosphorylated too and then form a complex with Smad 4. The complex then moves inside the nucleus and transduces signals. Furthermore, Smad 6 and 7 have been proposed to have an inhibitory effect on the specific Smads<sup>23-28</sup>.

According to the report of Ito et. al.<sup>29,30</sup> insulin immobilized by a carrier is not taken up by the cell, but continues to bind with the receptor and transduces signals. Considering these findings with the previously reported data of the BMP signaling pathway, it is possible to hypothesize the situation in the immobilized group as follows. When rhBMP-2 is chemically bonded to succinylated type I atelocollagen, the molecular size is increased. After rhBMP-2 binds with the receptor, the complex is not taken up by the cell but continues to transmit stimulation signals. As a result, the BMP-IA gene and specific Smad 1 and 5 genes continue to be expressed at high levels. Moreover, the specific Smad 8 and the inhibitory Smad 6 and 7 genes that were expressed at low levels at baseline were upregulated through stimulation by BMPR-IA in response to rhBMP-2 stimulation. From these observations, one may speculate that when the differentiation factor rhBMP-2 is immobilized, it continues to send signals for a prolonged period. On the other hand, in the non-immobilized group, expression of the BMPR-IA and Smad 1 and 5 genes was down-regulated 1 h after addition of non-immobilized rhBMP-2. Ito et. al.<sup>30</sup> reported that receptors stimulated by soluble (non-immobilized) insulin are activated transiently (several minutes to tens of minutes), and the complex is taken up by the cell and inactivated through down-regulation. The same phenomenon may have occurred in the non-immobilized group in the present study. It is possible that BMPR-IA and Smad 1 and 5 gene expression was reduced through down-regulation after rhBMP-2 stimulation.

In order to analyze the bone induction in vivo, immobilized rhBMP-2/atelocollagen and non immobilized rhBMP-2/atelocollagen were implanted in subcutaneous pockets of the rats. Interestingly, in rhBMP-2/atelocollagen implants were completely fully with new bone formation and cell proliferation. Whereas, in non immobilized rhBMP-2/atelocollagen implants showed new

hard tissue at the carrier periphery with only collagen membrane of the carriers in its center.

In conclusion, our results showed that intracellular signaling continued for prolonged period when rhBMP-2 was immobilized to succinylated type I atelocollagen. Moreover, immobilized rhBMP-2 stimulated alkaline phosphatase activity by ST2 cells in vitro and increase the bone induction in vivo. Because of this, we believe that immobilizing rhBMP-2 is an efficient method to increase bone induction.

#### Acknowledgment

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