

Delayed Expression of Circulating TGF- β 1 and BMP-2 Levels in Human Nonunion Long Bone Fracture Healing

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Background: The healing process of bone fracture requires a well-controlled multistage and sequential order beginning immediately after the injury. However, complications leading to nonunion exist, creating serious problems and costs for patients. Transforming growth factor-beta 1 (TGF- β 1) and bone morphogenic protein 2 (BMP-2) are two major growth factors involved in human bone fracture healing by promoting various stages of bone ossification. In this study, we aimed to determine the role of these factors during the fracture healing of human long bones and assess their impacts on nonunion condition.

Materials and Methods: We performed a comprehensive analysis of plasma TGF- β 1 and BMP-2 levels in blood samples from 10 patients with proved nonunion and 10 matched patients with normal union following a predetermined time schedule. The concentrations of TGF- β 1 and BMP-2 were measured at each time point using a solid-phase ELISA.

Results: TGF- β 1 and BMP-2 levels were detectable in all patients. For all patients, a maximal peak for TGF- β 1 was found at 3-week. In normal union group, TGF- β 1 showed a maximal peak at 2-week while nonunion group had a delayed maximal peak at 3-week. Plasma levels of BMP-2 for all patients and for normal union group reached a maximal peak at 1-week, but nonunion group showed a delayed maximal peak at 2-week. In general, plasma TGF- β 1 or BMP-2 level was not significantly different between normal union and nonunion groups.

Conclusion: The expression levels of TGF- β 1 and BMP-2 appeared to be delayed in nonunion patients which could play an important role in developing an early marker of fracture union condition and facilitate improved patient's management. (J Nippon Med Sch 2017; 84: 12–18)

Key words: circulating TGF- β 1, BMP-2, human long bone, fracture healing, nonunion

Introduction

In fracture healing, a well-controlled multistage and sequential process begins immediately after the injury. This process integrates complex components including various cells, cytokines, extracellular matrix products and growth factors¹⁻⁴. Of the growth factors, transforming growth factor-beta 1 (TGF- β 1) and bone morphogenic protein 2 (BMP-2) are two major growth factors involved in human bone fracture healing by promoting various stages of

bone ossification.

TGF- β 1 is a member of the TGF- β superfamily which has been claimed to play an important role in the process of fracture healing^{5,6}. In several animal models TGF- β 1 contributed to the healing process through enhancement of extracellular matrix remodeling⁶⁻⁹ and increased cartilage and callus formation¹⁰. In addition, circulating level of TGF- β 1 was reported to be elevated during human bone fracture healing¹¹⁻¹³ and decreased levels were sug-

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gested to predict delayed union and nonunion¹⁴.

BMP-2 is a member of a subgroup of TGF- β superfamily and triggers various signaling events which in turn stimulate chondrogenesis, osteogenesis, angiogenesis and extracellular matrix remodeling leading to fracture healing¹⁵. BMP signaling appears to induce chondrocyte differentiation and maturation. BMP-2 stimulated a form of type II procollagen specific for cartilage and a chondrocyte-specific marker termed alpha 10 integrin¹⁶. The results showed that compared to TGF- β 1, BMP-2 had superior ability to induce re-differentiation of chondrocytes. Also, the expressions of type IIB relative to type IIA procollagens and alpha 10 relative to alpha 11 integrin subunits were found to be good markers of chondrocytes differentiation state¹⁶. BMP-2 deficiency has been implicated in the delayed union or nonunion and recombinant BMP-2 and BMP-7 have been clinically used in the treatment of delayed union or nonunion^{15,17,18}. In addition a possibility of involvement of a BMP-related genetic predisposition in impaired fracture healing has been reported¹⁹.

Complications of fracture healing include delayed union or nonunion and have been observed in 5–30% of all patients with fracture²⁰. Subsequently, early detection and treatment of such cases could prevent serious impacts and costs for patients²¹. Studies on the role of TGF- β 1 and BMP-2 proteins in human long bone healing process are limited and further results are needed to establish the possibility of their application as early markers of union versus nonunion or their use in the treatment of delayed union or nonunion.

In this study, we performed a comprehensive evaluation of plasma levels of TGF- β and BMP-2 proteins in patients with long bone fracture during the healing process and assessed their impacts on healing failure or nonunion condition. Such knowledge could contribute to the understanding of molecular basis of the fracture healing and facilitate improved patient's management.

Materials and Methods

Patients

We studied patients with bone fracture injury who referred to our emergency medicine department between January 2010 and December 2012. A consecutive total of 136 patients (97 males and 39 females) were registered. Informed consents were obtained from all participants and the study was approved by the Institutional Review Committee of the Nippon Medical School. Patients with pathological fractures or more than two fractures or sig-

nificant tissue damage (Gustilo's classification type III open fractures) and age under 18 years old were excluded from the study.

All patients were closely observed after injury and during the course of healing. To avoid bias, we further limited the patients to those with lower limb fractures. Data on patient's age, sex, smoking status (5 cigarettes or more/day), diabetes mellitus, anatomical site of fracture, type of fracture, treatment modality and mean duration of healing (bone union) were recorded. All patients were followed-up during the course of the study using clinical and radiological examinations.

Normal union was defined as complete bone healing within less than 6 months after injury. Nonunion was defined based on conventional definition as no bony healing obtained in 6 months after the fracture evidenced by specific radiological features showing bone ending changes¹¹. During the course of the study, we experienced 10 patients with lower limb fracture who developed nonunion. For comparison purpose, we selected 10 patients with matched conditions from the group who had normal physiological union. Thus, our study is based on a total of 20 patients (13 males and 7 females).

Blood Samples

We collected blood samples from each patient in EDTA collection tubes following a predetermined time schedule. Briefly, blood samples were collected at the time of injury (0 week) and subsequently at 1, 2, 3, 4, 6, 8 and 12 weeks after injury. To prevent circadian effect, all samplings were done in the morning between 10:00 and 12:00. The samples were centrifuged according to the manufacturer's instruction accompanying with TGF- β 1 and BMP-2 immunoassay kits and caution was exercised for complete platelet removal. Aliquots of plasma supernatants were separated and stored at -80°C until use.

Measurements TGF- β 1 and BMP2

The concentrations of TGF- β 1 and BMP-2 proteins in the plasma were measured using commercially available immunoassay kits (Quantikine, RD Systems, Minneapolis, MN, USA) which were based on a solid-phase enzyme linked immunosorbant assay (ELISA). The TGF- β 1 kit had been designed to detect biologically active form of the protein. The BMP-2 kit had been specially designed to measure relative mass values for naturally occurring BMP-2 in bone tissue extracts which in fact is closer to the plasma condition rather than the serum. Plasma samples from all matched patients were analyzed with the same assay to prevent inter-assay variability.

Table 1 Demographic characteristics of the matched normal union and nonunion patient

Characteristics*	Normal union No.	Nonunion No.
Mean age (yrs ± SD)	37 ± 17	39 ± 17
Male/female	7/3	6/4
Smoker/non-smoker	5/5	5/5
Site of fracture		
Femur shaft	4	6
Tibia shaft	6	4
Type of fracture		
Wedge	6	5
Complex	4	5
Open fracture	3	6
Surgical treatment		
Nailing	7	8
Plate fixation	3	2
External fixation	5	6

Abbreviation: SD, standard.

*There was no significant difference between normal union and nonunion groups in each category.

Statistical Analysis

Mann-Whitney U-test was used to compare continuous variables between the groups. All statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA). Data is presented as mean ± SD. A p value <0.05 was considered as significant.

Results

The patients' demographic characteristics are shown in **Table 1**. The mean age in normal union group was 37 ± 17 and in nonunion group was 39 ± 17. Of the total patients, 13 (65%) were males and 7 (35%) were females. The ratio of male per female was 2.3 and 1.5 in normal union and nonunion groups respectively. The frequency of smoking habit was equal in both normal union and nonunion groups. Fracture of femur and tibia shafts occurred each in 10 cases and their frequencies were comparable in both groups. Open fracture type I and II Gustilo's classification occurred in 3 (33%) normal union and 6 (67%) nonunion patients. No patient had diabetes mellitus. All patients underwent surgery and had comparable frequencies in their treatment modalities between normal union and nonunion groups. The mean follow-up time for all patients was 26.1 ± 9.54 months (21.8 ± 0.67 months in normal union and 30.4 ± 16.7 months in nonunion groups). The Mean duration of healing (bone union) was 2.7 ± 0.67 months in normal union versus 13.2 ± 7.45 months in nonunion group.

Overall, there was no significant difference in terms of patient's age, sex, smoking habit, anatomical location of fracture, types of fracture and treatment modalities between normal union and nonunion groups. Additionally, we did not find a significant difference between normal union group and nonunion group in terms of the injury severity score (ISS) and timing of surgery. The ISS for both normal union group (range: 5–50) and nonunion group (range: 10–43) was 21.9.

In all patients, TGF-β1 and BMP-2 levels were readily detectable. Considering all patients, TGF-β1 reached a maximal peak at 3-week, slightly declined at 4-week and stayed at a similar level (**Fig. 1A**). In normal union group, TGF-β1 (range: 24.04–37.96 ng/mL) reached a maximal peak at 2-week, considerably declined at 8-week and elevated again (**Fig. 1B**). In contrast, in nonunion group, TGF-β1 (range: 11.68–36.82 ng/mL) reached a delayed maximal peak at 3-week, slightly declined at 4-week, rebounded at 8-week and declined thereafter (**Fig. 1C**).

Plasma levels of BMP-2 for all patients reached a maximal peak at 1-week, considerably declined at 4-week, slightly elevated and stayed at a similar level (**Fig. 2A**). In normal union group, BMP-2 (range: 32.95–32.91 pg/mL) resembled the pattern as for all cases (**Fig. 2B**). In contrast, in delayed union group, BMP-2 (range: 38.00–37.12 pg/mL) reached a delayed maximal peak at 2-week, declined at 4-week, elevated at 6-week, and fluctuated thereafter (**Fig. 2C**).

In general, TGF-β1 or BMP-2 level was not significantly different between normal union and nonunion groups, however a peak plasma value for TGF-β1 at 2-week and for BMP-2 at 1-week was determined.

Discussion

Limited studies exist on circulating TGF-β1 or BMP-2 in patients with bone fracture in which sequential measurements at different time points during the healing process had been employed. These studies have reported contradictory results. Some studies have measured TGF-β1 levels in serum and/or fracture hematoma from patients with normal bone fracture healing and delayed union versus healthy controls^{12–14}. Reduced serum level of TGF-β1 was associated with delayed union or nonunion suggesting TGF-β1 as a marker of delayed fracture healing¹⁴. Another study indicated that serum TGF-β1 level was significantly higher in normal healing patients versus healthy controls during early healing period and at 24 weeks and with lesser extent in nonunion group versus

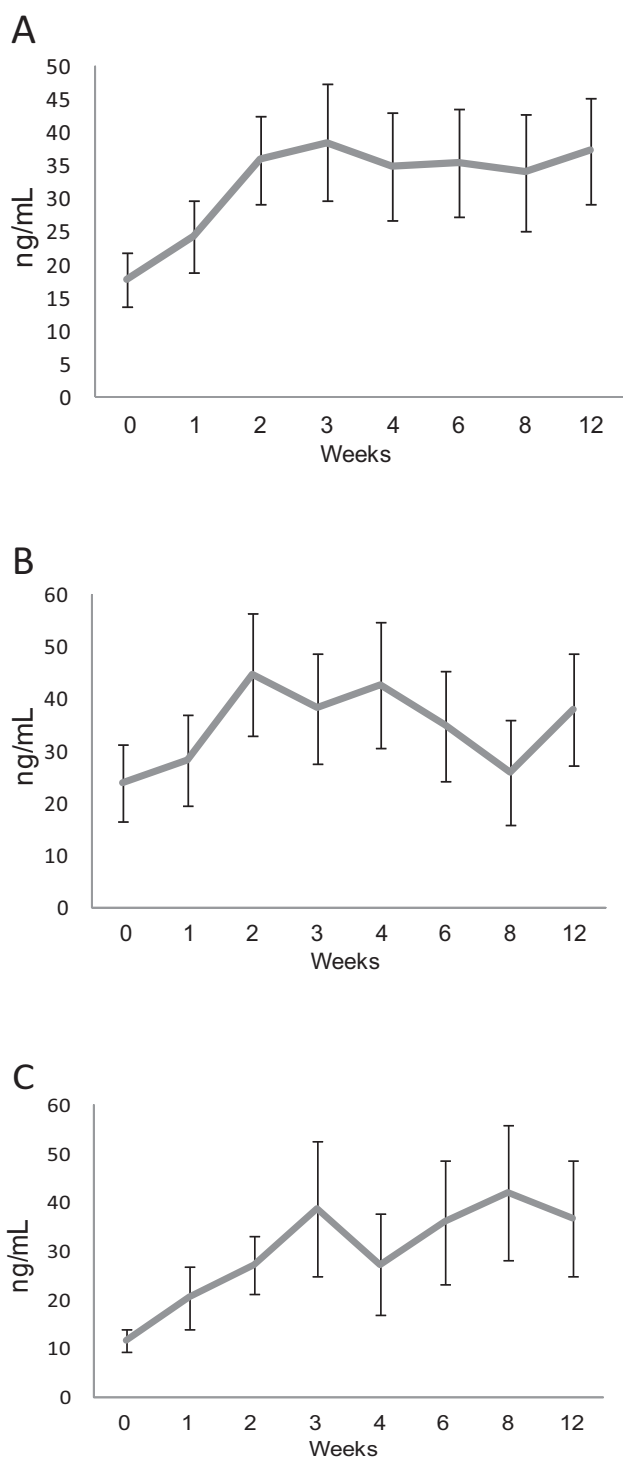


Fig. 1 Plasma levels of TGF- β 1 for all patients (A), and for normal union group (B) showed a maximal peak at 3-week and 2-week respectively while that for nonunion group (C) showed a delayed maximal peak at 3-week as compared to normal union group.

control patients¹¹. Also, TGF- β 1 levels were significantly higher in fracture hematoma as compared to peripheral serum of the patients but no significant difference was

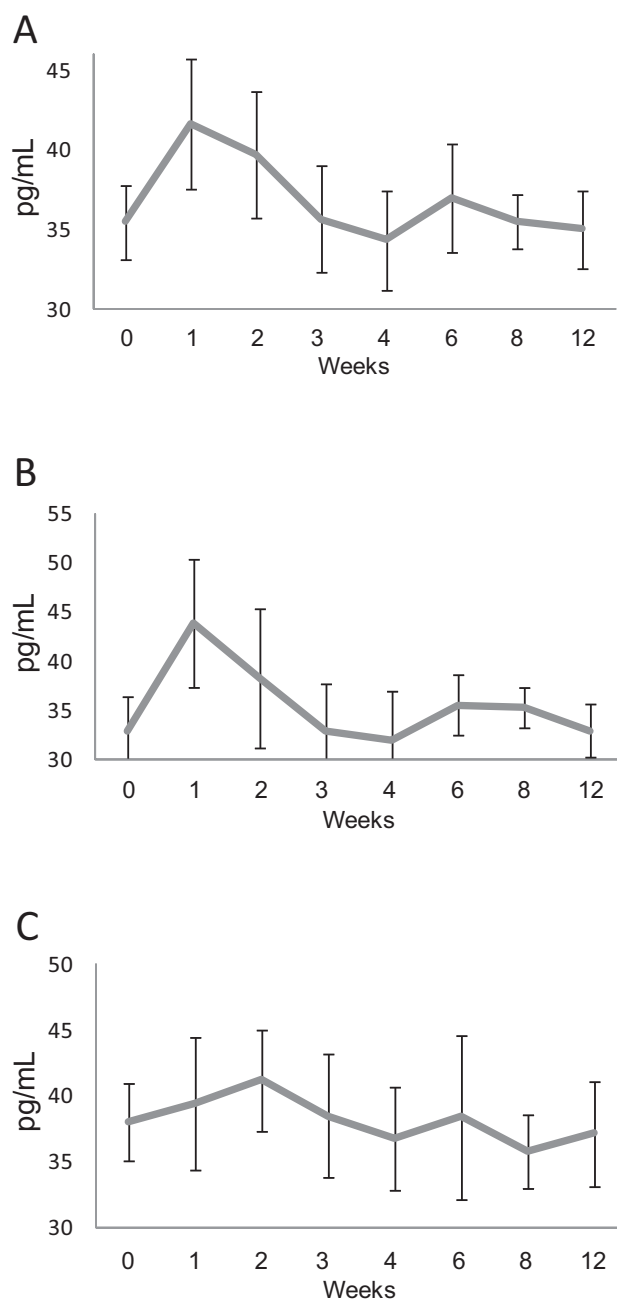


Fig. 2 Plasma levels of BMP-2 for all cases (A) and normal union group (B) show a maximal peak at 1-week while that for nonunion group (C) show a delayed maximal peak at 2-week.

found between patients with normal healing and those with delayed fracture healing. In our study, we also found a higher level of TGF- β 1 in normal union group as compared to nonunion group however the difference did not reach to a significant degree. Nevertheless, this reduced level of TGF- β 1 could contribute to the nonunion condition. Serum concentrations of TGF- β 1 varied depending upon smoking status, age, gender, diabetes mellitus and chronic alcohol abuse at different times¹², sug-

gesting precautionary measures in the application as a predictive marker of fracture healing. We used a set of strict criteria to match patients in the normal union and nonunion group thereby avoiding the effect of such factors.

On the BMP-2 levels, a deficiency of BMP-2 was found in nonunion fracture healing²². In contrast, another study found no significant difference in plasma levels of several BMPs including BMP-2, -4, -6, -7, and -9 between normal union and delayed union in a group of patients with diaphyseal tibia or femur fracture. The authors measured plasma BMP level only at two time points; at the injury time and at least 2 years after the bone healing²³. We measured plasma BMP-2 levels at several time points during the critical period of fracture healing starting from the injury time and up to 12 weeks after the injury. Consistent with the previous results, we also found a higher expression level of BMP-2 in normal union as compared to nonunion group but the difference was not significant. However, a reduced BMP-2 level could delay the healing process leading to the nonunion condition.

Regulation of fracture healing through the BMP-2 signaling pathway and/or the TGF- β signaling pathway using G-protein-coupled receptor kinase 2-interacting protein-1 (GIT1) knockout mice showed that decreased BMP-2 signaling exhibited delayed fracture healing²⁴. A better capability for BMP-2 than TGF- β 1 was demonstrated for stimulation of chondrocyte re-differentiation¹⁶. Moreover, TGF- β 1 could inhibit BMP-2 and BMP-7 signaling via upregulation of Ski-related novel protein N (SnoN) suggesting a possible mechanism for the failure of BMP therapy²⁵. Further studies showed biphasic effects of TGF- β on BMP in which TGF- β 1, in addition to its action as an inhibitor, could enhance osteoblast differentiation induced by BMP through reducing the negative feedback loop mediated by noggin²⁶. This might be useful in improving fracture healing. We found a delayed expression of BMP-2 in the nonunion group. As a hypothesis, the interactions between TGF- β 1 and BMP-2 signaling could be the case in our study in which TGF- β 1 could inhibit BMP-2 expression and promote nonunion condition. Accordingly, strategies to interfere with the inhibitory effect of TGF- β 1 signaling on BMP-2, for example by reducing the noggin-mediated negative-feedback loop, might be able to help enhance BMP-2 effect on osteoblast differentiation and improve fracture healing.

Overall most studies did not show a significant difference in the TGF- β 1 or BMP-2 level between normal union and nonunion patients. Our study results were also

consistent with those studies. Some factors may have contributed to the lack of difference between normal union and nonunion including difficulties to measure the existing traces amount of TGF- β 1 protein (nanograms level) and even lesser amount of BMP-2 protein (picograms level).

Recombinant human BMP-2 protein has been clinically used to enhance bone healing^{18,27,28}. So far, BMP-2 and BMP-7 has been approved for clinical use in open fractures of long bones, nonunion or spinal fusion. Clinical trials in humans have shown beneficial effect of BMP-2 on healing fractures in tibia bone. In one study, the authors noted that patients receiving recombinant BMP-2 could bear weight sooner than the controls²⁹. They concluded that recombinant BMP-2 can be used safely in patients with open tibial fractures and intramedullary nailing with no staged bone-grafting. Other studies showed that in cases with fracture of tibia and extensive bone loss at tibial diaphysis, recombinant BMP-2/allograft was clinically useful and safe substitute to autogenous bone graft³⁰. On the contrary, in a randomized clinical trial, recombinant BMP-2 did not significantly accelerate fracture healing¹⁸. Collectively these studies signify the usefulness of recombinant BMP-2 in accelerating fracture healing.

Although our study did not find a significant difference in TGF- β 1 or BMP-2 levels between normal union and nonunion groups which is consistent with previously reported findings, however we found a delayed maximal peak for both TGF- β 1 and BMP-2 proteins in nonunion patients as compared to the normal union patients. The peak plasma value of TGF- β 1 at 2-week may be explained by the fact that TGF- β 1 is released from the granules of platelets during the clotting process soon after trauma³¹. These findings suggested important roles for TGF- β 1 and BMP-2 proteins in fracture healing which could also facilitate as a marker for early detection of nonunion development. A major limitation of our study is the low number of patients. However, to the best of our knowledge only few studies have evaluated sequential human blood samples to simultaneously measure both TGF- β 1 and BMP-2 levels during fracture healing in the same patients and moreover we employed strict matching criteria which allowed us to compare patients with normal union and nonunion conditions.

In conclusion, the expression levels of TGF- β 1 and BMP-2 appeared to be delayed in nonunion patients. In addition, the delayed peak plasma value of TGF- β 1 at 2-week and of BMP-2 at 1 week could be useful in developing an early marker of fracture union condition. These

findings may contribute to the understanding of the mechanism(s) regulating fracture healing and facilitate improved patient's management.

Acknowledgement: This work was supported by a grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan No.: 24592752 to Dr. Yoshiaki Hara. The authors wish to thank Seiko Egawa for expert technical assistance.

Conflict of Interest: The authors declare no conflicts of interest.

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(Received, March 7, 2016)

(Accepted, September 29, 2016)