Circulating bone morphogenetic protein levels and delayed fracture healing

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Abstract

Objective Despite adequate treatment 5–30 % of bone fracture patients experience delayed union. During normal fracture union, bone morphogenetic proteins (BMPs) induce healing through a sequential cascade of events. Improved fracture healing after BMP-2 or -7 supplementation in patients with impaired fracture union suggests a deficiency of one or more of these factors. We postulated that low levels of circulating BMPs may result in delayed bone healing. The aim of this study was to quantify differences in levels of circulating BMP-2, -4, -6, -7, and -9 in patients that have demonstrated normal or delayed fracture healing. **Patients and methods** Blood samples were collected from an unselected cohort of 65 patients that had been treated for a diaphyseal tibia or femur fracture. Patients were divided into a group with fracture healing within nine months after injury and a group with delayed fracture union. BMP plasma concentrations were quantified using ELISAs and compared between these two groups.

Results Circulating plasma levels of BMP-2, -4, -6, and -7 did not differ between 34 patients with normal fracture healing and 31 patients with delayed fracture healing. Also the median BMP-9 plasma levels were not statistically different between the two groups of patients. However, the distribution in the patients with normal union showed a

wider range (72–2496 pg/ml) compared with the delayed union group (120–816 pg/ml).

Conclusion In general, circulating BMP concentrations are not statistically different between patients who demonstrated normal or delayed fracture healing. High circulating BMP-9 levels seem to be associated with faster fracture healing, but are apparently not decisive.

Introduction

Successful bone fracture healing depends on factors such as sufficient growth factors, adequate bone matrix and mechanical stability [1, 2]. Despite adequate treatment, delayed or non union occurs as a complication of fracture healing in 5–30 % of all patients with fractures [3]. Apparently, these patients lack or have decreased levels of one or more of the essential factors needed in fracture healing. Early recognition and treatment of impaired fracture healing could prevent significant impact, with prolonged therapies, and subsequent costs for patients [4].

Bone morphogenetic proteins (BMPs) are a subgroup of the transforming growth factor- β (TGF- β) superfamily and induce a sequential cascade of events, leading to fracture healing by stimulation of chondrogenesis, osteogenesis, angiogenesis, and remodelling of extracellular matrix [5]. More than 20 different types of BMPs have been identified in humans. In vitro assays and animal models showed that BMP-2, -4, -6, -7, and -9 exhibit high osteogenic activities [6–9]. Although their efficacy is still controversial, recombinant BMP-2 and -7 are being used clinically as bone growth factors in the treatment of impaired fracture healing [5].

Improvement of fracture healing after supplementation with BMPs in patients with impaired fracture union suggests a decreased expression or otherwise induced deficiency of

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one or more of these factors. Previously, significant differences in circulating TGF- β concentrations were found between patients with physiological and disturbed fracture healing [10]. However, to our knowledge, no data exist on systemic measurement of BMPs in relation to delayed fracture healing in humans. Earlier studies demonstrated a possible BMP-related genetic predisposition for impaired fracture healing (e.g. in the BMP inhibitor noggin) [11–13]. Consequently, low basal levels of BMPs should be determinable even years after the fracture has healed. The aim of this study was to compare circulating levels of BMP-2, -4, -6, -7, and -9 between patients with normal and with demonstrated delayed fracture healing.

Patients and methods

Study population

We retrospectively studied a cohort of patients treated in the Leiden University Medical Centre (LUMC) between January 2003 and December 2008 for diaphyseal tibia or femur fractures. Exclusion criteria were pathological fractures, trauma involving more than two fractures or injuries, significant soft tissue damage (type III open fractures according to Gustilo's classification), periprosthetic fractures, and age under 18 years. Patients were enrolled in 2011 and 2012 when healing had been completed. Data on age, gender, treatment, smoking and diabetes mellitus previous to and during fracture healing were retrieved from medical records. Smoking status was defined as smoking during fracture healing or non-smoking, regardless of quantity. Plain radiographs were re-examined to extensively determine fracture characteristics. Healing time was defined as the time between injury and the time of painless full weight bearing with radiological signs of consolidation. Patients eligible for inclusion were recruited by telephone and given written information. This study was approved by the Medical Ethics Committee of the LUMC.

Blood samples and BMP measurements

After informed consent blood samples were obtained in December 2011 and January 2012 between 10:00 and 12:00 to avoid circadian influences. The samples were processed following a standardised protocol. After centrifugation, aliquots of EDTA plasma samples were stored at -80°C until the time of analysis. BMP plasma concentrations were quantified using enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions. BMP-2, -4, -6, and -7 were analysed using Human BMP ELISA Kits (RayBiotech, Norcross, GA). Because an ELISA kit for BMP-9 was not available from this manufacturer, we developed an in-house BMP-9 ELISA. Nunc maxisorp plates were coated with mouse anti-

human BMP-9 antibodies (MAB3209, 1 $\mu\text{g}/\text{ml}$ in PBS, R&D systems, Minneapolis, MN) overnight at room temperature (RT). The plates were washed between all steps with PBS containing 0.05 % Tween-20 (Merck Millipore). Plates were blocked with 5 % Tween-20, 0.05 % sodium azide in PBS for one hour, followed by incubation with plasma samples (50 μl diluted with 1 % BSA [PAA, Germany]/PBS) or standard (recombinant human BMP-9, R&D systems) for two hours at room temperature. Detection was performed with biotinylated goat anti-human BMP-9 antibodies (BAF3209, 0.2 $\mu\text{g}/\text{ml}$ in 1 % BSA/PBS 2 h, RT), streptavidin-HRP and a substrate reagent pack (all R&D systems). Both the capture and detection antibody detect human BMP-9 with less than 0.1 % cross-reactivity with BMP-1, -2, -3, -4, -5, -6, -7, -8, and -10 according to the manufacturer.

Statistical analyses

Patients were divided into two groups according to the course of their fracture healing. Normal fracture union was defined as fracture healing within nine months after injury. Delayed union was defined as a healing time longer than nine months. Demographics, fracture characteristics and circulating BMP levels were compared between patients with normal fracture union and patients with delayed fracture union. The chi-squared test was used for comparing categorical data, and the Mann–Whitney *U* test and Student's *t*-test for comparing continuous data between patient groups. The median values were used for subdivision of patients with low and high BMP levels, except for BMP-7, where non-detectable versus detectable was used. *P*-values < 0.05 were considered statistically significant.

Results

Study population and patient characteristics

During the study period 167 patients were treated for a diaphyseal tibia or femur fracture, from which 142 met the inclusion criteria. Because contact information was missing or out-of-date for 49 patients, only 93 patients could be retrieved and invited. Of these, a total of 65 patients were recruited: 34 patients with fracture union within nine months after injury (normal union) and 31 patients with fracture union longer than nine months after injury (delayed union). Demographic and fracture characteristics did not vary significantly between the groups with normal healing versus delayed union (Table 1). The mean time of fracture healing was 26.7 ± 7.8 weeks for patients with normal healing versus 59.5 ± 19.7 weeks for patients with delayed union. Blood samples were collected from all recruited patients, on average 4.9 years after bone healing (range 2.01–8.56 years).

Table 1 Demographic and fracture characteristics of patients with demonstrated normal (within nine months) or delayed fracture healing of a femoral or tibia shaft fracture

Characteristics	Normal union (n=34)	Delayed union (n=31)	Univariate <i>p</i> -value
Patients			
Age, mean years ($\pm$ SD)	40.7 (15.8)	43.3 (14.2)	0.481
Male gender	14	11	0.638
Non-smoker	28	20	0.184
Diabetes mellitus	2	1	0.610
Fracture characteristics			
Anatomical region			0.834
Femur shaft	5	4	
Tibia shaft	29	27	
Type of fracture			0.216
Simple	20	18	
Wedge	5	1	
Complex	9	12	
Open fracture, Gustilo I or II	5	5	0.874
Conservative treatment	12	5	0.079
Fixation technique			0.254
Intramedullary nailing	18	19	
Plate fixation	2	5	
External fixation	2	2	
Duration of fracture healing, mean in weeks ($\pm$ SD)	26.7 (7.7)	59.5 (19.7)	<0.0001

BMPs in fracture healing

Circulating plasma levels of BMP-2, -4, -6 and -7 were not significantly different between patients with normal fracture healing and patients with delayed fracture healing (Table 2). The plasma levels of BMP-9 did not show a normal distribution (Fig. 1, Table 2). Although the statistical test based on the mean (Student's *t*-test) indicated a significant difference ($p=0.042$), the test based on the median (Mann–Whitney *U* test) did not ($p=0.231$).

Combined BMP parameters

To determine whether the association between BMP levels and delayed fracture healing could depend on a

combination of BMPs instead of a single BMP, the patients were divided into low or high BMP (based on the median value of each BMP separately). For BMP-7, which was not detectable in all samples, detectability was used instead of below/above median. The chi-square and *p*-values of crosstab analyses of

Table 2 Median circulating levels of five types of BMPs (pg/ml and range) between patients with demonstrated normal (within nine months) or delayed fracture healing

Type of BMP	Normal union (n=34)	Delayed union (n=31)	<i>p</i> -value
BMP-2	657 (542–2109)	641 (467–2036)	0.641
BMP-4	640 (372–2410)	674 (395–1584)	0.618
BMP-6	699 (135–2103)	554 (135–1379)	0.323
BMP-7	236 (146–2196)	299 (90–2127)	0.836
BMP-9	312 (72–2496)	288 (120–816)	0.231

Fig. 1 Scatter plot for BMP-9 plasma circulating concentrations (pg/ml) between patients that have demonstrated normal or delayed fracture healing. Each patient is indicated by a square. Horizontal bars represent mean values

BMP combinations versus consolidation groups are shown in Table 3. None of the BMP combinations (low/low, intermediates, high/high) showed significant associations with consolidation. The highest related combinations were BMP-2 & -9, BMP-6 & -9 and BMP-6 & -7 (Table 3).

Discussion

To our knowledge this is the first study analysing possible differences in circulating levels of BMPs between patients with normal and delayed fracture healing. The results revealed a possible association of high BMP-9 plasma levels with normal fracture healing times. All the other investigated BMPs, i.e. BMP-2, 4, -6, and -7 were not significantly associated with fracture healing time. Our data on BMP-2, -4, -6, and -7 seem comparable to those reported for circulating levels of TGF- β 1, for which, surprisingly, also no clear relation between low serum levels with delayed bone union could be established [14]. Although both studies contain too small patient groups to conclusively exclude effects of circulating levels of TGF- β or it's family members BMP-2, -4, -6, and -7 on fracture healing, the clinical use as predictive markers of delayed union of these parameters seems limited. The differences between the patient groups and the range of the levels are simply not large enough to be distinctive.

BMP-9 is the only BMP with lower plasma concentrations in patients with delayed fracture healing. However, also for this parameter its possible clinical use as prognostic indicator of delayed union cannot be confirmed based on our data. The large overlap between patients with normal and delayed bone union impedes the use of BMP-9 as a prognostic marker for bone union. Still, the more than five-fold difference between some patients is intriguing. Especially because in vitro assays have indicated BMP-9 as one of the most potent inducers of bone formation [6–9]. The

highly increased osteogenic activity observed with BMP-9 may be due to the fact that it is not affected by BMP antagonists such as noggin and BMP-3 [15, 16].

Genetic predisposition caused by variations in the BMP-9 gene could be a source of difference in protein expression. In a recent study some single nucleotide polymorphisms (SNPs) for BMP-2 and BMP-7 genes were shown not to be associated with a greater risk of fracture non-union, whereas two specific genotypes of a SNP in the BMP inhibitor noggin were [11]. Genetic variations in the BMP-9 gene are associated with susceptibility to ossification of the posterior longitudinal ligament [16]. Because the NCBI database contains more than 124 established SNPs in the BMP-9 gene, presumably some of these mutations are associated with low or high circulating BMP-9 levels. Additional studies are required to evaluate this further.

The local expression or accumulation of BMPs at the site of action does not have to be reflected or influenced by circulating levels of BMPs. In the case of BMP-9, hepatocytes seem to be the major cellular source of BMP-9. Circulating BMP-9 could play an important role in bone formation during fracture union instead of, or additional to, BMPs released locally in the fracture by, for instance, platelets [17, 18].

Conclusion

This study compared levels of circulating BMPs (-2, -4, -6, -7, -9) between patients with demonstrated normal and delayed fracture healing. Solely high circulating BMP-9 levels were associated with faster fracture healing. This finding supports a potential important role for BMP-9 in fracture healing.

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Conflict of interest The authors declare that they have no conflict of interest.

Table 3 Chi-square tests and *p*-values of combinations of two circulating BMP-levels in patients with normal or delayed union of a femoral or tibia shaft fracture

BMP	Measure	BMP-2	BMP-4	BMP-6	BMP-7	BMP-9
BMP-2	χ^2		0.001	1.133	0.365	2.512
	<i>p</i>		0.975	0.287	0.546	0.113
BMP-4	χ^2			0.026	0.041	0.617
	<i>p</i>			0.872	0.840	0.432
BMP-6	χ^2				2.597	2.763
	<i>p</i>				0.107	0.099
BMP-7	χ^2					1.262
	<i>p</i>					0.261

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