Elevated transforming growth factor-beta 1 (TGF-β1) levels in human fracture healing

Kambiz Sarahrudi a, Anita Thomas b, Mehdi Mousavi c, Georg Kaiser a, Julia Köttstorfer a, Mathias Kecht a, S. Hajdu a, S. Aharinejad b

a Department of Traumatology, Medical University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria
b Laboratory for Cardiovascular Research, Center of Anatomy and Cell Biology, Medical University of Vienna, Waehringerstr 13, A-1090 Vienna, Austria

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Abstract

Introduction: Transforming growth factor-beta 1 (TGF-β1) is a regulatory protein, involved in bone fracture healing. Circulating TGF-β1 levels have been reported to be a predictor of delayed bone healing and non-union, suggesting active relationship between tissue and circulating TGF-β1 in fracture healing. The purpose of this study was to analyse TGF-β1 local and serum concentrations in fracture healing to further contribute to the understanding of molecular regulation of fracture healing.

Patients and methods: Serum samples of 113 patients with long bone fractures were collected over a period of 6 months following a standardised time schedule. TGF-β1 serum concentrations were measured using ELISA. Patients were assigned to 2 groups: Group 1 contained 103 patients with physiological healing. Group 2 contained 10 patients with impaired healing. Patients in both groups were matched. One patient of the group 2 had to be excluded because of missing match partner. In addition, fracture haematoma from 11 patients of group 1 was obtained to analyse local TGF-β1 concentrations. 33 volunteers donated serum which served as control.

Results: TGF-β1 serum concentrations increased during the early healing period and were significantly higher in patients with physiological healing compared to controls (P<0.04). Thereafter, it decreased continuously between weeks 2 and 8 and fell again after week 8. TGF-β1 serum concentrations in patients with physiological healing were significantly higher at week 24 compared to controls (P<0.05). In non-unions, serum concentrations differed significantly from those of controls at week 6 (P<0.01). No significant difference in between patients with physiological and impaired fracture healing was observed. Fracture haematoma contained significantly higher TGF-β1 concentrations than peripheral serum of the patients (P<0.017).

Conclusion: Elevated levels of TGF-β1 in haematoma and in serum after bone fracture especially during the entire healing process indicate its importance for fracture healing.

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TGF-β1 seems to enhance the bone remodelling in rabbits with bone defect\(^1\) and local application of TGF-β1 has been shown to accelerate fracture healing.\(^1\) The presence of TGF-β1 in callos has been reported in human and animal fracture models.\(^5,\)\(^19\)\(^-\)\(^23\) In addition, evidence exists that serum concentrations of TGF-β1 are increased during the process of bone healing.\(^6\) Circulating TGF-β1 levels were found to be a predictor of delayed bone healing and non-union suggesting active relationship of its circulating levels to fracture healing process.\(^6\) To our knowledge, only few data exist on systemic and local measurement of TGF-β1 with regard to fracture healing in humans so far. The aim of our study was to analyse the local and systemic levels of TGF-β1 expression after bone fracture in patients with physiological and impaired fracture healing for better understanding of the role of this cytokine in the process of human fracture healing.

Patients and methods

This study was approved by the Ethic Committee of the Medical University of Vienna and performed in accordance with the ethical standards in the Declaration of Helsinki. A consecutive series of 113 patients with meta-/diaphyseal fractures of long bone (humerus, femur, lower leg and forearm) treated surgically at our institution between April 2006 and April 2008, were included. Patients gave informed consent to be enrolled in the study, and were 18–90 years old. Exclusion criteria were open fractures type III according to the Gustilo classification, multiple fractures, previous bone operations, pre-existing bone diseases except for osteoporosis, renal/liver insufficiency, malignant tumours, long term steroid treatment, immunosuppression and long term treatment with non-steroidal anti-inflammatory drugs. Patients were assigned in 2 groups according to their course of fracture healing. The first group contained 103 patients (male \(n = 50\), female \(n = 53\), mean age: \(54.2 \pm 20.4\)) with normal fracture healing. Ten patients with impaired fracture healing (delayed- or non-union) belonged to the second group. Three of the 10 patients developed a hypertrophic type of delayed union. Seven patients developed an atrophic type of delayed union. Demographics presented in Table 1.

The diagnosis of bony consolidation or delayed union was based on exercise-induced pain and conventional X-rays or computed tomography. Delayed union was defined as failed fracture healing without radiological signs of bony consolidation after 4 months postoperatively. Non union was defined as the absence of complete consolidation at 6 months after surgery. A corresponding patient with normal fracture healing and a healthy control was matched to each patient with delayed fracture healing. Table 1 presents the demographics of patients and the matching criteria. One of the 10 patients with delayed fracture healing had to be excluded, because no corresponding matching partner with adequate fracture healing could be found in our study cohort. Therefore, 9 patients with impaired and 9 patients with normal fracture healing were included in the final analyses.

In addition, fresh fracture haematoma was obtained from 11 patients of group 1 intra-operatively to analyse local TGF-β1 concentrations. Furthermore, 33 healthy volunteers (16 males, 17 females, mean age: \(37.1 \pm 11.65\) years) donated one blood sample as control.

All patients were followed up for at least six months after the operation. The follow up examination was based on clinical and radiological examination at 1, 2, 4, 6, 8, 12, 24 weeks after trauma.

Blood samples

Peripheral venous blood was obtained from each patient at 1, 2, 4, 6, 8, 12, 24 weeks after surgery and stored at \(-80^\circ\) C until analysis. TGF-β1 serum concentration was measured in 11 patients immediately after trauma at hospital admission. Each control individual donated one blood sample. Fracture haematoma was obtained at surgery. Haematoma was removed manually before any manipulation or irrigation, avoiding contamination by blood in the operating field, and placed in sterile containers. These specimens were centrifuged immediately and the resulting supernatant was stored at \(-80^\circ\) C until assayed.

Measurement of TGF-β1

TGF-β1 concentrations were measured by a commercially available antibody (Quantikine, RD Systems, Minneapolis, MN, USA) in enzyme-linked immuno sorbent assay (ELISA). All analytical steps were performed according to the manufacturer’s recommended protocol. The TGF-β1 assay detects specifically the biologic active form of the protein. Concentrations are presented as mean of duplicate measurements. To avoid interassay variability, samples of the corresponding matching partner were analysed with the same assay. The comparison of the measurements utilising different Kits for the same time points of the study measurements indicates the low range of variability of the assays.

Statistical analysis

Comparisons between groups of continuous variables were performed by using non-parametric ANOVA (Wilcoxon rank-sum test for two variables or Kruskal–Wallis-Test for more than two variables). For statistical comparison of a serum value at a certain time point between the non-union group and the matched unions nonparametric Mann–Whitney U test for unpaired samples were used. Statistical analyses were performed using the SAS system for Windows, v 9.1 and the Enterprise Guide, v 4.1 (SAS Institute, Inc., Cary, NC). Data are presented as means ± SEM. The statistical significance level was set at \(P < 0.05\).

Table 1

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\(^a\) Age (≥10 years).

\(^b\) According to ASIF classification.

\(^c\) Extent of soft tissue damage according to Gustilo classification.
Results

TGF-β1 serum concentrations in patients with physiological and impaired fracture healing

TGF-β1 serum concentration immediately after injury (measured in 11 patients) was 14,171.2 ± 5642.66 pg/ml. TGF-β1 serum concentrations were at a minimum level at 1 week (29,178.0 ± 1364.29 pg/ml) and increased to reach a maximum level (36,334.0 ± 1688.3 pg/ml) at 2 weeks after trauma (P = 0.0001). Serum concentrations decreased continuously after week 2 and reached another minimum concentration (31,932.4 ± 1397.0 pg/ml) at week 8 after trauma. After week 8, a continuous increase of the TGF-β1 serum concentrations was observed. A second peak of the TGF-β1 serum concentration was observed at week 24 after trauma (35,267.7 ± 2220.3 pg/ml) (Fig. 1). In patients with impaired fracture healing TGF-β1 serum concentrations were at a minimum level (27,339.7 ± 2973.45 pg/ml) at week 1 and increased to reach a first peak at week 2 (34,265.8 ± 4337.3 pg/ml), which was followed by a clear decline at week 4 after trauma (27,939.6 ± 3327.7 pg/ml). Between week 4 and 6 a significant increase of the TGF-β1 serum concentrations were observed (P = 0.03). TGF-β1 serum concentrations were highest at week 6 after fracture (43,294.1 ± 6949.5 pg/ml). Thereafter, a continuous decline of the serum levels was observed for the rest of the observation period (Fig. 1).

Comparison of TGF-β1 serum concentrations in patients with normal/impaired healing

Comparison of TGF-β1 serum concentrations between the matched patients with normal and impaired healing revealed no statistically significant difference for the entire observation period (Fig. 2).

Comparison of TGF-β1 serum concentrations between controls and patients

Comparison of TGF-β1 serum concentrations of patients with normal fracture healing and controls (29,735.3 ± 1328.4 pg/ml) revealed significant differences at weeks 2 (P = 0.04) and 24 (P = 0.05).

At these time points significantly higher TGF-β1 concentration were observed in patients with normal healing compared to controls. Comparison between the TGF-β1 serum concentrations of patients with impaired fracture healing and controls showed significantly higher TGF-β1 serum concentrations in patients with impaired healing at week 6 (P = 0.01).

To exclude that the differences in TGF-β1 serum levels between the patients and the controls are related to the age difference between the both groups (mean age: 35.6 vs. 54.2) an additional analysis with an age matched group was performed. This analysis revealed no age related difference in the TGF-β1 serum level between both matched groups and confirmed the reported results.

Comparison of TGF-β1 concentration in fracture haematoma and in serum of patients

Mean TGF-β1 concentration measured in fracture haematoma was 28,157 ± 6282.6 pg/ml. Mean TGF-β1 serum concentrations was 14,171.2 ± 2132.7 pg/ml. Fracture haematoma contained significantly higher TGF-β1 concentration than serum (P = 0.017) (Fig. 3).

Discussion

Various studies demonstrated that fracture repair is not a local process but is rather associated with systemic reactions that might partly be attributable to the uptake of bioactive molecules from the fracture site. In the present study, local and systemic
concentrations of TGF-β1 in patients with long bone fractures were analysed to elucidate the role of this osteogenic cytokine in the bone healing process. Previous studies showed characteristic alterations in serum concentrations of numerous enzymes and growth factors during fracture healing. 

Consistently, we found significant alterations in local and systemic distribution of TGF-β1 at certain time points after fracture of long bones. TGF-β1 concentrations in fracture haematoma were significantly higher than serum concentrations within the first hours after trauma indicating local release of TGF-β1 during the immediate response in concert with previous studies. 

Our data show a considerable fluctuation of the systemic TGF-β1 concentration within the first weeks of fracture healing. Whilst the mean post-traumatic serum TGF-β1 level in those 11 patients with immediate post-traumatic measurement was 14,171 pg/ml, the mean TGF-β1 serum concentration of the rest of the patients as well as half of the control-baseline of the healthy volunteers was almost twofold high at one week after trauma. Due to the fact that TGF-β1 is released from granulas of platelets during the clotting process, high local TGF-β1 concentrations in fracture haematoma of our patients do not appear surprisingly. 

An earlier study showed that TGF-β1 is released by platelets into the fracture haematoma, and then synthesised by osteoblasts and chondrocyts throughout the healing process. 

This explains the following increase of serum TGF-β1 concentrations within the first 2 weeks after trauma in our patients. Increased expression of TGF-β1 as well as other cytokines early after fracture was reported in other studies. 

This increase may partly be attributable to the absorption of cytokines from the fracture site into the circulation. 

On the other hand, the significant increase of serum TGF-β1 concentrations together with other cytokines such as PDGF, VEGF and M-CSF might indicate a systemic response to fracture. Supporting influence of systemic parameters on bone formation is well known and was demonstrated in previous studies. 

Maximum serum TGF-β1 concentrations during the intramembranous bone formation phase might give evidence for the chemotactic effect of TGF-β1 on bone cells. It is well known that an increasing number of osteoclasts, chondroblasts and immature progenitor cells invade the fracture area during the phase of intramembranous bone formation. 

Moreover, TGF-β1 is reported to stimulate bone formation by inducing differentiation of subperosteal mesenchymal cells into osteoblasts, which synthesise and release TGF-β1 by themselves at the proliferations stage and again exert stimulating effects on osteoblasts in an autocrine fashion. 

TGF-β1 concentrations started to decrease and reached a plateau between weeks 4 and 8. We believe that this continuous decrease of TGF-β1 serum concentration might be due to the increasing gain of mechanical stability of the fracture, as suggested by other clinical studies. 

For the later course, our data demonstrate that TGF-β1 serum concentrations in patients with bone fractures remain elevated during the remodelling phase; and this seems to be necessary to activate osteoblasts during the remodelling phase. On the other hand, osteoblasts activate TGF-β1 during the remodelling phase which might explain significantly high TGF-β1 serum concentration at week 24. 

Moreover, the expression pattern observed in our patients is in agreement with previous animal and human studies. 

These findings indicate that not only the local presence of the osteogenic growth factors but also their systemic presence is necessary to support fracture healing. Therefore, we suggest that whenever osteogenic growth factors are clinically or experimentally utilised for the enhancement of fracture healing, they should be used locally and systemically. 

Another question addressed in our study was whether TGF-β1 expression differs in patients with impaired fracture healing from those with physiological fracture healing. Since previous studies showed decreased serum concentrations of TGF-β1 with increasing age and in females we generated 2 homogenous groups with 9 patients to reduce the influence of treatment modalities, gender and age. Therefore, to each patient with impaired fracture healing a patient with physiological fracture healing was matched. As previously mentioned continuous decline of TGF-β1 serum concentrations during the plateau phase in patients with normal fracture healing was assumed to be caused by an increase of the mechanical stability of the fracture. Elevated TGF-β1 serum concentrations at week 6 in patients with impaired healing reflects the opposite course compared to normally healed patients and might be due to a lack of mechanical stability at that time. 

TGF-β1 serum concentrations of both groups were very similar for the rest of the observation period in our study. In contrast to results reported by Zimmermann et al we observed no significant differences in the TGF-β1 concentrations of patients with physiological and disturbed fracture healing. To exclude the only possible explanation for this discrepancy another analysis, i.e. only in patients with atrophic type of non union, was performed. However, the results did not reveal a significant difference. 

Finally, this study provides prospectively collected data on systemic levels of TGF-β1 over the entire period of fracture healing in a large collective of patients and data on local TGF-β1 concentrations in a smaller collective of patients with physiological fracture healing which may contribute to the understanding of molecular regulation of fracture healing. One limitation of this study is the small number of patients with impaired fracture healing. However, strictly chosen matching criteria enabled us to compare the data of patients with impaired fracture healing with those who had physiological healing. 

 Elevated levels of TGF-β1 in haematoma and in serum after bone fracture indicate its involvement in the human fracture healing. Significant differences in TGF-β1 levels of patients with physiological and impaired fracture healing could not be observed. 

Definitely further studies with higher number of patients with impaired fracture healing are needed to clarify the role of TGF-β1 in fracture healing. 

Conflict of interest statement 

All authors disclose any financial and personal relationships with other people or organisations that could inappropriately influence this work. 

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