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BASIC SCIENCE

The systemic angiogenic response during bone healing

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Abstract
Introduction Angiogenesis is known to be a critical and closely regulated step during bone formation and fracture healing driven by a complex interaction of various cytokines. Delays in bone healing or even nonunion might therefore be associated with altered concentrations of specific angiogenic factors. These alterations might in turn be reflected by changes in serum concentrations.
Method To determine physiological time courses of angiogenic cytokines during fracture healing as well as possible changes associated with failed consolidation, we prospectively collected serum samples from patients who had sustained surgical treatment for a long bone fracture. Fifteen patients without fracture healing 4 months after surgery (nonunion group) were matched to a collective of 15 patients with successful healing (union group). Serum concentrations of angiogenin (ANG), angiopoietin 2 (Ang-2), basic fibroblast growth factor (bFGF), platelet derived growth factor AB (PDGF-AB), pleiotrophin (PTN) and vascular endothelial growth factor (VEGF) were measured using enzyme linked immunosorbent assays over a period of 24 weeks.
Results Compared to reference values of healthy uninjured controls serum concentrations of VEGF, bFGF and PDGF were increased in both groups. Peak concentrations of these cytokines were reached during early fracture healing. Serum concentrations of bFGF and PDGF-AB were significantly higher in the union group at 2 and 4 weeks after the injury when compared to the nonunion group. Serum concentrations of ANG and Ang-2 declined steadily from the first measurement in normal healing fractures, while no significant changes over time could be detected for serum concentrations of these factures in nonunion patients. PTN serum levels increased asymptotically over the entire investigation in timely fracture healing while no such increase could be detected during delayed healing.
Conclusion We conclude that fracture healing in human subjects is accompanied by distinct changes in systemic levels of specific angiogenic factors. Significant alterations of these physiologic changes in patients developing a fracture nonunion over time could be detected as early as 2 (bFGF) and 4 weeks (PDGF-AB) after initial trauma surgery.

Keywords Fracture healing · Endochondral bone formation · Angiogenesis · Nonunion

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Introduction

Whereas angiogenesis—the sprouting of new capillaries from pre-existing blood vessels—in cancer or inflammatory disorders is part of a pathologic development and therefore, potential target for therapeutic interventions, under certain conditions it plays an indispensable role in maintaining or restoring physical inviolability. One of these conditions is bone formation during development or skeletal regeneration after fractures. Bone formation for skeletal regeneration is initiated by an inflammatory reaction and an aggregation of mesenchymal cells at the fracture site [44]. Mesenchymal precursor cells differentiate into a chondrocyte lineage [3, 11, 22] and start deposing extracellular matrix molecules such as collagen-2 and proteoglycans to form the cartilage callus [19]. The cartilage callus provides preliminary stability to the fracture zone. For transformation of the cartilage scaffold into bone tissue invasion of new blood vessels is essential. Matrix metalloproteinases (MMPs) cause a loosening of the extracellular matrix to allow ingrowth of new blood vessels into the callus. This process is stimulated by angiogenic cytokines.

Among these, crucial functions of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), angiogenin (ANG), angiopoietin 2 (Ang-2), platelet derived growth factor AB (PDGF-AB), pleiotrophin (PTN) have been described in the literature for regulation of new blood vessel formation during early fracture healing. Expression analyses in animal models showed all of these factors to be locally up-regulated during fracture healing [5, 25].

Furthermore, evidence exists, that fracture healing is not only regulated by this local release of bioactive molecules, but is also influenced by systemical changes of various cytokines, hormones and growth factors [21]. In earlier studies, we could show that, osteogenesis in adults is accompanied by specific changes of serum concentrations of messenger molecules [47, 48, 50]. By measuring serum levels of endothelial stimulating angiogenic factor (ESAF) one group was able to show an increased systemic angiogenic activity after a tibia fracture, although they were not able to compare their results between patients with successful fracture healing and nonunion patients [23]. These detectable changes might be attributable on one hand to the absorption of locally acting cytokines into the bloodstream but on the other hand might also be part of a systemic response to the fracture in order to support osteogenesis. Enhancement of—even heterotopic—bone formation by influence of systemic parameters is a clinically well known fact [29, 43]. In reverse a perturbed systemic regulation might exert adverse effects on the bone healing process, thus leading to delayed or even nonunion. In preceding studies we were able to show that TGF-β1-concentrations as well as serum concentrations of several members of the matrix metalloproteinase system from nonunion patients differ from those with normal bone healing [14, 50]. A recent study showed an increased systemic angiogenic activity in response to a sustained fracture even on cellular level with mobilization of endothelial precursor cells into the peripheral bloodstream in human subjects after a tibial fracture [22]. Systemic messenger molecules might also contribute to recruitment of mesenchymal precursor cells from distant locations to the fracture site, which was demonstrated by Shirley et al. [40].

These findings further underline the importance of a systemic view on angiogenesis during fracture healing. Better understanding of the pathophysiological processes leading to delayed fracture healing or nonunion might open new diagnostic or even therapeutic approaches to disturbed fracture healing. Thereby, systemic measurements represent a convenient minimal-invasive way to study physiological or pathological regulatory functions of angiogenic molecules in human subjects in vivo.

Goal of this study was therefore, to determine normal time courses of angiogenic factors as well as detection of possible alterations during the development of a fracture nonunion. The selection of measured angiogenic cytokines was based on the findings of local importance of each factor as well as the feasibility of detection within human serum.

Materials and methods

Recruitment parameters, sample collection schedule, the matching process and patient demographics have already been published in detail earlier [14].

Shortly, 137 consecutive patients receiving an osteosynthetic procedure for the treatment of a long bone fracture were enrolled prospectively in this study. For all patients, radiographic proofs of a long bone fracture, age between 18 and 80 years as well as informed and written consent were required prior to inclusion into the study. In order to achieve a collective as homogenous as possible, patients with multiple trauma, extensive soft tissue damage, open fracture type II or III according to the Gustilo-classification, postoperative respirator ventilation for longer than 24 h, septic complications or more than two fractures were excluded. Further exclusion criteria, were the existence of systemic diseases such as hypo- or hyperthyroidism, diabetes mellitus, advanced liver disease, chronic inflammatory diseases, malignancy and extreme obesity as well as long-term medication with immunosuppressive drugs.

All enrolled patients, were invited to attend follow-up examinations at standardized intervals: 1 and 2 weeks after trauma, bi-weekly for the first 3 months and once 6 months after trauma. Due to the design of our study no pre-trauma

baseline sample could be obtained. The first post-trauma sample was collected between 2 and 4 days after trauma in all patients. Reference values present data from healthy human subjects as supplied by the kit′s manufacturer or previous studies [49] and are summarized in Table 1.

If no bony consolidation of the fracture could be detected on conventional radiographs and the patient continued to report exercise induced pain 4 months after trauma, a computed tomography was performed. On the basis of these findings, patients with radiographic and clinical evidence of failed fracture healing and formation of an atrophic nonunion were assigned to the nonunion group. Nonunion patients underwent revision surgery including debridement, bone autografting and reosteosynthesis given that the standard criteria for operability were fulfilled.

A corresponding patient from the residual collective with proper fracture healing was matched to each nonunion group member by the following criteria: age (±5 years), sex, localization of fracture, type of fracture (according to the AO/ASIF-classification), type of osteosynthesis and the habit of smoking. These matched control patients comprised the union group. Out of the 137 enrolled patients, 17 were classified as suffering from fracture nonunion. Two of these patients had to be excluded, because no matching partner was available. Thus, 15 patients in each group could be included in the final analysis. Revision surgery for the treatment of nonunion was never performed earlier than 4 months after the initial trauma.

Sample acquisition

Venous blood samples from all patients were collected at follow-up examinations. Samples were drawn between 8 and 11 a.m. after an overnight fasting period. Serum was separated from other blood components, aliquoted and stored at −80°C.

Measurement of serum concentrations

Serum concentration of PTN were measured with an in-house developed enzyme linked immunosorbent assay (ELISA), which was performed on Nunc-Immuno Maxisorp plates (Nunc, Roskilde, Denmark) that had been coated overnight at room temperature with anti-PTN (AF-252-PB; R&D Systems, Minneapolis, MN; 50 ng/well), washed three times with 0.05% Tween 20 in PBS (washing buffer), blocked for 1 h at room temperature with 1% BSA in PBS, and washed with washing buffer. Wells were incubated for 2 h at room temperature with samples, standards, or blanks (300 μl, diluted with PBS), washed three times with washing buffer, and incubated for another 2 h at room temperature or overnight at 4°C with biotinylated anti-PTN (BAF-252; R&D Systems, Minneapolis, MN; 50 ng/well). After washing three times with washing buffer, wells were incubated for 20 min at room temperature with a 1:20,000 dilution of a 1.25 mg/ml solution of streptavidin–peroxidase (catalog no. 43-4323; Zymed, Burlingame, CA), washed three times with washing buffer, and then incubated for 30 min at room temperature with 100 μl of tetramethylbenzidine–H_2O_2 (TMB Substrate kit, catalog no. 34021; Pierce, Rockford, IL). The reaction was stopped by the addition of 50 μl of 0.5 M H_2SO_4, and the yellow dye was measured at an absorbance of 450 nm. The assay was linear for 0.05–1.5 ng of PTN with a detection limit of ~0.02 ng. Recombinant human PTN for standard solutions was obtained from PeproTech (Rock Hill, NJ).

Serum levels of VEGF, ANG, Ang-2, bFGF and PDGF-AB were quantified on commercially available ELISA (‘Quantikine® ELISA Kits’; R&D Systems, Minneapolis, MN, USA). Assays were performed according to the manufacturer’s instructions and suggested serum dilutions. All samples were measured in duplicates. To avoid data corruption by interassay variability, all samples from each nonunion patient and the respective matching partner were assessed simultaneously on the same plate.

Table 1 Reference serum concentrations of the investigated cytokines from normal human subjects

	n	Mean (pg/ml)	SD	Range (pg/ml)
Angiogenin	40	360	NA	196–496
Angiopoietin-2	60	2,494	1,341 pg/ml	1,065–8,907
bFGF	65	2.21	NA	0–6.9
PDGF-AB	31	20.1	NA	10.5–29.5
Pleiotrophin	58	420	240 pg/ml	0–1,000
VEGF	37	220	NA	62–707

NA not available, *SD* standard deviation

Statistical analysis

The results of 15 patients from each group were included in the statistical analysis. A study group size of a total of 30 patients (15 patients per group) was calculated to be necessary for group comparison using a pretrial power analysis based on the results of the first three pairs of patients. The power has been calculated exemplarily for bFGF as one of the major angiogenic cytokines of the current study.

For the detection of significant changes over time the nonparametric Friedman-test was applied. The Wilcoxon signed rank test for paired samples was used for comparison between the two groups at each time point of the observation period. Results are presented as mean ± standard error of mean.

$P < 0.05$ was considered significant. The study was conducted upon formal approval of local ethics committee and

in accordance with the declaration of Helsinki. Written informed consent was obtained from all patients prior to inclusion in the study.

Results

For all investigated factors time courses of serum concentrations could be determined over the entire investigation period (Fig. 1). In patients with successful bone healing all angiogenic factors showed significant changes over time.

Compared to the respective reference values, measured cytokine serum levels were higher for bFGF, PDGF, PTN and VEGF, whereas Ang-2 levels were considerably lower. Measured values for ANG were well within the range of the given reference.

Analysis of the time courses of the serum concentrations revealed three different types of curve progressions:

ANG and Ang-2 decreased steadily after trauma. The second group comprises VEGF, FGF basic and PDGF-AB. These factors reached peak concentrations 2 weeks after fracture timely coincident with the histologically defined period of matrix degradation and vascularization of the cartilage callus with subsequent continuous decrease and approximation to reference values. In contrast to the two aforementioned groups, serum concentrations of PTN increased asymptotically over time.

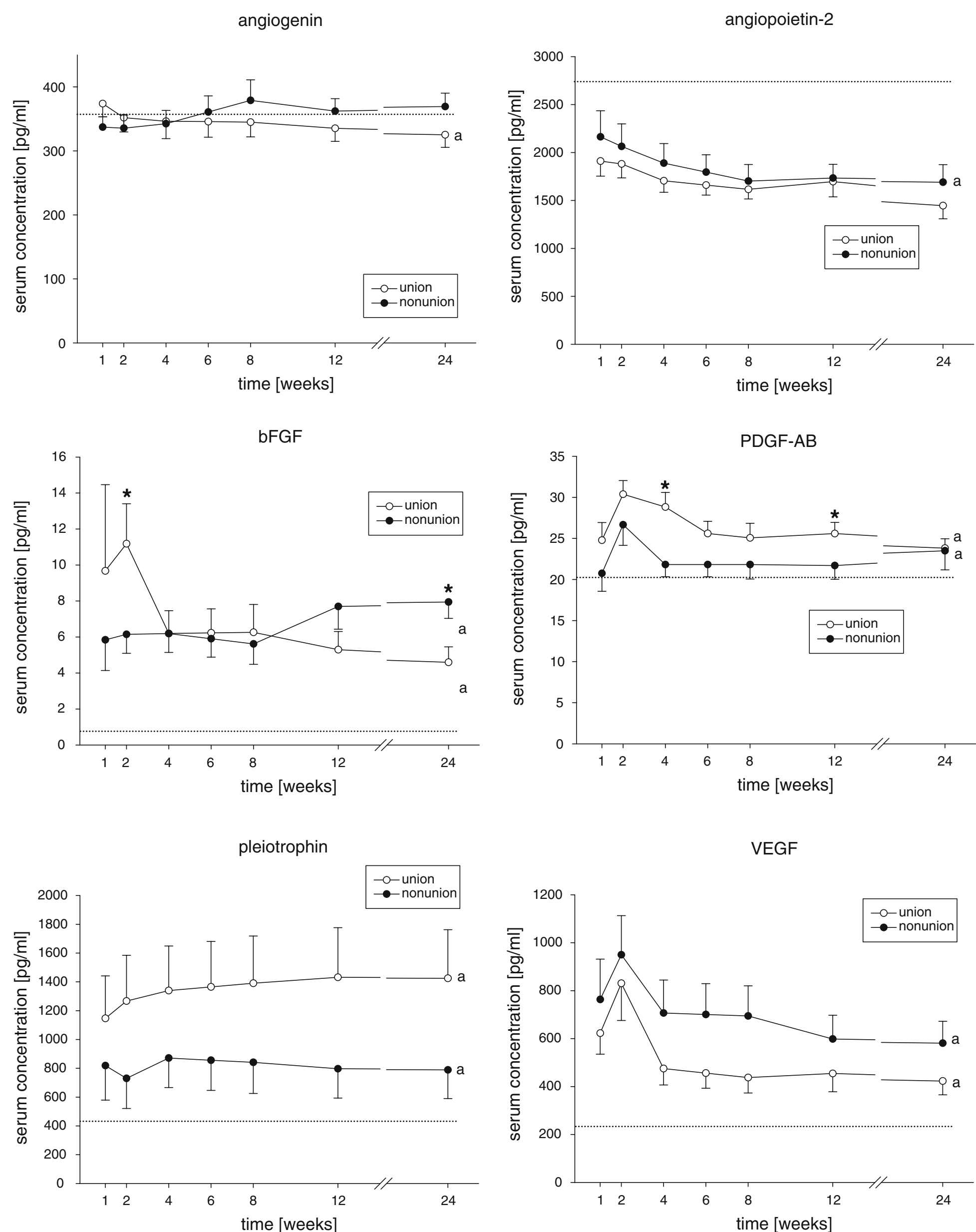

Fig. 1 Time courses of serum concentrations of angiogenic factors in patients with proper and failed fracture healing. * $P < 0.05$ for comparison between union and nonunion group; $^{a}P < 0.05$ for changes over time within one group. *Dashed line* illustrates reference values

In comparison with the physiological time courses of the investigated angiogenic agents several differences could be found in patients who developed a fracture nonunion: whereas Friedman-analysis revealed significant changes over time for all six cytokines in the union group, this could not be proven for ANG and Ang-2 in nonunion patients, though, mean values for these two factors did not differ greatly from those of patients with normal fracture healing.

The peak of bFGF serum concentrations which was seen at 2 weeks after trauma in patients with proper bone healing was lacking in patients who developed a nonunion. This difference was statistically significant at this time point ($P = 0.011$). In contrast to the union group where bFGF concentrations decreased continuously after the initial peak and leveled off near to the reference value after 6 months, in nonunion patients serum bFGF increased slightly at 2 months after surgery leading to significant differences between the groups at the end of the observation period.

In addition to changes in bFGF time courses, mean PDGF-AB serum concentrations were lower in nonunion patients over the entire investigation period, reaching the level of significance at 4 ($P = 0.015$) and 12 weeks ($P = 0.041$) after trauma.

In contrast, time course of VEGF serum concentrations in resembled each other closely in both groups with slightly higher serum values in the nonunion collective. However, this difference was not statistically significant.

Compared to the constant increase of PTN over time during the physiologic fracture healing period in unions, serum concentrations of this protein did not deviate greatly from the first post-trauma value in the nonunion group. Missing statistical significance, the difference of serum concentrations between unions and nonunions in support of constantly higher levels during normal fracture healing seems at least noticeable.

Discussion

Pre-existing data, mostly derived from histological examinations of tissues from experimental animal models, demonstrated distinct stages of fracture repair, which are characterized by the involved cellular phenotypes, composition of the extracellular matrix and expression of various enzymes and messenger molecules [4, 28, 36, 43, 46]. For understandable ethical reasons, multiple surgical explorations of a fracture site and tissues excisions are not possible in human subjects. However, clinical and experimental observations suggested that the local process of bone regeneration is associated with systemic reactions that might partly be attributable to the uptake of bioactive molecules from the fracture site [8, 9, 31].

We therefore analyzed serum samples of patients for cytokines known to be involved in osteogenesis as a surrogate for their local activity at the area of bone formation. In our preceding studies, we were already able to show characteristic changes in serum concentrations of various growth factors, enzymes and hormones during different types of bone formation, such as distraction osteogenesis, osteotomy and fracture healing as well as formation of a fracture nonunion [14, 47, 48, 50]. One striking finding was a lack of adequate TGF-β1 serum concentrations in patients without fracture healing 4 weeks after trauma when compared to subjects with timely fracture healing [50].

A critical step during the process of fracture healing is vascular ingrowth into the cartilage callus. This process requires loosening of the collagenous matrix by proteases, namely MMPs and is itself a prerequisite for the transformation of the soft callus into calcified bone tissue. An altered balance of the MMP/TIMP system in favor of proteolytic activity as shown in our earlier investigation may be involved in the pathophysiological processes leading to fracture nonunion [14]. Like the overall process of bone formation, time and location of this angiogenic process must also be closely regulated by cytokines, growth factors and hormones.

To determine if the period of angiogenesis during maturation of the fracture callus is also associated with an altered systemic angiogenic activity we used this already established methodology on angiogenic factors that are known to be involved in the regulation of new blood vessel formation especially during endochondral osteogenesis.

Our own current analysis focused on the proangiogenic factors ANG, Ang-2, bFGF, PDGF-AB, PTN and VEGF. Expression analyses in animal models showed all of these factors to be up-regulated during fracture healing [5, 25].

VEGF

VEGF is known to be one of the major players in promoting angiogenesis during bone formation [10]. It has been shown to play a crucial role in regulating osteogenesis not only by mediating new blood vessel formation, but also by promoting cell differentiation of hypertrophic chondrocytes, osteoblasts, endothelial cells and osteoclasts [29]. Active VEGF is contained in the extracellular matrix of the cartilage precursor. During matrix remodeling MMPs such as MMP-9 degrade extracellular matrix and release VEGF. A second pathway to an increased release of VEGF seems to be the TGF-β1 induced expression of VEGF by osteoblast-like cells within in the fracture callus [38] and in vitro [7]. In our study peak levels of VEGF were present at 2 weeks after the fracture and were increased fourfold compared to reference concentrations. This corresponds to the results presented by Street et al. [41], where plasma levels

of VEGF were shown to be elevated after trauma. Whereas, our earlier results on MMPs cannot easily be correlated with the demonstrated time course of VEGF concentrations in terms of their association in the VEGF release from extracellular stores, comparison with TGF-β1 serum concentrations shows a convincing analogy [14, 50]. Both cytokines show elevated serum concentrations already at 1 week after trauma, peak levels were reached at 2 weeks and at 4 weeks return to baseline is achieved. Although, this correlation does not prove the dependence of VEGF release on the stimulation of TGF-β1 it suggests an association of these two factors within the process of angiogenesis. However, from the results of our current study showing no significant difference between systemic VEGF in timely versus delayed fracture healing one can argue that, at least at the systemic level, TGF-β1 induced release of VEGF seems not to be not disturbed during the process of delayed fracture healing.

Basic fibroblast growth factor (FGF basic, FGF-2, bFGF)

FGF basic was also shown to stimulate expression of VEGF, independently from the TGF-β1 pathway [39]. Stimulation of angiogenesis during bone healing by local administration of bFGF was demonstrated in a variety of animal studies including nonhuman primates and a clinical application is already discussed [12, 32, 37]. However, underlying mechanisms of bFGF mediated enhancement of fracture healing are still unclear. As already suggested by Saadeh et al. [39]—based on the synchronous courses of serum concentrations during fracture healing—we suggest a close interconnection between the three cytokines, bFGF, VEGF and TGF-β1. The initial peak serum concentration of bFGF 2 weeks after trauma, which was missing in the nonunion group leads to the important conclusion, that early disruption of the angiogenic progress may hinder the physiological process of osteogenesis.

Pleiotrophin, PTN; osteoblast stimulating factor 1, OSF-1; heparin-binding growth-associated molecule, HB-GAM

Pleiotrophin is a member of a newly identified family of developmentally regulated, secreted, heparin-binding proteins with proven angiogenic potency. It is highly expressed during embryogenesis and plays an important role not only in early growth and differentiation, but also in tumor growth and metastasis and PTN serum levels have been shown to correlate with tumor growth and disease stage [20, 49]. Under physiological conditions, its expression in human adults is minimal [49]. Increased expression could be detected in osteoarthritis. The importance of PTN for bone development has been shown by Imai et al. [18] since, PTN over-expression in a transgenic mice model resulted in a phenotype characterized by increased bone thickness. Although local expression of the angiogenic factor PTN throughout the entire process of bone regeneration was demonstrated in rats [35], the specific effect of this molecule on osteogenesis remains controversial. For example, PTN showed negative effects on mouse bone defect healing [27] and lack of PTN did not affect normal bone physiology in rodents [26]. Tare et al. [42] showed that these multiple effects of PTN on the formation of bone were dependent on the concentration and the timing of its presence. Our results support the importance of PTN in regulation of fracture healing. Compared to the relatively low serum concentrations in non-injured adults systemic PTN values show a prolonged increase during the long-term process of physiological fracture healing and remodeling. In nonunion patients serum PTN still ranged above the upper standard deviation of the reference level. However, there was no increase of systemic PTN over time and serum concentrations were constantly lower than in the union group. Although the difference to patients with proper bone healing was not significant, which might be due to either relatively high standard deviations or insufficient group sizes, this tendency towards reduced systemic PTN concentrations might also demonstrate an early onset of an unphysiological process at least partially regulated by PTN.

Platelet derived growth factor (PDGF)

Platelet derived growth factor is known to be released from α-granules of platelets within the fracture hematoma. A major function might be its attribute to the inflammatory response by stimulating granule release from neutrophils and monocytes [45]. After that first step it is additionally expressed by mature chondrocytes [16] and might also attribute to osteogenesis by its known chemotactic and mitogenic activity on cells of mesenchymal origin such as fibroblast or endothelial cells [13] and its ability to stimulate collagen synthesis [33]. Platelet derived growth factor also shares a structural similarity with VEGF suggesting an involvement in the process of angiogenesis [30]. In addition PDGF was already examined in a human in vivo study and showed an increased gene expression during bone repair [1]. Together with this strong evidence of an increased expression of PDGF at the site of fracture healing, locally administered PDGF demonstrated a convincing positive effect on bone healing in a rabbit model of tibial osteotomies [34].

Our data adds evidence to these findings: increased expression of PDGF is mirrored in the peak serum concentration at 4 weeks after sustaining of the fracture and significantly decreased serum concentrations are associated with unsuccessful fracture healing. It is worth mentioning, that at the same time point TGF-β1 concentrations were

also significantly lower in the nonunion group indicating similar regulatory functions or even co-regulation of PDGF and TGF-β1 during early steps of fracture repair [50].

Angiogenin (ANG)

Angiogenin belongs to the pancreatic RNase superfamily and is a potent inducer of new blood vessel formation. Similar to VEGF, ANG seems to be involved in early stages of bone healing. It exerts its activity by binding to actin and thereby raising the proteolytic activity of plasminogen and tissue plasminogen activator. Main target of proteolysis are extracellular matrix molecules and basement membranes. Degradation of these structures enables ingrowths of newly formed blood vessels [2, 17]. Actions of ANG within the fracture healing process are not yet well-investigated. From our study, results with maximum serum concentrations within the first week we conclude that its main period of action seems to be the initial stages of the osteogenic process. As suggested elsewhere [6], ANG can be seen as a marker of endothelial damage caused for example by the trauma during surgery which might explain is continuous decrease during fracture repair. Nonunion formation did not significantly alter ANG serum concentrations.

Angiopoietin-2 (Ang-2)

In growing human bone, Ang-2 was shown to be co-expressed with VEGF with maximum levels of expression at the sites of hypertrophic cartilage in endochondral bone formation [15]. Furthermore, it seems to be under a conjoint control of TNF-α signaling together with MMPs which underlines the association of matrix degradation and angiogenesis during endochondral osteogenesis [25]. Interestingly serum analysis showed an early maximum of Ang-2 concentrations, even before matrix turnover or hypertrophic chondrocytes can be expected within the soft callus. Future research might therefore focus on additional actions of this molecule at early stages of the fracture healing process. Co-expression of Ang-2 with VEGF might be reflected in the pathophysiological process of fracture nonunion. Together with VEGF and in contrast to all other investigated angiogenic factors, Ang-2 was elevated in patients with unsuccessful fracture healing. Although, a clear tendency was recognizable, differences did not reach the level of significance at any given time point.

Taken together our study results underline the strong association of the local process of angiogenesis during fracture healing with the systemic activity of angiogenic cytokines. The specific time courses of the investigated molecules can be correlated to the histologically defined stages of fracture repair in general and especially during vascularization of the cartilage callus. Major findings of our current study are significantly decreased serum concentrations of bFGF and PDGF in patients with unsuccessful bone healing which could be detected during fracture repair as early as 2 and 4 weeks after trauma. Markedly, although, not significantly decreased serum values of PTN in delayed fracture repair presented an additional interesting finding. However, care has to be taken when interpreting the results in terms of a generally decreased angiogenic activity in delayed fracture healing. It must be emphasized that because this is an association study, the measured factors truly are serum markers and no direct cause effect relationship between their function in vivo and in vitro can be established by the study. Further, serum concentrations of other angiogenic factors like VEGF, ANG and Ang-2 were not significantly altered by the process of delayed bone healing. Disadvantages of our model are already described in detail in our preceding publications [14]. Most of these cutbacks are based on the limitations that apply when correlating systemic to local concentrations. In addition to the concentration of the substance at the point of interest its systemic concentration can be influenced, among others, by the rate of uptake into the bloodstream, protein binding, degradation or inactivation herein. As a systemic release of osteogenic and angiogenic factors is known to contribute to osteogenesis—such as, excessive bone formation following traumatic brain injury—it has also to be considered that not only uptake from the fracture site but also a release at a distant site might contribute to the measured serum concentration. Furthermore, care should be taken not to over-interpret 24 week values in nonunion patients: due to the revision surgery which was performed at non-standardized time points later than 16 weeks after trauma the sample collective is rather inhomogeneous and certainly not easily comparable to patients without a secondary operation.

An additional drawback of our survey might be the lack of a true pre-operative baseline value. Of course, the ideal way to study changes in bone turnover would be comparison between pre- and post-fracture samples. This study design has, however, not been feasible in our clinical setting. Using a large cohort from the Malmö OPRA (osteoporosis prospective risk assessment) study Ivaska et al. [19] were the first to analyze pre- versus post-fracture serum values in elderly women. The authors focussed on typical bone formation and resorption markers like osteocalcin, type I collagen telopeptides and collagen pro-peptides revealing no significant alterations between pre- and post-fracture serum levels when sampling was performed within 4–6 h after injury. Thereafter, and up to 1 year after surgery, the post-fracture samples varied significantly from the pre-fracture status and—due to the conclusions of this study—should not be used as baseline values. No comparable data for pre- and post-fracture serum concentrations of the factors measured in our recent survey exist so far in the

literature. In our current study, the first sample was obtained within a range of up to 4 days after the injury. Therefore, we found it not adequate to pronounce the first postoperative serum sample as reliable baseline value and focussed the illustration of our data on mean absolute serum values along the observational period. For both illustration and comparison reasons we—instead—referred our results to a one-time measurement of serum samples in larger groups of normal healthy adult human subjects known from the literature and pronounced these data as reference values.

Nevertheless, the presented data are of significant scientific importance because we were able to show for the first time an analysis of systemic angiogenic factors in human subjects in vivo during bone regeneration. Due to their descriptive nature we cannot—based on our data—contribute to the elucidation of underlying mechanisms and biochemical pathways involved in angiogenesis, but we can provide a solid base when interpreting mechanistic data from animal studies in terms of their transferability to the human body. However, when performing such comparisons it has to be considered that fracture consolidation in small rodents is usually achieved within 3 weeks indicating that similar stages of the osteogenic processes are reached considerably earlier than in human subjects.

Further, from our data, it seems certainly overconfident to argue in terms of bFGF and PDGF-AB as predictive factors for delayed or failed fracture healing or to draw conclusions about clinical relevance. However, it is of high interest that significantly decreased concentrations of these two angiogenic factors can be detected in patients developing delayed fracture healing later on as early as 2 and 4 weeks after trauma. It might be worthwhile to further investigate systemic occurrence of these factors at these specific time points in larger surveys.

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