The influence of low molecular weight heparin on the expression of osteogenic growth factors in human fracture healing

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Abstract

Purpose Anticoagulant therapy with low molecular weight heparins (LMWH) and mechanical compression is considered the gold standard for the prevention of thrombosis. However, evidence exists that LMWHs impair bone metabolism. The aim of this study was therefore to analyse alterations in the expression of M-CSF, VEGF and TGF- β 1 after treatment with enoxaparin in patients with long bone fracture to investigate the effect of LMWH on human fracture healing.

Methods A total of 81 patients with long bone fractures were included in the study and divided into two groups. One group comprised patients who received enoxaparin and the other group, patients who did not receive enoxaparin postoperatively. Growth factor levels were analysed in patients' serum and different groups were retrospectively compared.

Results M-CSF serum concentrations were found to be significantly higher only at 48 weeks after surgery in enoxaparin. Mean overall VEGF serum concentration was higher in patients with enoxaparin. TGF- β 1 serum concen-

trations were higher at 48 weeks after surgery in patients with enoxaparin.

Conclusion This is the first comparative systemic measurement of M-CSF, VEGF and TGF- β 1 serum levels in patients with and without enoxaparin after long bone fracture. Significant differences of the expression of the growth factors after enoxaparin therapy were only observed at week 48 after surgery for M-CSF and TGF- β 1.

Introduction

Deep-vein thrombosis is one of the most dreaded complications after surgery. In particular, immobilised patients with bone fractures are at a high risk of developing thrombosis. The pharmaceutical prevention of thrombosis with heparin began in the 1930s [1]. Today, anticoagulant therapy and mechanical compression are considered the gold standard for patients undergoing surgery [2]. However, there is evidence that anticoagulants impair bone metabolism. Although low molecular weight heparins (LMWH) are the thromboprophylactic agents of choice in orthopaedic and trauma surgery, it is widely believed that LMWHs may impair fracture healing. However, only a few animal studies with conflicting results have evaluated the impact of anticoagulation on fracture healing and the mechanisms are not yet fully understood. The results of the animal studies vary from no impairment of bone healing [2] to impairment of the healing process [3]. To our knowledge, based on literature research, there is no data on the role of LMWHs on fracture healing in humans. The aim of the study was therefore to analyse alterations in the expression of the osteogenic growth factors TGF- β 1, M-CSF and

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VEGF after treatment with enoxaparin inpatients with long bone fracture to verify a possible effect of the treatment with LMWH on human fracture healing.

Patients and methods

This study was approved by the Ethics Committee of the Medical University of Vienna and performed in accordance with the ethical standards in the Declaration of Helsinki. Patients gave informed written consent to be enrolled in the study. The recruitment parameters, sample collection schedule, matching process, patient demographics and exclusion criteria of this study have been previously published in detail [4]. In brief, a consecutive series of 113 patients with meta-/diaphyseal fractures of long bone (humerus, femur, lower leg and forearm) treated surgically were included. In order to have a homogenous study group and due to the strict selection criteria 32 patients with incomplete data were excluded from further investigation. Finally, the data of 81 patients were analysed. Patient's serum was collected following a standardised time schedule. M-CSF, VEGF and TGF- β 1 levels were then measured in patient's serum. Patients were then assigned into two groups according to their course of postoperative anticoagulation. Group 1 consisted of 76 patients who received postoperative anticoagulation therapy with enoxaparin (40 mg in 90%, 60 mg in 7% and 20 mg in 3% of the patients). Group 2 consisted of five patients without postoperative anticoagulation. All patients were followed-up for 12 months after the operation. Follow-up examination was based on clinical and radiological examination at one, two, four, six, eight, 12, 24 and 48 weeks after trauma. A total of 43 male and 38 female patients formed the study population. Mean age of the patients was 59.3 (range 17–90). Fracture consolidation was achieved in all patients.

Blood samples

Peripheral venous blood was obtained from each patient at one, two, four, six, eight, 12, 24 and 48 weeks after surgery and stored at -80°C until analysis. These specimens were centrifuged immediately and the resulting supernatant was stored at -80°C until assayed.

Measurement of growth factors

M-CSF, VEGF and TGF- β 1 concentrations were measured by a commercially available antibody (Quantikine, RD Systems, Minneapolis, MN, USA) in enzyme-linked immunosorbent assay (ELISA). All analytical steps were performed according to the manufacturer's recommended protocol. The assays detect specifically the biologically

active form of the protein. Concentrations are presented as mean of duplicate measurements.

Statistical analysis

The Mann-Whitney U-test was used to compare patients with and without enoxaparin therapy, because Kolmogorov-Smirnov testing indicated non-normal distribution of data. Spearman's correlation coefficient (enoxaparin and no enoxaparin) and test were used to determine associations between numerical variables. Statistical analyses were performed using the SPSS for Windows 17.0. Data are presented as means $\pm$ SEM (standard error of the mean). Statistical significance was considered at $p < 0.05$.

Results

M-CSF serum concentrations were found to be higher at the sixth and 48th weeks after surgery; however, this difference was statistically significant only at 48 weeks after surgery in those patients who did not receive enoxaparin (4733.5 ± 992.5 pg/ml) compared to those patients with enoxaparin (1568.7 ± 198.3 pg/ml) ($p = 0.014$). However, the mean overall serum concentrations did not significantly differ between both patient groups. Figure 1 demonstrates the time course of M-CSF serum concentrations in both groups.

The mean overall VEGF serum concentration was higher in patients on enoxaparin (660.7 ± 31.8 pg/ml) compared to the patients who did not receive enoxaparin (613.5 ± 97.3 pg/ml) ($p = 0.043$). Figure 2 demonstrates the time course of VEGF serum concentrations in both groups.

TGF- β 1 serum concentrations were found to be higher at 48 weeks after surgery in patients taking enoxaparin ($33,880.5 \pm 54,87.1$ pg/ml) compared to patients who did

Fig. 1 M-CSF serum concentrations (mean $\pm$ SEM) in patients with long bone fractures with (enoxaparin) or without (no enoxaparin) enoxaparin therapy. The asterisk indicates a significant difference in the serum concentrations between both groups ($p = 0.014$)

Fig. 2 VEGF serum concentrations (mean±SEM) in patients with long bone fractures with (enoxaparin) or without (no enoxaparin) enoxaparin therapy

not receive enoxaparin ($6,266.7 \pm 39,54.2$ pg/ml) ($p=0.017$). Mean overall TGF- β 1 serum concentrations did not significantly differ between both patient groups. Figure 3 demonstrates the time course of TGF- β 1 serum concentrations in both groups.

The clinical and radiological routine examinations revealed no difference in the time and quality of fracture consolidation between both groups.

Discussion

Animal studies have clearly demonstrated that hypocoagulation with warfarin, heparin and acetylsalicylic acid impairs fracture healing [5–7]. However, the findings of in vivo animal studies of the effect of LMWHs on fracture healing remain contentious and no conclusive findings can be drawn from these studies [6, 8, 9]. Nevertheless, in vitro studies on bone cells [10–12] and in vitro studies on bone metabolism [6, 13–17] demonstrate that LMWHs decrease

bone formation and, therefore, could potentially delay fracture healing. Evidence exists that LMWHs reduce cancellous bone volume [15, 17, 19] in a dose-dependent manner [15] and reduce the biomechanical strength of bone [14, 15]. In vitro studies have shown that the differentiation of osteoblasts from mesenchymal stem cells is inhibited by heparin and LMWHs, albeit to different extents [11]. Moreover, there is evidence that heparin and LMWH down-regulate genes that are necessary for the maturation of the osteoblasts [11, 12]. But not only bone formation can be affected by LMWHs, also fracture haematoma and angiogenesis can be influenced by these substances [8, 18–21]. Increased size of fracture haematoma after LMWH therapy was observed in a study on rabbits leading to a reduced cell proliferation until the resorption of the haematoma [8]. In addition, angiogenesis seems to be compromised by LMWHs. VEGF is known to be an important factor for angiogenesis and bone formation [22]. Moreover, VEGF is actively involved in the process of fracture healing [23, 24]. Oncological studies have shown that LMWHs prolong survival time not only by having antithrombotic properties but also by their anti-angiogenic effect [18–20]. In an animal model on angiogenesis the use of LMWHs suppressed the VEGF-induced angiogenesis [21]. The experimental data on the role of LMWHs on fracture healing give reason to assume that these agents compromise the fracture healing process. However, clinical data derived from human studies do not exist so far. Therefore, we analysed the expression of important osteogenic growth factors M-CSF, VEGF and TGF- β 1 during the fracture healing process with and without the use of LMWHs to detect a possible impact. Numerous studies have previously shown that the growth factors measured in our study play a cardinal role in the regulation of human fracture healing [4, 24–26]. Interestingly, our results could not confirm the results of the experimental studies as we did not observe a significant decrease in the expression of the analysed growth factors due to the use of enoxaparin. There certainly were significant alterations of the M-CSF and TGF- β 1 levels due to the use of enoxaparin. However, the different expression pattern was evident very late at 48 weeks after trauma. It is clear that the fracture healing process is not affected any more at one year after trauma. In addition, the levels of M-CSF and TGF- β 1 were influenced in different ways. While M-CSF levels were significantly low, TGF- β 1 levels were significantly high in patients who received enoxaparin. Considering the negative influence of the LMWHs on fracture healing as described in the literature, it would be logical to conclude that enoxaparin suppresses the expression of TGF- β 1. It is clear that the alteration of M-CSF and TGF- β 1 level one year after trauma, in patients who already achieved bony consolidation at least four to six

Fig. 3 TGF- β 1 serum concentrations (mean±SEM) in patients with long bone fractures with (enoxaparin) or without (no enoxaparin) enoxaparin therapy. The asterisk indicates a significant difference in the serum concentrations between both groups ($p=0.017$)

months after trauma, does not have any influence on the healing process. Furthermore, we could not observe any significant differences between the VEGF serum levels of the patients with and without enoxaparin at any time point. We merely observed that the overall VEGF serum concentrations were significantly higher in patients who received enoxaparin. In addition, the clinical and radiological examination of the patients did not show any difference with regard to fracture healing between either group.

In summary, the study is limited by the low number and heterogeneity of the patient group. The strength of the study is the prospective manner of data collection. As far as we know, these are the first comparative systemic measurements of M-CSF, VEGF and TGF- β 1 serum levels in patients with and without enoxaparin therapy after long bone fracture. Significant differences of the expression of M-CSF and TGF- β 1 after anticoagulation with enoxaparin in patients with long bone fractures were observed only at 48 weeks after surgery without any influence on the healing process. No significant difference of the VEGF expression was observed.

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