

Femoral neck fracture is accompanied by local changes in the content of transforming growth factor-beta1, interleukin-1beta and collagenase activity

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Abstract

The aim of the study was to evaluate the relationship between the time from femoral neck fracture and the content of transforming growth factor-beta1 (TGF- β 1), interleukin-1beta (IL-1 β) and collagenase activity in bone samples of the femoral neck. The material consisted of 42 cancellous bone samples from the femoral neck collected from patients after the femoral neck fracture during hip replacement procedure. The content of TGF- β 1, IL-1 β in bone samples was measured with the use of enzyme-linked immunoassay (ELISA) and collagenase activity was measured with spectrofluorimetry. The mean content of TGF- β 1/total protein was 2.29 pg/ μ g (range from 0.9 to 4.0). The mean content of IL-1 β was 4.93 fg/ μ g (range from 1.4 to 12.5). The mean activity of collagenase was 49.08 nU/ μ g (range from 5.6 to 113.7). The content of TGF- β 1 and IL-1 β decreased after the injury. In case of TGF- β 1 the difference was statistically significant ($p < 0.05$). The activity of collagenase was statistically significantly increasing in relation to time from the fracture ($p < 0.05$). We found no correlation between the content of TGF- β 1, IL-1 β and the activity of collagenase and the age and the sex of the patients. Also, no significant discrepancies were found between the examined cytokines in relation to the bone loss of the femoral neck according to Singh's scale. These results confirm mutual changes of activity between examined cytokines in the area of fractured bone.

Keywords: Collagenase, Femoral Neck Fracture, Interleukin-1, Time, Transforming Growth Factor-beta

Introduction

A fracture of bone initiates a cascade of healing processes. The basis of these changes are local factors such as cytokines and growth factors. Acting locally, at the site of the fracture, they are responsible for the regulation of bone remodelling both in physiological processes and during fracture healing¹. Such local factors include: transforming growth factor-beta (TGF- β), bone morphogenetic proteins (BMPs), insulin-like growth factor-1 (IGF-1), interleukin-1

(IL-1) or enzyme collagenase. Changes in local amounts and activities of those factors during healing processes were documented in numerous experimental models, osteoblast cultures and *in vivo* experiments².

Very little research data is available on the amount of the before-mentioned cytokines in bone in humans. There is no clear data on the changes in the amount of TGF- β , IL-1 β or the activity of collagenase in such an important site from the clinical point of view as the proximal femur in response to fracture. There is no research either on the change in those chemicals according to the patient's age, sex and the degree of bone loss of the proximal femur.

The aim of the study was a prospective measurement of time-dependent changes of the content of TGF- β 1, IL-1 β and the activity of collagenase in bone samples of the femoral neck in patients after femoral neck fracture and the evaluation if the fracture results in changes of the local content of the cytokines.

The authors have no conflict of interest.

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Materials and methods

Samples of cancellous bone from the femoral neck were collected from patients with femoral neck fracture during hemiarthroplasty (37 patients) or total hip replacement (5 patients) procedures. The research project was accepted by the Warsaw Medical School Bioethics Committee. The study included 42 patients with femoral neck fracture – 34 females and 8 males. Each of them signed a consent form. The median age was 80.2 years (from 63 to 95 years). In 25 cases the surgery involved the left hip and in 17 the right hip. The mean time from fracture to the surgery was 8 days (from 0 to 39 days). Twenty-one patients (50%) were operated on within 6 days after fracture. The most common reasons for delaying the operation were: transferring from other hospitals (11 patients) and poor general health that required pre-operative treatment (13 patients). Patients with no clear history of the exact date of the injury were excluded from the study. All the patients were walking independently before the fracture. Thirty-three patients (78%) were using walking aids prior to the injury.

The white blood cells (WBC), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), alkaline phosphate (ALP) and osteocalcin level in serum were measured at admission. Only those patients with the level of the above-mentioned markers within normal range were included in this study.

Singh's scale³ was used to assess bone loss in proximal femur. Bone loss correlates with the presence of strands of bone trabeculae on the X-ray of the proximal femur. Along with "the worsening of the X-ray" and the diminished amount of trabeculae, the patient was qualified to a lower group in the five-grade Singh's scale. The presence of all basic strands of bone trabeculae on AP X-ray corresponds to group five, and in group one all basic trabeculae are missing. All X-rays were evaluated by one surgeon (the first author). Densitometry of the proximal femur was not performed because of the pain caused by the femoral neck fracture. According to Singh's classification 10 hips (23.8%) were classified as group II; 16 (38.1%) as group III; 12 (28.6%) as group IV and 4 (9.5%) as group V.

The exclusion criteria were as follows: pathological femoral neck fracture, cancer of any location, a general medical condition that could affect the bone metabolism such as thyroid disease, type-I diabetes, rheumatological diseases, long-term steroid therapy, non-steroid anti-inflammatory drug intake before the operation and post-inflammatory changes of the hip.

Methodology

The samples of cancellous bone were collected during arthroplasty. Each sample (volume of 0.5 cm³) from the femoral neck was collected away from the amputation saw working area and were localised 5 mm distally from the fracture on the medial side of the femoral neck. Following the biopsy, the material was rinsed three times in phosphate-buffered saline solution

(PBS) at 4°C. Then the bone samples were homogenised mechanically and with the use of ultrasounds in Labsonic U device (Brown, USA) at 0°C for 15 minutes.

Assay procedures

Total protein assay

The content of total protein in bone samples was measured with the use of Bicinchoninic Protein Assay Reagent (BCA, Pierce, Beijerland, The Netherlands). BCA assay is the modified Lowry procedure⁴. In an alkaline environment, proteins reduce copper ions Cu⁺² to Cu⁺¹, which together with bicinchoninic acid create a colourful water-soluble compound that demonstrates the highest absorbance with the light length $\lambda=562$ nm^{5,6}.

Transforming growth factor-beta 1 assay

The concentration of TGF- β 1 was determined with the use of the Quantikine Human TGF- β 1 Immunoassay Test (R&D Systems, Minneapolis, USA). The test is based on the enzyme-linked immunoassay (ELISA)⁷. Quantikine Human TGF- β 1 Immunoassay contains a microplate coated with mice monoclonate anti-TGF- β 1 antibodies. The latent form of TGF- β 1 in the material inspected was activated with 2.5 N acetic acid solution in 10 M of urea. After ten minutes' incubation at room temperature the samples inspected were neutralised with 2.7 N NaOH solution with 1 M buffer. The absorbency intensification was read within 30 minutes with the use of Reader 210 (Organon Teknika, The Netherlands) with the light length $\lambda=450$ nm.

Interleukin-1 β assay

The concentration of interleukin-1 β was estimated on the basis of the Quantikine Human IL-1 β Immunoassay Test (R&D Systems, Minneapolis, USA), also based on the immunoenzymatic method ELISA⁸. Quantikine Human IL-1 β Immunoassay contains microplates covered with monoclonate mouse antibodies anti IL-1 β . The conjugate solution consists of anti IL-1 β antibodies coupled with horseradish peroxidase. The absorbency intensification was read within 30 minutes with the use of Reader 210 (Organon Teknika, The Netherlands) with the light length $\lambda=450$ nm.

Collagenase activity assay

Collagenase activity was assayed fluorimetrically with the use of a synthetic substrate called succinite-Gly-Pro-Leu-Gly-Pro-AMC (Bachem, Biochemica GmbH Heidelberg, Germany). Affected by collagenase, Suc-Gly-Pro-Leu-Gly-Pro-AMC undergoes hydrolysis at the Leu-Gly bond. The released Gly-Pro-AMC, affected in turn by superfluous X-prolylo-dipeptidyl-aminopeptidasis (X-Pro-DAP) undergoes hydrolysis into Gly-Pro and to 7-amido-4-methylcoumarone

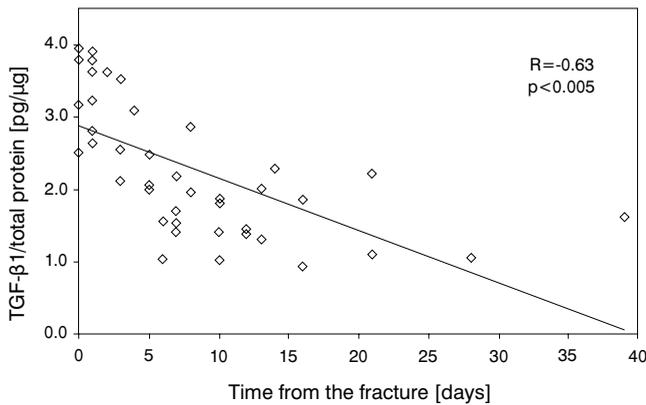


Figure 1. Correlation between the time from the fracture and the TGF- β 1/total protein content.

(AMC) free group⁹. The quantity of the released methylcoumarone is directly proportional to the proteolytic activity of the enzyme in the biological material under investigation.

Statistic analysis

For continuous data such as the concentrations of the substances examined, the variability range, dispersions and means as well as normal distribution errors have been marked.

For categorical values, such as sex, time, frequency of incidence and its Bernoulli distribution, errors have been marked. Correlation co-efficients between the quantities analysed were measured. The data from analysed subgroups were compared with the use of the student t-test. The data was statistically significant at the level below 0.05.

Results

The mean content of TGF- β 1/total protein was 2.29 pg/ μ g (range from 0.9 to 4.0 pg/ μ g; \pm 2.29). The mean content of IL-1 β was 4.93 fg/ μ g (range from 1.4 to 12.5 fg/ μ g; \pm 0.37). The mean activity of collagenase was 49.08 nU/ μ g (range from 5.6 to 113.7 nU/ μ g; \pm 6.64).

We observed a significant drop in the TGF- β 1/total protein content along with the lengthening of the period between the fracture and the surgery. The mean content of TGF- β 1 during the first 6 days from the fracture was 2.92 pg/ μ g (SD \pm 0.85) and was statistically significantly higher in comparison to the patients operated after 6 days from the fracture where the mean content of TGF- β 1 in that group was 1.66 pg/ μ g (SD \pm 0.48; p <0.05). The correlation was statistically relevant (Figure 1).

The content of IL-1 β /total protein tended to decrease along with the lengthening of the period of time between the fracture and the surgery (Figure 2). However, this drop was not statistically relevant. The mean content of IL-1 β in bone samples

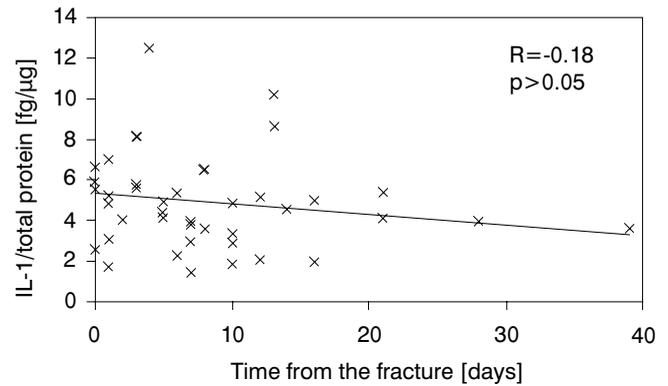


Figure 2. Correlation between the time from the fracture and the IL-1 β /total protein content.

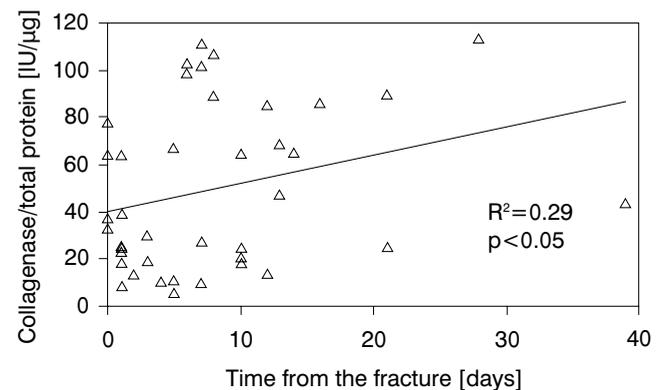


Figure 3. Correlation between the time from the fracture and the collagenase/total protein activity.

obtained from patients operated on during the first 6 days from the fracture was 5.59 fg/ μ g (SD \pm 1.86) and was higher in comparison to patients operated after one week from the fracture, where the mean content of IL-1 β was 4.27 fg/ μ g (SD \pm 1.67). The difference was not statistically significant.

A reverse tendency was observed in the changes of the collagenase/total protein activity. There was a statistically relevant increase along with prolonging of the time from the fracture and the activity of collagenase (Figure 3). The activity of collagenase increased in relation to the time from the fracture, especially in the first few days after the fracture. In patients operated on in the first 6 days after the fracture the mean activity of collagenase was 37.98 nU/ μ g (SD \pm 25.57) and was statistically significantly lower in comparison to patients operated on after 6 days from the fracture (p <0.05). The mean collagenase activity in the latter group was 69.74 nU/ μ g (SD \pm 35.81).

There was no correlation between the content of TGF- β 1, IL-1 β and the activity of collagenase and the age of the

Bone loss of the femoral neck according to Singh's classification	The mean content of TGF- β 1 [pg/ μ g]	The mean content of IL-1 β [fg/ μ g]	The mean activity of collagenase [nU/ μ g]
II	1.83; \pm 0.23	4.47; \pm 0.83	51.64; \pm 13.38
III	2.18; \pm 0.24	4.71; \pm 0.65	54.84; \pm 13.31
IV	2.94; \pm 0.26	5.72; \pm 0.74	50.03; \pm 9.79
V	2.17; \pm 0.13	4.41; \pm 0.58	55.74; \pm 21.40

Table 1. The correlation between the bone loss of the femoral neck according to Singh's scale and the content of TGF- β 1, IL-1 β and the activity of collagenase.

patients. Correlation coefficients between the content of TGF- β 1, IL-1 β and the activity of collagenase and the age of the patients were: -0.09 -0.07 -0.26 respectively.

Also, no significant discrepancies were found between the examined cytokines in relation to the sex of the patients. In the case of TGF- β 1 the mean content of TGF- β 1 for women was 2.36 pg/ μ g; \pm 0.16, while for men it was 2.02 pg/ μ g; \pm 0.37; (student t-test: -0.84). The analysis of the correlation between the content of IL-1 β and the sex of patients showed that the mean content of IL-1 β in women and men was: 4.88 fg/ μ g; \pm 0.72; and 5.14 fg/ μ g; \pm 3.29 respectively; (student t-test: 0.22). The difference was not statistically significant. As far as collagenase activity is concerned, there were no important discrepancies between either males and females. In females, the mean collagenase activity was 47.81 nU/ μ g; \pm 6.27 and in males it was 54.31 nU/ μ g; \pm 11.78; (student t-test: 0.49).

We found no statistically significant differences between the content of TGF- β 1, IL-1 β and the activity of collagenase in hips classified to different groups of bone loss according to Singh's scale. The relation between the content of TGF- β 1, IL-1 β and the activity of collagenase was shown in Table 1.

Discussion

In our study the age, the sex of the patients and the bone loss of the neck of the femur have no correlation with the content of TGF- β 1, IL-1 β or collagenase activity. The only factor that influenced the content and activity of the examined cytokines was the time from the fracture to the operation, when the samples were collected. In bone samples taken from patients operated on within different periods of time from the fracture the level of TGF- β 1 and collagenase activity changed inversely proportional in relation to one another.

The fracture resulted in the activation of healing processes and the increase of the contents of TGF- β 1 and IL-1 β . Both TGF- β 1 and IL-1 β were decreasing proportionally to the amount of time from the fracture. This confirms the

metabolic activity of this growth factor as one of the most important remodelling and bone repair factors¹⁰. Our results may suggest that a fracture might be a possible factor initiating the increase of the TGF-beta content in bone.

The results of experimental research confirm those findings. Lind proved that TGF- β 1 accelerates fracture healing by chemotaxis and osteoblast stimulation¹¹. Also Sun et al.¹² proved the special role of TGF- β 1 in the primary healing period. Tatsuyama et al.¹³ noted the presence of antibodies against TGF- β 1 and BMP-2 as soon as the third day after the fracture of tibia in rats. Si et al.¹⁴ received similar results. During their research on the physiology of bone healing in rabbits, they noted an increased expression of m-RNA for BMP-2 in the first post-fracture period. They observed the increased levels of m-RNA for TGF- β 1 in later healing periods, during chondrogenesis and the formation of callus. In their research on bone healing in rats, Matsumoto et al.¹⁵ noticed increased levels of TGF- β 1 between the 7th and the 14th day from the fracture. Tielinen et al.¹⁶ presented different findings. Based on their research on rats, they claimed that TGF- β 1 does not accelerate the healing of bone defects in rats. Although most experimental research on cytokines' function was performed on animal models, according to Andrew et al.¹⁷, TGF- β -m-RNA in humans is similar to that in animals.

In experimental models of femoral fracture healing in rats, the increased levels of TGF- β and IGF-1 observed in the early healing period activated the production of metalloproteinases and increased their activity in the later process of fracture healing¹⁸. A similar relationship was noticed in our study performed in humans. Although the methodology of those two studies differs significantly, higher levels of TGF- β 1 in the first post-fracture period were noted in both of them. The level of TGF- β 1 was declining in the later post-trauma time, which was accompanied by a proportional increase of collagenase activity.

According to Uusitalo et al.¹⁹, during the healing of a bone defect the level of m-RNA for metalloproteinase-9 and type-1 collagen increases. The increase in the activity of collagenase and m-RNA expression for that enzyme in the later healing period was also noted by Yamagiwa et al.²⁰. The above-mentioned observations explain higher collagenase activity in bone samples collected later after the fracture.

As for IL-1 β , different changes were noted. IL-1 β is one of the factors controlling the number of osteoblasts in newly formed callus via regulation of their apoptosis and maturation²¹. The level of this cytokine was higher in bone samples of the patients operated on in the first days from the fracture. This is confirmed by animal experimental research. Kon et al.²² noticed that right from the first post-fracture period in the initiation phase of fracture healing there is a significant growth of IL-1 β expression in tibia in mice. IL-1 and IL-6 regulate the process of fracture healing by influencing osteoclastogenesis and early recruitment and differentiation of osteoclasts' precursors²³. Spiliopoulou et al.²⁴ obtained similar results in his work. His research involved

patients post- hip fracture. He noted that the IL-1 β level in gluteal muscle samples was higher in patients after the fracture when compared to levels in people from the same age group who were not injured. The very same work showed higher local levels of IL-1 β in on the side of the fracture when compared to the healthy side.

To sum up, the results show varied levels of measured factors in the tissue examined in different timing from the fracture. They confirm the active role of TGF- β 1, IL-1 β and collagenase in bone remodelling processes after femoral neck fracture.

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