Plasma and drainage fluid levels of soluble receptor activator of nuclear factor- κ B (sRANK), soluble receptor activator of nuclear factor- κ B ligand (sRANKL) and osteoprotegerin (OPG) during proximal humerus fracture healing

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Abstract Fracture healing is an ordered process that restores the structural integrity of the bone. Soluble receptor activator of nuclear factor- κ B (sRANK), its ligand (sRANKL) and osteoprotegerin (OPG) are involved in bone remodelling, thus they may play a role in fracture repair. OPG, soluble RANK and RANKL levels were measured in plasma and in drainage fluid, collected from pre-surgery phase to healing in ten patients of both genders (age range 26–65 years) with proximal humerus fracture needing osteosynthesis. All patients showed fracture healing. No significant modifications

in the concentrations of sRANKL and OPG were observed, while sRANK showed a significant increase in drainage fluid 24 hours post-surgery compared with intra-surgery time. OPG levels were higher in plasma and drainage fluid than sRANK and sRANKL at each time point. Since there are no published data about sRANK involvement in fracture healing, our study represents the first preliminary indication about a local increase of this marker concentration immediately after surgery.

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Introduction

Proximal humerus fractures represent 4% of all fractures [1]. The incidence is about 73/10⁵, and some studies have predicted a three-fold increase over the next three decades [2]. These fractures are frequently observed in the elderly [3], although high speed trauma and athletic injuries are responsible in young patients. The mechanism of injury may be direct or indirect, but the overall fracture pattern results from a combination of the traumatic energy and the biomechanics of the injury [4].

The involvement of bone remodelling in fracture repair could implicate the RANK/RANKL/OPG system in the healing process, since this cytokine network regulates bone cell biology, osteoblast–osteoclast and bone-immune cross-talk, and maintenance of bone mass [5].

Although the findings reported in many studies suggest an important role for these cytokines as a local and paracrine factor in bone [6–8], it still remains unclear whether during fracture healing they circulate from bone to peripheral blood, what is the main source of these circulating proteins and, finally, if these circulating components are directly involved in the regulation of bone physiology.

Fig. 1 Preoperative images of an injured shoulder. X-ray images in lateral (a) and anteroposterior (b) positions and 3D CT scan (c)

Clinical studies showed that the RANK/RANKL/OPG system is involved in many skeletal and immune-mediated diseases characterised by increased bone resorption and bone loss [6, 9, 10]. In addition, RANKL and OPG expression have been involved in the fracture healing process in a mouse model of tibial fracture [11].

To our knowledge, a comprehensive evaluation of the components of the RANK/RANKL/OPG system at different times during fracture healing, and a comparison between their systemic and local concentration in drainage fluid has never so far been performed. For this reason in our study we selected a group of patients with proximal humerus fracture, undergoing a direct lateral surgical approach for the implantation of a plate into the proximal humerus [12] and evaluated the concentration of sRANK, sRANKL and OPG in plasma and drainage fluid at different time points before and after surgery. The aim of this study was to investigate the involvement of the RANK/RANKL/OPG system in the fracture healing process after the surgical treatment of proximal humeral fracture.

Materials and methods

Subjects

The subjects included in the study were recruited at I.R.C. C.S. Istituto Ortopedico Galeazzi and written informed consent was obtained from each patient.

The study population included ten subjects of both sexes, aged between 26 and 65 years, with proximal humeral fracture needing osteosynthesis. Exclusion criteria were presence of fractures resulting from a primary or metastatic

tumour, nonunions and malunions, haematochemical anomalies, bone, blood, hepatic, nephritic and pulmonary diseases, polytrauma, sepsis and treatment with anticoagulant or anti-osteoporotic drugs. At admission, in the pre surgery phase, peripheral blood from the cubital vein was collected. During surgery, blood was collected from the cubital vein and drainage fluid from the site of fracture. At 24-hours after surgery the drainage fluid was

Fig. 2 Postoperative X-ray image of an injured shoulder after the plate implantation

collected again, before drain tube removal. Blood collection from the cubital vein was also performed at selected time points from surgery, i.e. after 24 hours, 48 hours, one week, one month and the last time point, six months, representing fracture healing.

Clinical valuation and operative technique

Preoperative clinical examination was routinely performed with anterior-posterior (AP), lateral and axillary X-rays and through CT scan in case of four or more fragments shown in the X-rays (Fig. 1).

All operations were performed within one week of injury by the same surgical team and followed the recommendations of Gardner [12]. A longitudinal incision was made over the shoulder from the anterolateral apex of the acromion distally about 10–12 cm (the length of the incision depends on the fracture type and the need for a limited open reduction). After dissecting the subcutaneous tissue, the avascular raphe splitting the anterior and middle heads of the deltoid was incised and spread about 4–5 cm along its length distally. Once the axillary nerve was localised, the incision in the raphe was extended distally isolating the axillary nerve and its related vascular

Fig. 3 Results of plasma biomarker measurements for each considered time point; values are shown as mean and dispersion

structures. Good visibility of the whole fracture of the proximal humerus was then achieved, especially allowing recognition of both fragments of the greater tuberosity. The proximal humerus was then ready either to be reduced, pursuing the guidelines of the locking plate system in use.

After confirmation of the final surgical result by intra-operative radiographic assessment in an anteroposterior and axial views, the soft-tissue layers were closed with resorbable running sutures.

The average surgical time for the locked plating was 45 minutes (30–60 minutes). The average duration of the hospital stay after surgery was three days (range, 2–5 days). A clinical evaluation was performed at one and two weeks, and at one, three and six months.

Radiological checks were performed at one, three, six and 12 months. Figure 2 shows a postoperative X-ray.

Bone remodelling biomarkers measurement

Blood and drainage fluid were collected in plasma-EDTA tubes (BD Vacutainer Systems, Becton Dickinson, Franklin Lakes, NJ, USA). Plasma was separated by centrifugation no more than one hour after sampling, immediately aliquoted and frozen at -80°C until assayed.

Plasma and drainage fluid levels of OPG and sRANK were assayed with ELISA kits (R&D Systems, Minneapolis, MN, USA). Reported maximum intra- and interassay CVs were, respectively, 7% and 7% for OPG and 10% and 9% for RANK. The detection limits were 2.8 pg/ml for OPG and 2.34 pg/ml for RANK.

Plasma and drainage fluid sRANKL concentrations were measured with a duo-set ELISA kit (BIOMEDICA Medizinprodukte, Wien, Austria). Reported maximum intra- and interassay CVs were 9% and 6%, respectively, and the detection limit was 0.02 pmol/l.

Absorbance readings of each test were measured by means of spectrophotometers (MR-96A, Mindray Shuzen, China).

Statistical analysis

Data are showed as mean values and dispersion. A multivariate generalised linear model for repeated measures was then used to estimate between and within subject variability.

The relation of each parameter with time was investigated by a simple regression analysis. Comparisons between sRANK, sRANKL and OPG at two time points were performed by Mann-Whitney U-test (two tails $\alpha=0.05$). All statistical tests were performed with SAS software (SAS Institute, Cary, NC, USA) at a confidence level of 0.05; *p*-values lower than this threshold were considered significant.

Results

In all cases, after six months a good alignment of fracture fragments and good fracture healing were found with no loosening of implants.

Among bone remodelling plasma biomarkers, we observed high within-subject variability for sRANK, sRANKL and OPG.

Figure 3 shows the plasma values of sRANK, sRANKL and OPG at the different time points pre, intra and after surgery. Higher OPG levels with respect to sRANKL and sRANK were measured for all the considered time points. OPG showed no changes between every considered time point. Similarly, the sRANKL plasma value remained unchanged during the considered time points. On the contrary, sRANK plasma levels displayed a little increase 24 hours after surgery, which become more evident, although not statistically significant, 48 hours after surgery.

Regression analysis with concentration as the variable over time indicated no significant linear relation of concentrations in time for all the considered molecules.

Figure 4 shows the drainage fluid levels of sRANK, sRANKL and OPG at intra surgery and 24 hours post

Fig. 4 Results of drainage fluid biomarker measurements for the two considered time points; values are shown as mean and dispersion

surgery time points. A statistically significant increase ($p=0.0065$) was observed for sRANK 24 hours post surgery with respect to intra surgery, while sRANKL and OPG drainage levels showed no significant changes. As observed for plasma concentrations, OPG levels were also higher in drainage fluid than sRANKL and sRANK at both intra surgery and 24 hours post surgery time points.

Discussion

The expression pattern of the RANK, RANKL and OPG is particularly linked to the osteoarticular system, being involved in the osteo-immunological regulation of the bone system [5]. The possible use of these molecules as plasma biomarkers of bone remodelling is still poorly defined. Indeed, human reference ranges for plasma values of sRANK, sRANKL and OPG both in normal subjects and in patients with fractures have not been clearly established so far [13]. There are few reports about the modes of action of RANKL, OPG and none about RANK during fracture healing in humans. In these studies, following patients with fractures at different anatomical sites, no differences have been shown in these biological measurements between upper and lower limb [14, 15]. Our study represents one of the first investigations to define the involvement of RANK, RANKL and OPG in fracture healing and their possible roles as biomarkers to monitor this process. Moreover, the novelty of our research is the comparison of the systemic values of these molecules and their local, specific drainage fluid values, in order to better define their action in the healing process.

We collected a homogeneous population with the same anatomical site of fracture, and we monitored fracture repair for a long period after surgery.

Concerning the analysed biomarkers, we observed higher OPG levels both in plasma and drainage fluid at every considered time point with respect to the other two markers. Unlike RANK and RANKL, OPG is mainly produced at high level by cells of the osteoblastic lineage, but it can also be produced at very low level by immune and endothelial cells [16–19].

For both OPG and sRANKL there were no significant differences between local and systemic levels and their values were not altered by the fracture healing process after surgery at the considered time points.

On the contrary, sRANK showed a significant increase of the drainage fluid concentration 24 hours post surgery compared to intra surgery, which is reflected by a slight, although not significant, systemic increase 24 hours post surgery and 48 hours post surgery. At later time points (one week, one month and six months) after surgery, the systemic levels returned to normal.

These data suggest that, among the RANK, RANKL and OPG molecules, the main role during the fracture repair process is played by sRANK. Since RANK has a major role in osteoclast activity, the rapid and local increase of the levels of this cytokine after surgery could indicate an increase of the osteoclastic activity involved in the repair process. Moreover, our data indicated that sRANK has an acute effect, with a concentration peak 24 hours post surgery at the local site of fracture, then reflected by an increase at 48 hours post surgery at the systemic level, suggesting a role for this molecule in the early phase of bone remodelling and consequent osteosynthesis after fracture surgery. Interestingly, the increase in circulating levels of sRANK directly reflect the increase at the fracture site and this suggests a possible role of sRANKL as a useful tool to monitor fracture healing.

In conclusion, our study can be considered the first comprehensive evaluation of the RANK/RANKL/OPG system during fracture healing, in order to evaluate these biomarkers as a possible tool to monitor the bone healing process. Further investigation will be performed to improve the correlation of these parameters with clinical evaluation of surgery and post surgery recovery.

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References

1. Court-Brown CM, Garg A, McQueen MM (2001) The epidemiology of proximal humeral fractures. *Acta Orthop Scand* 72:365–371
2. Palvanen M, Kannus P, Parkkari J et al (2000) The injury mechanisms of osteoporotic upper extremity fractures among older adults: a controlled study of 287 consecutive patients and their 108 controls. *Osteoporos Int* 11:822–831
3. Burkhead WZ, Schienberg RR, Box G (1992) Surgical anatomy of the axillary nerve. *J Shoulder Elbow Surg* 1:31–36
4. DeFranco MJ, Brems JJ, Williams GR Jr et al (2006) Evaluation and management of valgus impacted four-part proximal humerus fractures. *Clin Orthop Relat Res* 442:109–114
5. Kong YY, Boyle WJ, Penninger JM (2000) Osteoprotegerin ligand: a regulator of immune responses and bone physiology. *Immunol Today* 21:495–502
6. Theoleyre S, Wittrant Y, Tat SK et al (2004) The molecular triad OPG/RANK/RANKL: involvement in the orchestration of pathophysiological bone remodelling. *Cytokine Growth Factor Rev* 15:457–475
7. Kearns AE, Khosla S, Kostenuik PJ (2008) Receptor activator of nuclear factor kappaB ligand and osteoprotegerin regulation of bone remodeling in health and disease. *Endocr Rev* 29:155–192
8. Boyce BF, Xing L (2008) Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Arch Biochem Biophys* 473:139–146
9. Hofbauer LC, Schoppet M (2001) Serum measurement of osteoprotegerin-clinical relevance and potential applications. *Eur J Endocrinol* 145:681–683

10. Rogers A, Eastell R (2005) Circulating osteoprotegerin and receptor activator for nuclear factor kappaB ligand: clinical utility in metabolic bone disease assessment. *J Clin Endocrinol Metab* 90:6323–6331
11. Kon T, Cho TJ, Aizawa T et al (2001) Expression of osteoprotegerin, receptor activator of NF-kappaB ligand (osteoprotegerin ligand) and related proinflammatory cytokines during fracture healing. *J Bone Miner Res* 16:1004–1014
12. Gardner MJ, Griffith MH, Dines JS et al (2004) A minimally invasive approach for plate fixation of the proximal humerus. *Bull Hosp Jt Dis* 62:18–23
13. Kudlacek S, Schneider B, Woloszczuk W et al (2003) Serum levels of osteoprotegerin increase with age in a healthy adult population. *Bone* 32:681–686
14. Marchelli D, Piodi LP, Corradini C et al (2009) Increased serum OPG in atrophic nonunion shaft fractures. *J Orthop Traumatol* 10:55–58
15. Lee JS, Ryu CH, Moon NH et al (2009) Changes in serum levels of receptor activator of nuclear factor-kappaB ligand, osteoprotegerin, IL-6 and TNF-alpha in patients with a concomitant head injury and fracture. *Arch Orthop Trauma Surg* 129:711–718
16. Tan KB, Harrop J, Reddy M et al (1997) Characterization of a novel TNF-like ligand and recently described TNF ligand and TNF receptor superfamily genes and their constitutive and inducible expression in hematopoietic and non-hematopoietic cells. *Gene* 204:35–46
17. Yun TJ, Chaudhary PM, Shu GL et al (1998) OPG/FDCR-1, a TNF receptor family member, is expressed in lymphoid cells and is up-regulated by ligating CD40. *J Immunol* 161:6113–6121
18. Malyankar UM, Scatena M, Suchland KL et al (2000) Osteoprotegerin is an alpha vbeta 3-induced, NF-kappa B-dependent survival factor for endothelial cells. *J Biol Chem* 275:20959–20962
19. Hofbauer LC, Heufelder AE (2000) The role of receptor activator of nuclear factor-kB ligand and osteoprotegerin in the pathogenesis and treatment of metabolic bone diseases. *J Clin Endocrinol Metab* 85:2355–2363