Immunohistological identification of receptor activator of NF-κB ligand (RANKL) in human, ovine and bovine bone tissues

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Receptor activator of NF-κB ligand (RANKL, also called ODF/TRANCE/OPGL) is the final factor of osteoclast differentiation. Osteoclastogenesis may be determined by its receptor RANK and the relative ratio of RANKL to its decoy receptor osteoprotegerin (OPG), and alterations in this ratio may be a major cause of bone loss in many metabolic and immunologic disorders.

In order to get a better insight into this complex regulatory system, this study aimed to determine where RANKL protein is located in bone tissues. RANKL was stained immunohistochemically in ex vivo human, ovine and bovine bone tissue.

RANKL was observed labelled in the membrane of osteoblasts, osteocytes and osteoclasts and their surrounding matrix. In cartilage, which was used as a negative tissue control, chondrocytes were not stained. The presence of RANKL protein in the membrane of osteoblasts and also the secretion of RANKL by osteoclasts has been hypothesised in earlier studies. In this study, RANKL protein was shown histologically for the first time in the membrane and in the long processes of osteocytes. The result strongly suggests the crucial involvement of osteocytes in terms of orchestrating bone remodelling by influencing differentiation and activation of osteoclasts.

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1. Introduction
1.1. Biological aspects
The past 15 years have witnessed an explosion in the field of bone biology. The scientific view on bone has evolved to an extremely complex organ regulated by a host of systemic and local factors. The integrity of the skeleton depends upon permanent bone remodelling. Morphogenesis and remodelling of bone is a physiologically controlled process that involves the synthesis of bone matrix by osteoblasts and the harmonised resorption of bone by osteoclasts [1]. Osteoclasts differentiate from haematopoietic precursors of the monocyte-macrophage lineage and resorb the bone matrix [2,3,4]. Several factors affect osteoclastogenesis at distinct stages of development. Besides macrophage colony-stimulating factor (M-CSF) [5], various interleukins, such as IL-1, IL-6, IL-11, transforming growth factor alpha and beta (TGF-α and TGF-β), tumour-necrosis factor alpha and beta (TNF-α and TNF-β), vitamin D₃, calcitonin, prostaglandin E₂ (PGE₂), and parathyroid hormone (PTH) [6,7], only the receptor activator of NF-κB ligand (RANKL, also called ODF/TRANCE/OPGL), a 317 amino acid long membrane-bound member of the TNF-ligand family, has been identified as the final and critical differentiation factor that specifies the osteoclast maturation programme and hence induces bone resorption [4,8,9]. Together with its receptor RANK and its decoy receptor osteoprotegerin (OPG), RANKL forms a cytokine system which seems to play a crucial role in mediating bone metabolism. The relative ratio of RANKL to OPG is a determinant in regulating all aspects of osteoclast functions: these include proliferation, differentiation, fusion, activation, survival and apoptosis.

Imbalances between “the players” of this system and thereby also of osteoclast activities can arise from a variety of hormonal changes or perturbations of inflammatory and growth factors, resulting in skeletal abnormalities characterised by decreased (osteoporosis) or increased (osteopetrosis) bone mass [10].

1.2. RANKL expression
RANKL localisation on osteoblasts, activated T-cells, synovial fibroblasts and even osteoclasts has been reported in previous studies [9,11–13]. RANKL also seems to be present in an active soluble form (sRANKL), which is derived from the cell-associated form by
1.3. Localisation of RANKL in human, ovine and bovine tissues
In order to understand the molecular mechanisms of bone resorption and osteoclast biology, it is necessary to clearly describe RANKL in bone tissue in a histological and morphological context. Since RANKL was first discovered in 1998 its essential role in bone metabolism strikingly gained importance for biomedical research. This study may form a basis to further investigate the very complex pathobiological functions of RANKL in bone metabolism and disease. The goal of this study was to visualise and localise RANKL in human, ovine and bovine bone tissues by the use of immunohistochemistry.

2. Materials and methods
2.1. Tissue
Human cancellous bone was obtained from the Department of Orthopaedic Surgery of the hospital in Davos with approval of the Ethic Commission of Graubunden (Nr. EK: 18/02). Bone fragments of the tibia and femur condyles were obtained freshly at surgery from five patients undergoing a total knee replacement and femoral heads of two patients undergoing total hip prosthesis transplantation. Ovine bone tissue from a four-month-old female Swiss alpine sheep, weight 56 kg, was freshly obtained from the Department of Experimental Surgery of the AO Research Institute, Davos. Bovine bone tissue was freshly obtained from a 14-month-old male from a slaughter house in Davos. Bone cores with a defined size of 5 mm² height and diameter were fixed at 4°C by immersion either for 18 h in 4% paraformaldehyde in 0.1 M PIPES buffer (piperazine-NN’-bis-2ethane sulphonic acid, pH 7.4) or in 70% ethanol and afterwards embedded in Technovit 9100New (Haslab, Ostermudingen, Switzerland).

2.2. Antibodies and immunohistochemistry
All immunohistochemistry was carried out at room temperature (20–25°C). RANKL immunostaining of the 6 μm thick microtome (Reichert-Jung Leica, Glattbrugg, Switzerland) tissue sections was carried out after deacrylation of the embedding medium for 2 x 30 min in 1-aceto-methoxy-ethane. The method was based on the immunolabelling of focal adhesions [15]. The blocking buffer, pH 7.4, contained 0.1 M PIPES (Fluka code 80636), 0.1% Tween 20 (Fluka code 93773), 1% bovine serum albumin (BSA) (British Biocell International (BBI), Cardiff, UK) and 1% goat serum (Sigma code S6898). The primary antibody, a monoclonal anti-TRANCE (TNFSF11), human clone 70525.11, purified mouse IgG2B (Sigma, Buchs, Switzerland product No. T2942, Lot-No. 90K0944) was used at a dilution of 1:100 in blocking buffer. Incubation was performed for 1 h in humid chambers. After six washes with blocking buffer, additional blocking was performed with 5% goat serum diluted in blocking buffer for 5 min. The sections were incubated for 2 h with a 5-nm gold-conjugated goat anti-mouse secondary antibody (Auroprobe EM, Code RPN 430 BS, Lot No. 193124) (Amersham Pharmacia Biotech, Bucks, UK) diluted 1:200 in blocking buffer. After another six washes with 0.1 M PIPES buffer, the sections were rinsed in distilled water for 2 min and on each specimen a droplet of silver enhancement solution (silver enhancing kit Cat. SEKL15; BBI, Cardiff, UK) initiator/ enhancer = 1:1 was added. Silver enhancement was performed for 8–12 min for scanning electron microscopy (SEM) imaging and for 30 min for light microscopy (LM) imaging. Dehydration through graded series of ethanol (70%, 80%, 96%, 100%) and clearing in xylene for 5 min each was performed and the sections were mounted in Eukitt for LM.

2.3. Negative control
In order to prove the specificity of the immunoreactions, negative controls were carried out by omitting the primary antibody. As negative tissue control, hyaline cartilage from the same species was used.

2.4. Microscopical evaluation
The stained specimens were analysed with a Zeiss Axioplan brightfield LM fitted with an Axiocam and processed with Axioplan and Axiovision. For SEM imaging the specimen were coated with 20 nm of carbon by thread evaporation (CED 030, Baltec, Buchs, Liechtenstein). Samples were analysed with a Hitachi S-4700 Field Emission SEM (FESEM) fitted with an Aurata yttrium aluminium garnet (YAG) backscattered electron (BSE) detector, and operated in BSE detection mode [16].

3. Results
3.1. RANKL expression in human, ovine and bovine bone tissues
RANKL protein appeared to be expressed by osteoblasts, osteocytes and osteoclasts in all human, ovine and bovine tissue samples. RANKL protein was determined histologically for the first time in osteocytes. RANKL signals were expected along the bone trabeculae where osteoblasts and osteoclasts are located (Fig. 1(a)). Fig. 1(a) shows three multinucleated osteoclasts, one of which is located in its Howship lacuna. Silver enhanced gold particles, suggesting the presence of the transmembrane form of RANKL in the membrane of different bone cells, was seen in 70% ethanol fixed bone samples out of a femur head of a 81-year-old female (Fig. 1(b)) but also in ovine (Fig. 1(c)) and bovine (Fig. 1(d)) bone tissues. RANKL signals were present in osteoblasts along the bone trabeculae, in osteoclasts and also in osteocytes, which are located within the mineralised bone matrix.