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.

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 D_3, calcitonin, prostaglandin E_2(PGE_2), 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

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metalloproteatic cleavage at positions 140 and 145 [8, 14]. RANKL is a component of a very complex cytokine network. Hormones and cytokines that modulate the production of RANKL are likely to be major regulators of osteoclastogenesis. It has to be mentioned that the osteoclastogenesis regulating factor OPG inhibits osteoclast formation as a RANKL decoy receptor, but furthermore does not affect the up and down regulation of RANKL [7].

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 mm^2 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, Ostermundingen, 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 × 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 Autrata 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.

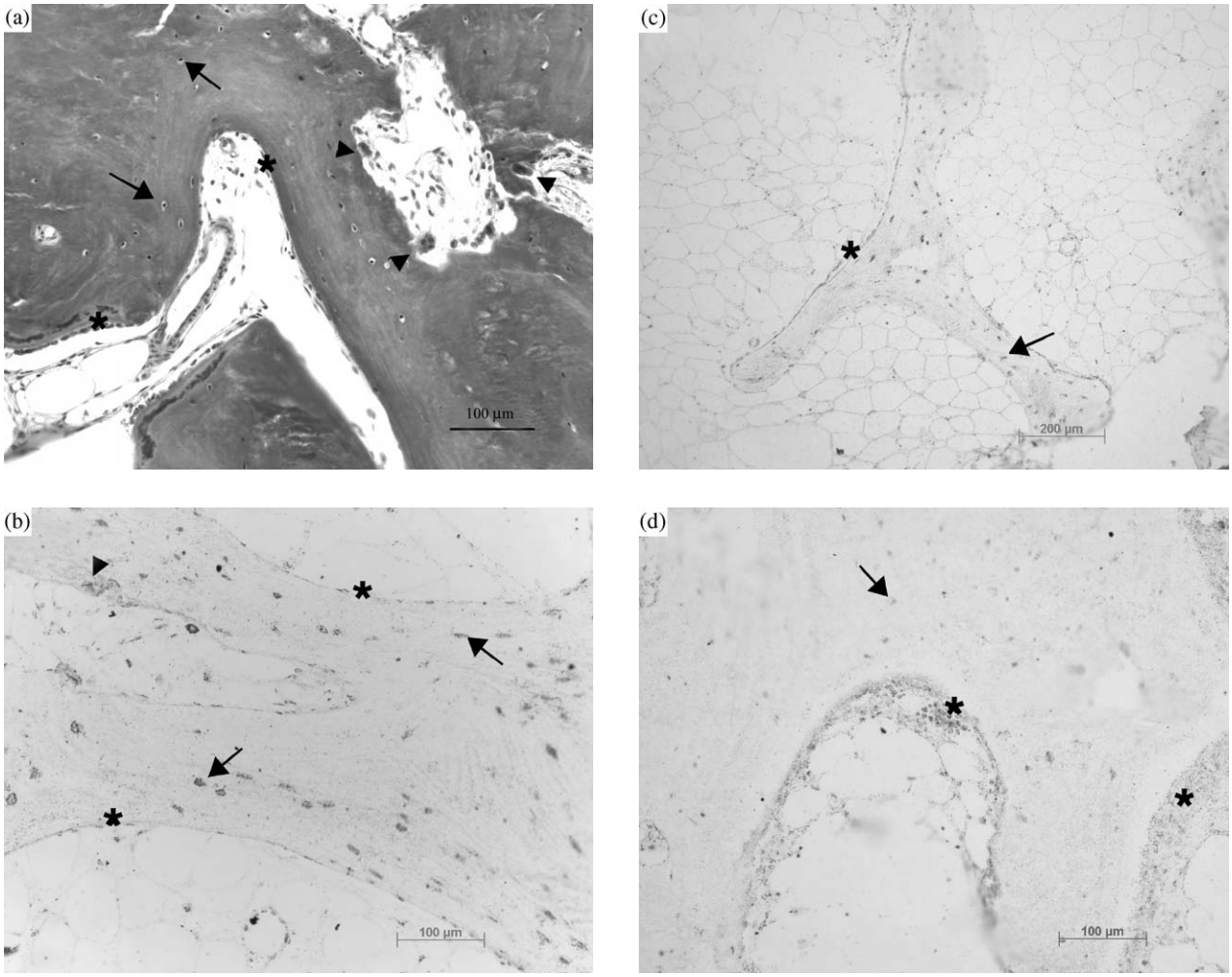

Figure 1 (a) Masson Goldner morphology-staining on human bone out of the knee of a 73-year-old female undergoing a total knee replacement showing several multinucleated osteoclasts (arrowheads), osteoblasts (stars) along the bone trabeculae and osteocytes (arrows) in the mineralised bone matrix. The bone was fixed in 70% ethanol and embedded in Technovit 9100New. RANKL immunolabelling on human bone out of the femur head of an 81-year-old female patient undergoing a total hip replacement (b), ovine (c) and bovine (d) bone tissue sections. The bone was fixed in 70% ethanol and embedded in Technovit 9100New. Immunolabelling for RANKL protein shows a clear positive signal on osteoblasts (stars), osteocytes (arrows) and on an osteoclast (arrowhead). Specificity of these labellings could be proven with negative controls (data not shown).

The immunoreactions on the membranes of the cells were, however, of different intensities.

Osteocytes in all samples appeared to be stained very clearly. The silver-enhanced gold particles were present on the membranes of the cell, appearing in a ring-like form, and even the osteocytic processes reaching relatively far through the calcified matrix were stained for RANKL protein (Fig. 2(a)). Secretion of RANKL (sRANKL) occurs not from all cells that express transmembrane RANKL, but in some sections staining around certain cells could be observed. Possible sRANKL staining around a lining cell was also imaged in a 70% fixed bone sample out of the femur head of a 50-year-old male who underwent a total replacement operation of his hip (Fig. 2(b)). In a 4% paraformaldehyde fixed bone sample out of a human femur head of an 81-year-old female patient, an osteoclast located in a Howship lacuna was imaged (Fig. 2(c)). This osteoclast showed a stronger signal in its membrane and in the surrounding tissues compared to other osteoclasts outside the lacunae.

3.2. No RANKL protein staining on human chondrocytes

In contrast to the examined bone tissue, RANKL protein could not be identified in human cartilage tissue close to the bone border. Fig. 3 shows the cartilage/bone border in a bone core section from knee bone of a 73-year-old female with varus gonarthrosis, who underwent total knee replacement. The specificity of the immunolabelling is shown by immunostained tissue sections, where the primary antibody was omitted (Fig. 3(b)).

In cartilage tissue no cells were shown to display RANKL protein expression, whilst within bone tissue osteocytes and osteoblasts showed a clear immunostaining signal for RANKL. Cartilage could therefore be used as the negative tissue control in this human tissue investigation for RANKL protein. Indeed, closer investigation of the tissue sections revealed a striking difference between chondrocyte and osteocyte staining. SEM imaging of the same sections confirmed these results (Fig. 4). Fig. 4 (a)–(c) show RANKL signal on human, ovine and bovine osteocytes with silver

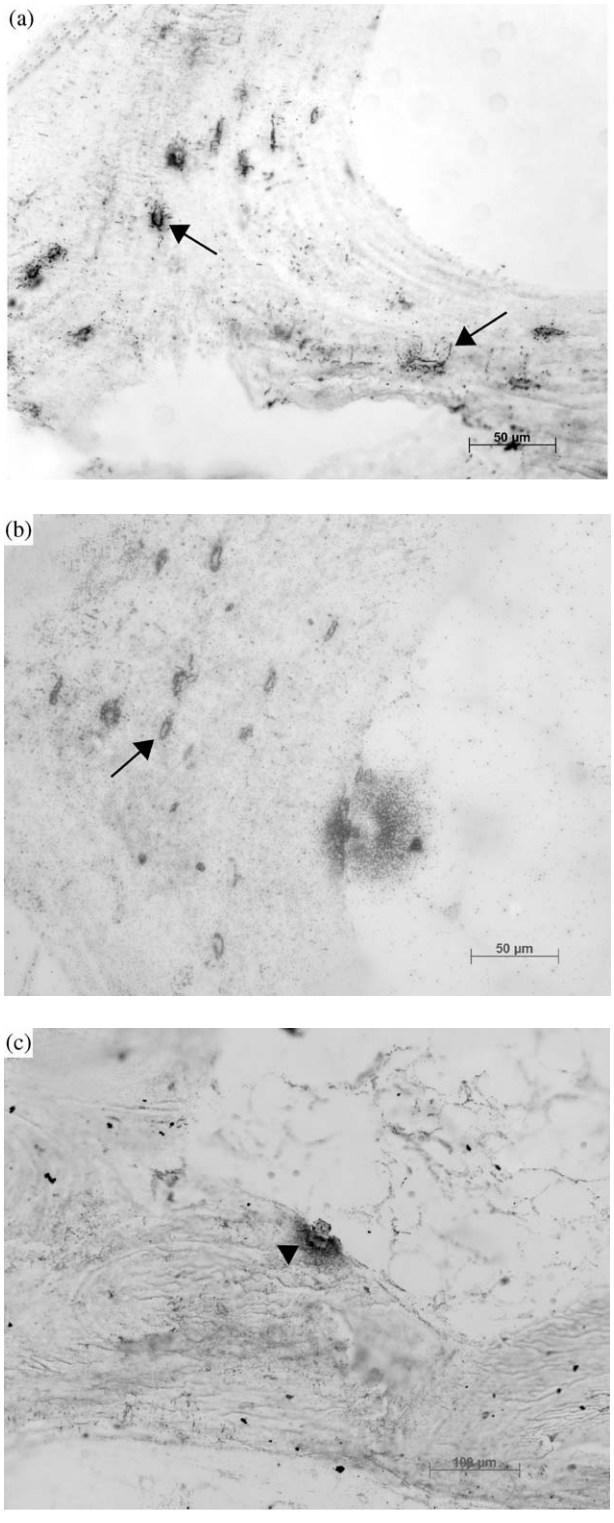

Figure 2 Bone tissue immunostained for RANKL protein. (a) A positive signal for RANKL is detected on the membrane of osteocytes and on osteocytic processes (arrow) and on. (b) sRANKL labelling around another cell – possibly a T-lymphocyte – stained osteocytes and their processes are marked with arrows. The bone tissue sections out of the femur head of a 50-year-old male undergoing total hip replacement were fixed in 70% ethanol and embedded in Technovit 9100New. (c) Labelled osteoclast (arrowhead) sitting in a Howship lacuna showing sRANKL. The bone tissue out of the femur head of an 81-year-old female patient undergoing total hip replacement was fixed in 4% paraformaldehyde and embedded in Technovit 9100New.

enhanced gold conjugated secondary antibody as marker. In Fig. 4(d), chondrocytes from the same tissue section as imaged in Fig. 4(a) are shown. None of the chondrocytes showed any RANKL labelling.

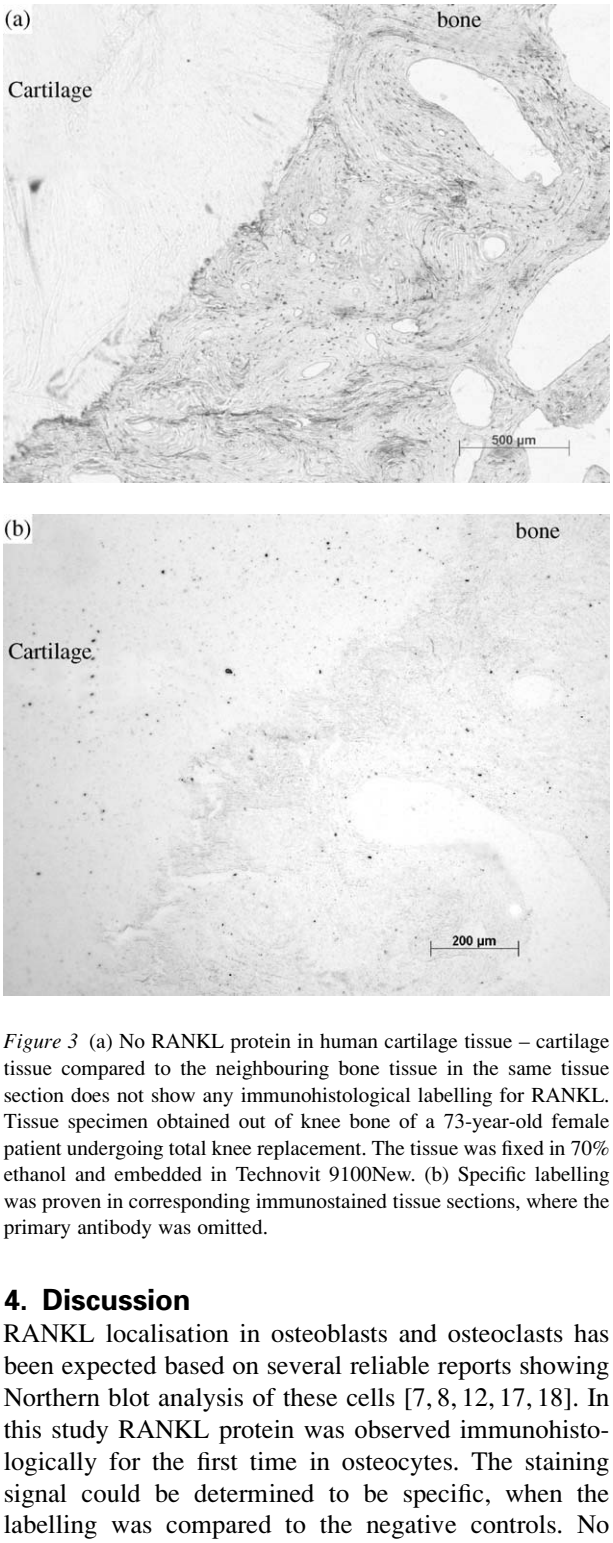

Figure 3 (a) No RANKL protein in human cartilage tissue – cartilage tissue compared to the neighbouring bone tissue in the same tissue section does not show any immunohistological labelling for RANKL. Tissue specimen obtained out of knee bone of a 73-year-old female patient undergoing total knee replacement. The tissue was fixed in 70% ethanol and embedded in Technovit 9100New. (b) Specific labelling was proven in corresponding immunostained tissue sections, where the primary antibody was omitted.

4. Discussion

RANKL localisation in osteoblasts and osteoclasts has been expected based on several reliable reports showing Northern blot analysis of these cells [7, 8, 12, 17, 18]. In this study RANKL protein was observed immunohistologically for the first time in osteocytes. The staining signal could be determined to be specific, when the labelling was compared to the negative controls. No RANKL signal was seen in human cartilage tissue close to the examined bone tissue (Fig. 3), in SEM imaging no RANKL staining in chondrocytes was observed whereas osteocytes in the same tissue section were stained clearly (Fig. 4(a) and (d)), suggesting that chondrocytes are a cell type which do not express RANKL. Immunolabelling of RANKL on osteocytes in ovine and bovine tissues were demonstrated (Figs. 1(c,d) and 4(b,c)). The expression of RANKL protein was not homogeneously expressed in all osteocytes. This could be due to the fact that the cells are in various phases of activity [13] and do not all express the same amount of RANKL in their membranes. Cell-to-cell contact between osteoblasts/stromal cells and cells from the monocyte-macrophage lineage was determined to be

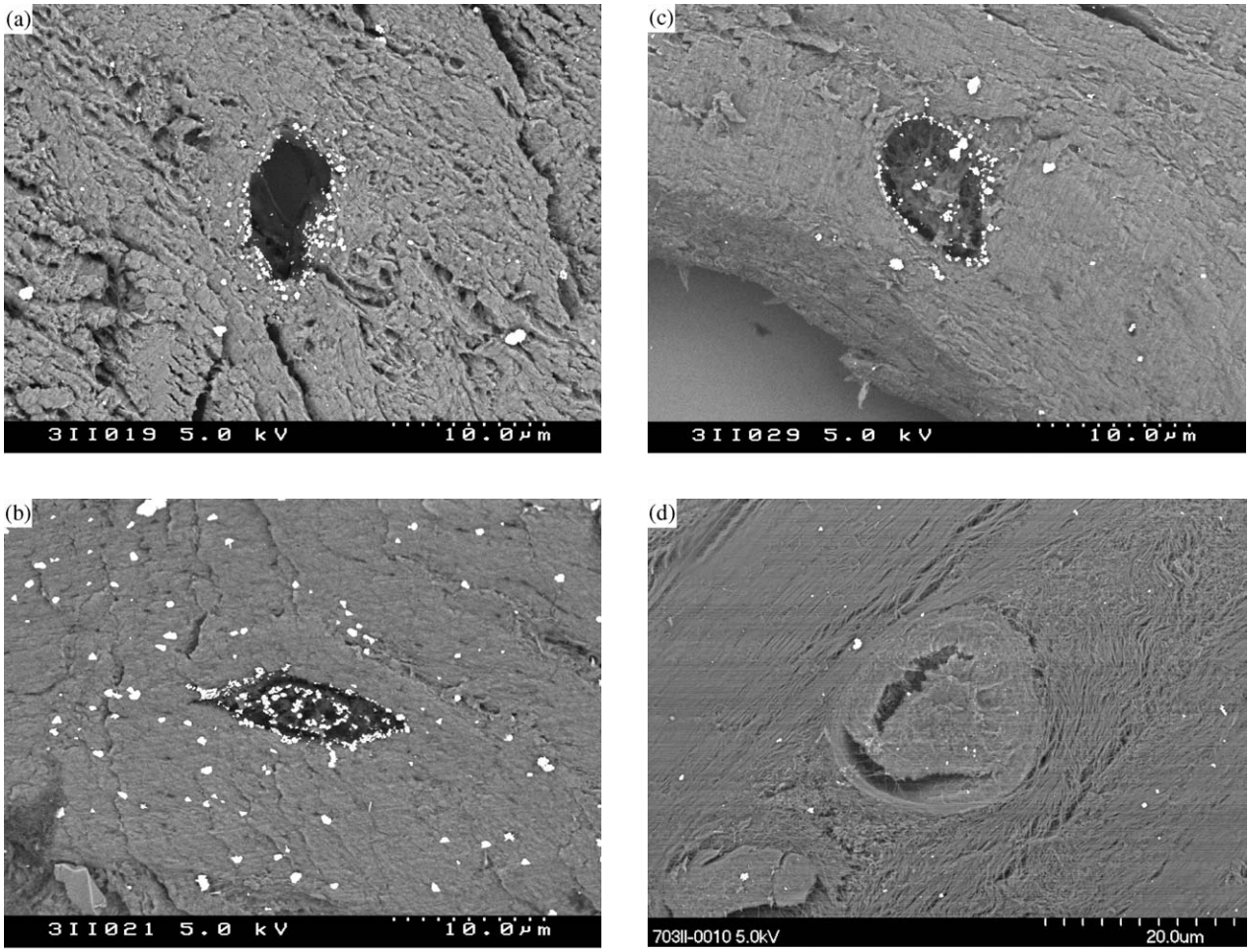

Figure 4 SEM images of human (a) and (d), ovine (b) and bovine (c) tissue sections immunolabelled for RANKL protein. Human bone was obtained out of the knee bone of a 73-year-old female patient with varus gonarthrosis undergoing a total knee replacement. Osteocytes are stained positively for RANKL protein in human, ovine and bovine bone tissues, (a), (b) and (c). In contrast, a chondrocyte of the same tissue specimen as in (a) does not show any immunolabelling for RANKL protein (d). All tissue specimens were fixed in 70% ethanol, embedded in Technovit 9100New and sectioned 5 µm. Gold conjugated antibody was visualised after silver enhancement for 12 min.

indispensable for osteoclast development by several scientists. Whether cell-to-cell contact is really needed for following osteoclastogenesis has not yet been confirmed. The production of the cleaved form of reactive soluble RANKL in some bone cells was described in several *in vitro* experiments [6–8, 19] and would be against the necessity of cell-to-cell contact. This *ex vivo* study, however, is suggesting that some cells do not produce soluble RANKL and suggests certain cells, such as osteocytes require cell-to-cell contact through their processes. It was shown earlier that viable osteocytes widely send their processes into the osteoblast layer outside the calcified bone matrix and can probably communicate with cells such as osteoclast precursors in the marrow [20]. The clear results of positive staining for RANKL in the dendritic processes of osteocytes (Fig. 2(a)) may give an indication on the involvement of osteocytes in orchestrating bone remodelling. Osteoclastogenesis occurs with the promotion of RANKL. Vitally, osteoclastogenesis has to be checked by certain control mechanisms, otherwise bone resorption would inevitably be excessive. It is likely that osteoclasts within their resorption lacunae secrete RANKL while ''not-active'' osteoclasts would not do that [17]. However, it is reported that inhibition of apoptosis of osteoclasts is dependent on the presence of osteoblasts or sRANKL [7, 21]. This is consistent to the diffuse RANKL labelling in Fig. 2(c). Whether this osteoclast is actively secreting sRANKL would have to be further investigated.

4.1. Speculations

RANKL plays a role in cell activation, proliferation, survival or death depending on the context in which it is expressed and the nature of the target cell (i.e. its receptor) [11]. When humans and animals do not use the bone, this causes a decrease of the bone mass and of the lamellae by increasing bone resorption. In this study, bone from patients who underwent hip or knee operations was used. It is likely that this bone tissue showed a negative record of bone mass (bone atrophy) and would therefore show a higher amount of RANKL. It is a philosophical question whether load and mechanical stress or the RANKL/RANK/OPG systems are primarily influencing osteoclastogenesis. Future studies should examine the regulation of expression and therefore the ratio of RANKL to OPG in bone tissue in response to mechanical strain, microdamage and load. If osteocytes turn out to be important cells for RANKL expression and thereby of osteoclast differentiation, this could be a hurdle on the way to developing clinical therapies. It will

be necessary to see osteocytes as major target cells using a RANKL-blocking drug. First, there are approximately 10 times as many osteocytes as osteoblasts in normal human bone [23] and second, it is not going to be trivial to reach osteocytes buried in the calcified bone matrix as easily as osteoblasts.

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