Receptor activator of nuclear factor kappa B ligand in an experimental intervertebral disc degeneration

Z. Mackiewicz[1,7], J. Salo[2], Y.T. Konttinen[1,3,6], A. Kaigle Holm[4], A. Indahl[5], J. Pajarinen[6], S. Holm[4]

[1]COXA Hospital for Joint Replacement, Tampere, Finland; [2]Department of Orthopedic Surgery, and [3]Department of Medicine/ Invärtes Medicin, Helsinki University Central Hospital, Helsinki, Finland; [4]Department of Orthopaedics, Sahlgrenska University Hospital, Göteborg, Sweden; [5]Department of Physical Medicine and Rehabilitation, Kusthospitalet, Stavern, Norway; [6]ORTON Orthopedic Hospital of the Invalid Foundation, Helsinki, Finland; [7]Vilnius University Institute of Experimental and Clinical Medicine, Vilnius, Lithuania.

Abstract

Objective

This study was designed to clarify the role of the receptor activator of nuclear factor kappa B ligand (RANKL) in the process of discus degeneration and spondylarthrosis. It was hypothesized that experimental discus lesion would initiate not only local bone remodelling but also increased osteoclast formation on a location remote to the injury site due to altered spinal biomechanics. It was speculated that these changes in vertebral bone remodelling could be reflected in an increased RANKL expression.

Methods

The presence of RANKL in the spine was studied in an experimental perforating lesion of the cranial endplate of L4 and the adjoining disc in six domestic pigs and in one human herniated disc. After three months, the experimental and contiguous control vertebrae, complete with intervertebral discs, were subjected for immunohistochemistry.

Results

This is the first study to show that RANKL was locally seen (produced) in osteoblasts, fibroblasts replacing annulocytes and mesenchymal bone marrow cells and, in part, apparently bound to the surface of osteoclasts and macrophage-like prefusion macrophages. Such RANKL induction was also seen at sites remote from the experimental lesion driving the whole process. More RANKL-positive cells were found in close proximity to the endplate than in the central parts of the vertebrae. Osteocytes in bone matrix and most bone marrow cells in the marrow microenvironment showed no RANKL staining. Human annulus fibrosus also contained RANKL, RANK and OPG.

Conclusion

We have demonstrated that RANKL is produced locally, also in soluble form, at the site of injury and also in intact vertebrae and bony structures likely due to altered biomechanics. It seems to be engaged in bone healing and remodelling, essentially proving our working hypothesis. These secondary bone changes could represent part of the degenerative spine disease (spondylarthrosis). RANKL inhibitors, like recombinant human osteoprotegerin (OPG), could be interesting drugs to test, not only in osteoporosis, but also in spondylarthrosis.

Key words

RANKL, osteoclasts, intervertebral disc degeneration, swine, experimental.

Zygmunt Mackiewicz, MD, PhD
Jari Salo, MD, PhD
Yrjö T. Konttinen, MD, PhD
Allison Kaigle Holm, PhD
Aage Indahl, MD, PhD
Jukka Pajarinen, BSc (Med)
Sten Holm, PhD

This study was supported by grants from the Invalid Foundation, evo grants, Sigrid Jusélius Foundation, Wilhelm och Else Stockmanns Stiftelse, the Finnish Research Foundation of Orthopaedics and Traumatology and Finska Läkaresällskapet.

Please address correspondence to:
Yrjö T. Konttinen, MD, PhD,
Institute of Clinical Medicine,
Department of Medicine,
Biomedicum Helsinki, P.O. Box 700,
FIN-00029 HUS, Finland.
E-mail: yrjo.konttinen@helsinki.fi

Please address reprint request to:
Sten Holm, MD, PhD,
Department of Orthopaedics,
Guldhedsgatan 10,
Sahlgrenska University Hospital,
S-413 46 Göteborg, Sweden.
E-mail: sten.holm@orthop.gu.se

Received on June 10, 2008; accepted in revised form on November 27, 2008.

Conflict of interest: Dr. Y.T. Konttinen has received EVO grants from the Invalid Foundation, the Sigrid Jusélius Foundation and Wilhelm och Else Stockmanns Stiftelse; Dr. J. Pajarinen has received grant support from the Finnish Research Foundation of Orthopaedics and Traumatology; the other co-authors have declared no competing interests.

Introduction

Bone remodelling is a cyclic physiological process that involves activation of osteoclasts to resorb bone, followed by reversal and osteoblast-mediated new bone formation in repeated activation-resorption-formation cycles. Osteoclasts are specialised multinuclear cells of the monocyte/macrophage lineage that are formed by fusion of haematopoietic precursors (1, 2). Osteoclast formation is stimulated by receptor activation of nuclear factor κB ligand (RANKL), which is present primarily as a membrane-bound protein on the surface of osteoblasts. RANKL has been shown to stimulate osteoclastogenesis and to promote their survival and activity (3, 4). Effects of RANKL are mediated via a specific receptor, known as RANK, which is expressed on haematopoietic osteoclast prefusion progenitors (5, 6) as well as on mature osteoclasts (7, 8). Thus, upon activation of the RANK (= receptor), genes required for osteoclast differentiation and activation are engaged and either up- or down-regulated (5). Both gain and loss of function gene experiments have established RANKL/RANK interactions as essential for osteoclast formation (9, 10). It was originally considered that osteoclast formation is driven by direct cell-to-cell contacts between RANKL+ osteoblasts and RANK+prefusion macrophages (or forming osteoclasts) (11).

Osteoclasts are multinucleated non-dividing cells of relatively short life-span and, therefore, factors that affect their formation, survival and activity have powerful effects on osteolysis. The ability of osteoclasts to degrade bone lies in their ability to secrete protons and specialised collagenolytic proteinases, in particular the cysteine proteinase cathepsin K, in the acidic microenvironment that underlies osteoclasts during bone resorption (12).

Degenerative diseases of the human spine are accompanied by vertebral bone remodelling, including bony end plate thickening and osteophyte formation. This means that the basic multicellular units are actively engaged in activation-reversal-formation cycles so that the macro- and micro-architecture of the verbtebrae and their appendicular structures, like facet joints, change (13, 14). It is not known, however, if remodelling also occurs in contiguous units. The hypothesis of the present work was that an experimental lesion in the endplate of a lumbar spine L4 vertebra leading to disc degeneration, engages the RANKL system. This RANKL involvement was speculated to be seen both at the local site of injury reflecting bone healing and remodelling as well as, due to altered biomechanichs at remote sites in the lumbar spine. The first aim of this study was therefore to analyze the expression of RANKL in a surgical experimental porcine model of lumbar intervertebral disc and endplate injury. The second aim of the study was to check if such a local injury is reflected in RANKL expression at sites remote from the local injury site.

Material and methods

Endplate and intervertebral disc injury surgical procedures

Ethical approval for the use of animals in this study was granted by the Animal Research Ethics Committee of Gothenburg. The experimental procedure has been described elsewhere (15). Briefly, domestic pigs (n=6), 4-5 months old, weighing 50-60 kg, were sedated by an i.m. injection of Ketalar (ketamine, 15-20 mg/kg; Parke-Davis, Gwent, UK) and after 10 minutes anesthetized with i.v. Hypnodil (methomidate chloride, 3-5 mg/kg of body weight; AB Leo, Helsingborg, Sweden) and Stresnil (azaperone, 0.1 mg/kg of body weight; Janssen-Celag, Sollentuna, Sweden). With the animal lying on its right side, the L3-L4 disc was exposed using a left retroperitoneal approach. The cranial endplate of the L4 vertebra was perforated using a 3.5-mm drill bit inserted from the lateral cortex at mid-height. Angulated at 45°, to reach the central part of the endplate, a single hole was drilled into the nucleus pulposus and the drill withdrawn. After 3 months the animals were sacrificed, their spine was excised, frozen, and later subjected to histological and immunohistochemical examinations of the injured and degenerated discs and paradiscal spine, which were compared to the adjacent control segment. Histological sections

were cut in perpendicular orientation and contained the remnants of the nucleus pulposus and annulus fibrosus (or in the control sample the intact disc itself), together with cartilaginous and bony vertebral endplates, spinal ligaments and some vertebral body bone marrow.

Human annulus fibrosus samples were collected from one patient operated for L5-S1 lumbar herniation and processed as described before (16). Briefly, these samples were frozen and stored until cut to 6-10 μm sections, which were placed on slides coated with formolgelatin as adhesive. Sections were first air-dried for one hour and then fixed for 5 minutes in cold (+4°C) acetone before staining.

Histological analysis

Samples were fixed in 4% neutral formaldehyde, decalcified, dehydrated and embedded in paraplast. 3-4 μm thick sections were cut and mounted on L-polylysine-coated slides for staining using haematoxylin and eosin, van Gieson, picrosirius and Safranin-O.

Immunohistochemical staining and staining controls

Monoclonal mouse anti-human RANKL IgG_{2b} (MAB626, 1.25 μg/ml), goat anti-human RANK IgG (0.25 μg/ml) and mouse anti-human OPG (osteoprotegerin) IgG1 1.25 μg/ml (R & D Systems, Minneapolis, Minnesota) were tested in pilot studies for their reactivity in porcine enzootic pneumonia caused by *Mycoplasma hyopneumoniae* and proliferative enteropathy caused by *Lawsonia intercellulare*. Synovial membrane-like interface tissue from revised totally replaced hip joints were used as positive human sample controls. Fixation used in these pilot studies was the same as was used in the actual experiments. RANK and osteoprotegerin antibodies did not cross-react or their immunoreactivity was destroyed by the sample processing protocol, whereas monoclonal mouse anti-human RANKL IgG_{2b} showed interspecies cross-reactivity. These same antibodies were used to stain the human annulus fibrosus cryostat sections, which at the same time served as positive sample controls.

Five μm thick paraffin sections were mounted on DAKO capillary slides (TechMate, DAKO), deparaffinized in xylene, rehydrated in graded ethanol series and 10 mM phosphate buffered 0.9 M saline, pH 7.4. For antigen retrieval the slides were placed in antigen retrieval buffer (DAKO, Glostrup, Denmark) and microwaved in a microwave processing labstation (MicroMED T/T Mega Histoprocessing Labstation, Milestone Inc, Atlanta, USA) for 10 minutes at +98ºC according to the manufacturer's program, kept at room temperature for 30 minutes, washed in phosphate buffered saline and stained automatically using the following protocol (cryostat sections entered directly this step after fixation): 1) monoclonal mouse anti-human RANKL IgG_{2b} (MAB626, R & D) 1.25 μg/ml, diluted in DAKO ChemMate antibody diluent for 1 hour; 2) secondary antibody containing both biotinylated goat anti-rabbit IgG and biotinylated goat anti-mouse IgG antibodies for 30 minutes; 3) peroxidase block for 30 minutes; 4) peroxidase-conjugated streptavidin 3 x 3 minutes; 5) horse radish peroxidase substrate buffer; and finally 6) H_2O_2 substrate working solution containing 3,3'-diaminobenzidine tetrachloride (ChemMate Detection Kit) for 5 minutes. Between each step, the sections were washed with DAKO ChemMate washing buffers three times and dried in absorbent pads. Replacement of the primary antibody with normal mouse IgG of the same isotype and concentration as the specific primary antibody but with irrelevant specificity, diluted in DAKO ChemMate antibody diluent, was used as negative staining control. After staining, the sections were removed from the machine, counterstained with haematoxylin or left without counterstaining, washed, dehydrated in ethanol series, cleared in xylene and mounted in a synthetic mounting medium (Diatex, Beckers Industrifäg, Märsta, Sweden).

Evaluation of the results

Semiquantitative microscopic assessment of immunohistochemical staining was performed under × 400 (high power fields) using five grades: – = no immunoreactivity; + = only a few immunoreactive profiles; ++ = some immunoreactive profiles; +++ = moderate numbers of immunoreactive profiles; ++++ = many immunoreactive profiles. Two histopathologists evaluated the results of histological and immunohistochemical staining independently using this predefined and very simple scoring system leading to very similar readouts and followed by a consensus session where the few and slight discrepancies were discussed and solved.

Results

General comments

In cross-sectional histological views of the degenerated disc, loss of the gel-like nature of the nucleus and brown pigmentation were clearly seen in the degenerated disc but not in the adjacent control disc. T2-weigted magnetic resonance imaging disclosed absence of MR signal in the degenerated disc nucleus, while the adjacent level showed high intensity in the nucleus region as has been earlier described (15).

Histological analysis

The experimental trauma was well repaired. No traces of the original trauma inflicted during the experimental drill procedure were found in the histological analysis, which in the experimental samples revealed signs of intervertebral disc degeneration. In the control tissues annulus fibrosus maintained its dense lamellar structure and nucleus pulposus showed no granular degeneration. Secondary bone changes were seen in the lumbar spine. Vertebrae disclosed osteophyte formation, fibrosis of the bone marrow cavity and loss of the predominantly longitudinal orientation of the bone trabeculae, mostly just beneath of endplate. Superficial bone erosions were seen immediately under periosteum in experimental, but not in control samples. Spinal ligaments had, to some extent, lost their strictly ordered fiber orientation and packaging in the experimental samples.

Immunohistochemical findings

The main finding of the present paper is that RANKL was found to be increased in experimental disc lesions *per se*, but also in the lumbar spine

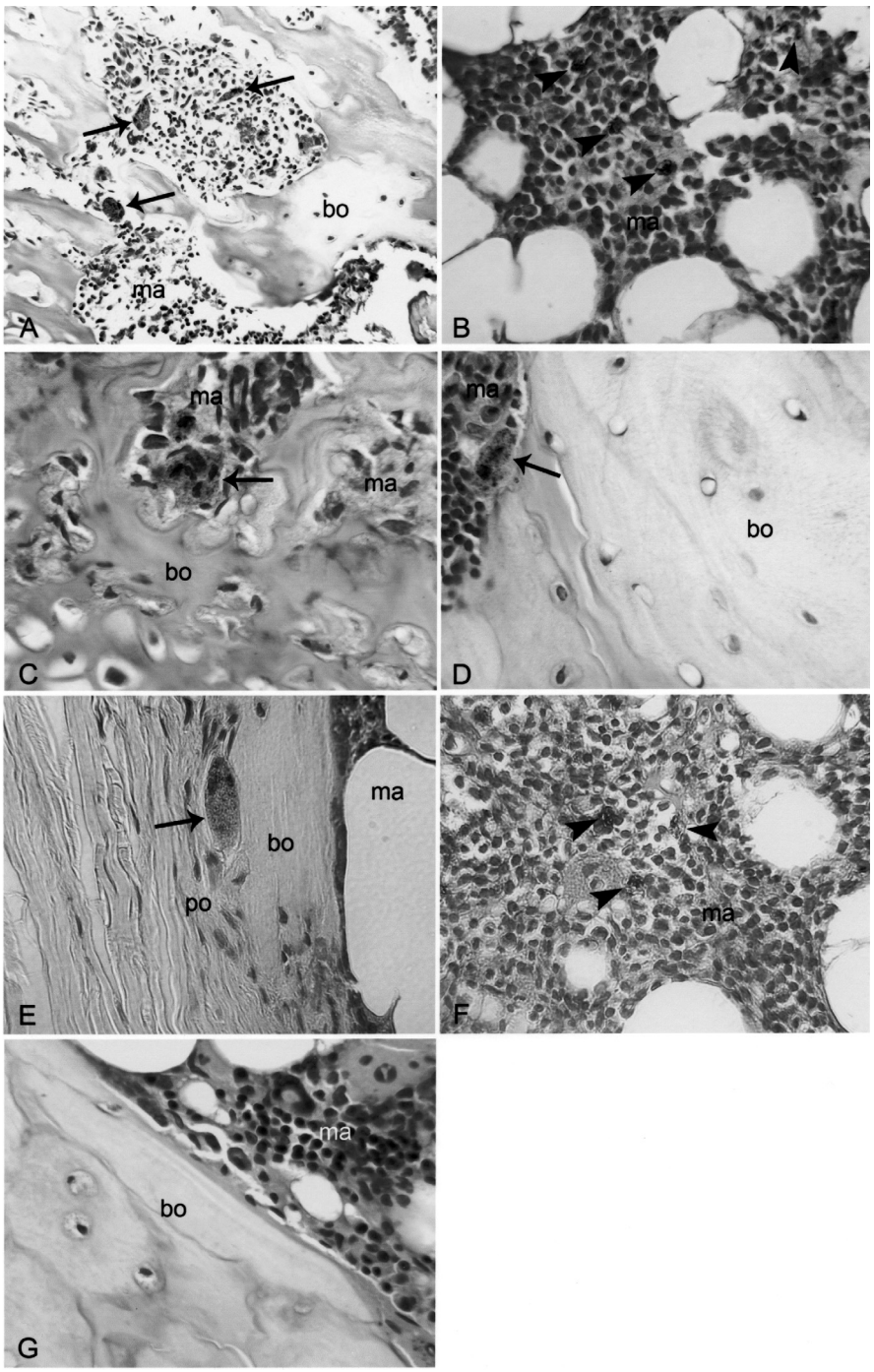

Fig. 1. Expression of immunoreactive RANKL in an experimental porcine model of intervertebral disc degeneration.
Panels **A**-**E**: degenerated samples, **F**: a contiguous control sample, **G**: negative control staining. **A**: expression of RANKL in some mononuclear cells (arrows) and multinuclear cells (arrow heads) in the bone marrow; **B**: RANKL in mononuclear cells (arrows) in the bone marrow; **C**: RANKL expression in osteoclasts (arrow head); **D**: RANKL in an osteoclast on the surface of a bone trabecula (arrow head); **E**: RANKL on the surface of compact cortical bone beneath periosteum (arrow head); **F**: RANKL in bone marrow of a contiguous control vertebra (arrows); **G**: no RANKL expression in negative control staining performed with non-relevant mouse IgG. Counterstained with haematoxylin. Original magnification x 200 (A); x 400 (B -G).
bo: bone; **ma**: bone marrow; **po**: periosteum

in general. Staining intensity of positive cells varied from cell to cell from faint to strong, experimental samples showing both higher proportion of positive cells and more intense staining. More RANKL-positive cells were found in close proximity to the endplate than in the central parts of the vertebrae. The osteocytes embedded deep in bone matrix and most bone marrow cells in the marrow microenvironment showed no RANKL staining.

RANKL was found in many mesenchymal cells known to be able to produce RANKL. In experimental samples RANKL immunostaining was mainly observed in bone marrow, where both multinuclear cells and some mononuclear cells (Fig. 1, panels A and B) were stained. RANKL-positive staining was also found in some osteoblasts at the interface between annulus fibrosus and bone (Fig. 2, panel A), but also in some fibroblast-like cells in the bone marrow (Fig. 2, panel B) and in some annulocyte-like cells in the peripheral part of annulus fibrosus (Fig. 2, panel C).

In addition to some mononuclear mesenchymal cells, RANKL ligand was somewhat surprising also found on multinuclear cells, which are not considered to be able to produce RANKL. However, as these cells have RANKL receptors, they are able to bind exogenous RANKL. Many multinuclear osteoclast-like cells were positive (Fig. 1, panels A and C). Multinuclear cells were often seen attached to the surface of bone trabeculae (Fig. 1, panel D) and to a lesser extent also to the external surface of the bone, just beneath periosteum (Fig. 1, panel E). Comparison of the L3-L4 level with the adjacent levels disclosed that RANKL+ cells were also seen in the levels remote from the experimental lesion at the cranial endplate of L4 but RANKL+ cells were found in somewhat lower numbers (Fig. 1, panel F, Table I). In addition, hardly any osteoclasts were seen on external bone surface beneath the periosteum. No RANKL-positive cells were seen in remote and thus undamaged intervertebral discs.

Staining controls confirmed the specificity of the staining. The immunohistochemical reaction was highly specific as negative controls were devoid of any staining (Fig. 1, panel G).

Human annulus fibrosus samples contained RANKL, RANK and OPG positive cells, whereas the negative staining controls were negative (Fig. 3).

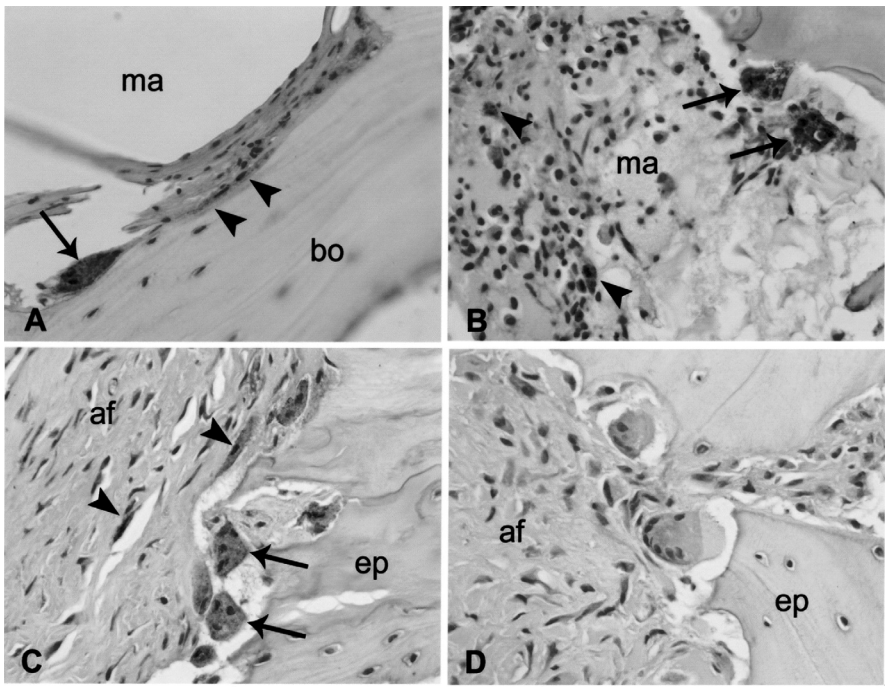

Fig. 2. Expression of immunoreactive RANKL in mesenchymal osteoblasts, fibroblast-like cells and annulocyte-like cells.
Panel **A**: Some RANKL positive osteoblasts have been marked with arrow heads. A close by osteoclast on bone surface (arrow) is also RANKL positive.
Panel **B**: Some fibroblasts in the bone marrow are RANKL positive (arrow heads) and again two close-by osteoclasts on bone surface are also RANKL-positive (arrows).
Panel **C**: Some annulocyte-like cells in the peripheral parts of annulus fibrosus are RANKL-positive and some osteoclasts (arrows) on the resorbing surface of the bony endplate are also RANKL-positive.
Panel **D**: A negative RANKL staining control. Original magnification x400.
af: annulus fibrosus; **bo**: bone; **ep**: end plate; **ma**: bone marrow.

Table I. Expression of RANKL in experimental intervertebral lumbar disc degeneration.

Localization	In degenerated samples	In control samples
Trabecular osteoclasts	+++	++
Bone surface osteoclasts	+	–
Bone marrow cells	+++	++

Score value: –: no positive profiles; +: a few positive profiles; ++: some positive profiles; +++: moderate numbers of positive profiles; ++++: many positive profiles.

Discussion

The first aim of this study was to analyze the expression of RANKL in a surgical experimental porcine model of lumbar intervertebral disc and endplate injury. As RANKL drives osteoclast formation as well as modulates their function, it is of interest that the expression of RANKL was clearly increased in tissues adjoining the damaged and degenerated intervertebral disc. RANKL binds to its signal transducing receptor of NFκB (RANK) (4, 17-20). In addition to its role in the precursor cell differentiation to osteoclasts, RANKL also augments osteoclast survival and activity (3, 5, 21-24). It thus seems that RANKL-driven osteoclast formation, survival and function was locally increased in the injured and subsequently degenerated intervertebral disc. We think this reflects host response to the local endplate trauma, resorption of damaged and dead bone and remodelling of newly formed bone produced for bone repair.

RANKL is normally found on the surface of bone-forming osteoblasts. In the present study it is described on the surface of one more mesenchymal cell, namely fibroblasts replacing annulocyte-like cells of annulus fibrosus often seen in close contact to macrophage-like cells and multinuclear osteoclasts. We think that this close cellular association allows direct cell-to-cell contacts and RANKL-RANK interactions, which promote osteoclast formation, survival and activity. Not surprisingly RANKL was also found in osteoblast-like bone lining cells, fibroblast-like connective tissue cells and mesenchymal marrow cells.

Perforation of bone tissue and intervertebral disc causes damage and thus initiates bone repair processes as well as bone remodelling. It is therefore not surprising that activation of RANKL/RANK system and osteoclastogenesis is seen in the early stages of healing of our expremental lesion. However, later when healing is already advanced, the loss of normal intervertebral disc structure is likely to cause changes in the distribution of mechanical forces and to impair stress absorbance. This later stage of our experiment resembled consequences of slowly developing disc degeneration. An aspect of interest is that at this stage RANKL/RANK system seems to play a role in the pathogenesis of the degenerative changes of the spine not only at the level of the experimental lesion but also at the level of adjacent vertebrae. In addition, all components of the RANKL complex (RANKL, RANK and OPG) were found in a degenerating human disc. However, it is not clear whether the RANKL/RANK system is also involved in disc degeneration *per se*.

Apart from its cell surface-bound membrane attached RANKL, it also occurs in soluble form (25) .To activate bone-destroying osteoclasts, RANKL must bind to its receptor on osteoclasts. It is therefore noteworthy that immunoreactive RANKL protein was also found on the surface of osteoclasts, apparently bound to these cell surface receptors. *In vivo* and *in vitro* the effect of RANKL is dependent on the balance between its cell membrane-bound receptor RANK and its inhibitor OPG (26). We believe that visualization of RANKL binding shows that at least some RANKL has escaped the neutralizing OPG pool and was bound to the surface of osteoclasts and its precursors, apparently driving

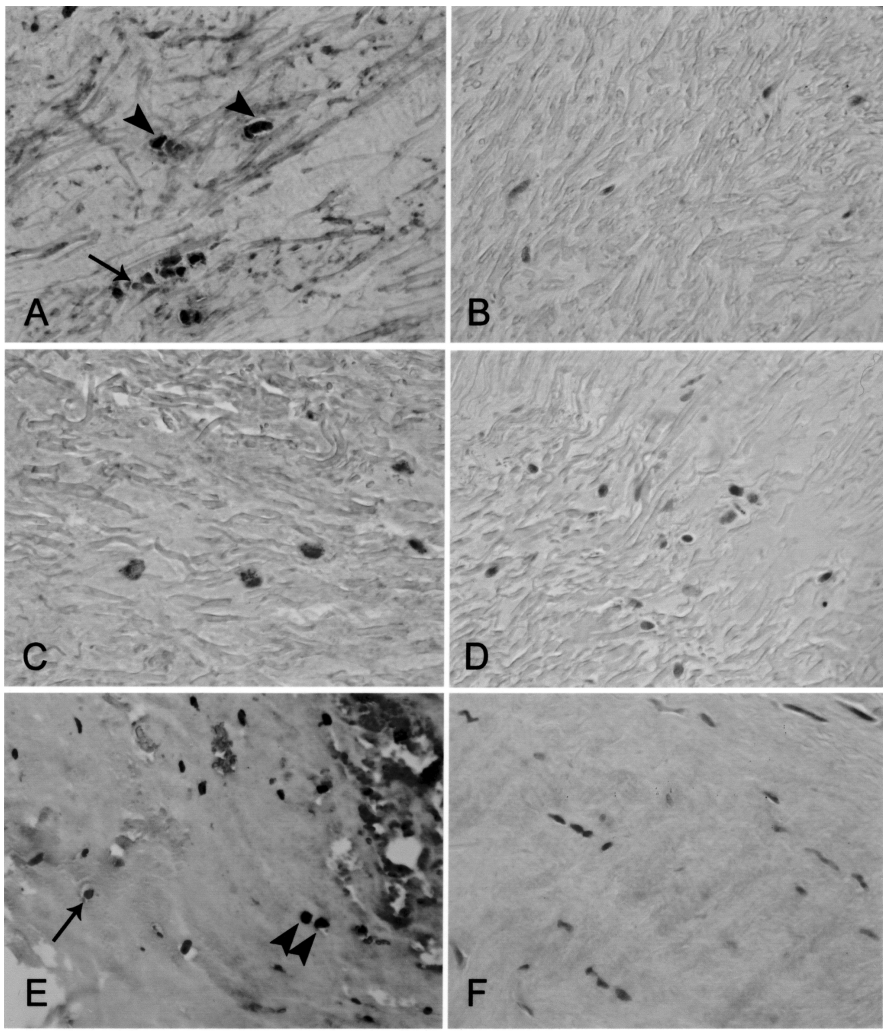

Fig. 3. Expression of immunoreactive RANKL, RANK and OPG in a degenerated human annulus fibrosus.
Panel **A**: RANKL positive cells and in panel **B** the corresponding negative control staining.
Panel **C**: RANK positive cells and in panel **D** the corresponding negative control staining.
Panel **E**: OPG positive cells and in panel **F** the corresponding negative control staining.
Original magnification x 400.

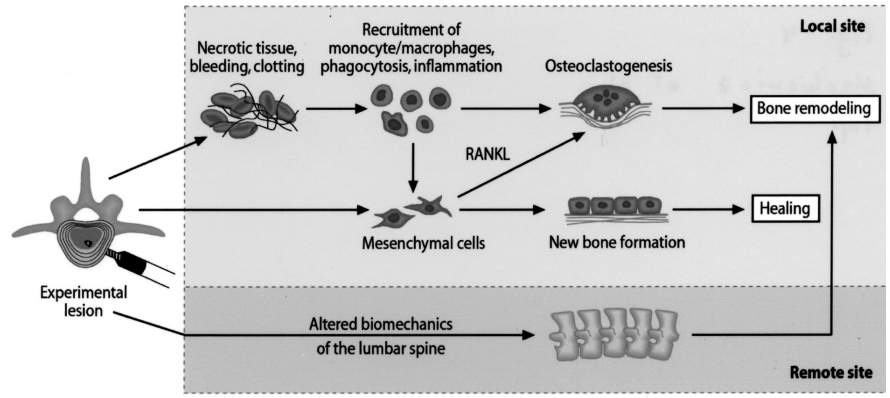

Fig. 4. A schematic figure about the initial endplate and disc trauma. It leads to activation of both local (upper box) and remote (lower box) processes. The local process is first dominated by injury and inflammation, but afterwards bone healing and remodelling ensue. It is the result of the direct local trauma. The remote process is initiated by the altered biomechanics of the lumbar spine. It leads to involvement of the basic multicellular units of the bone, the result of which is bone resorption and bone formation in form of remodelling.

cell fusion and osteoclastogenesis. This direct demonstration of RANKL on osteoclast surface was also important as, unfortunately, the antibodies against the various other components of the RANKL complex, which work very well in human tissues (27), did not recognize the porcine forms of RANK and OPG.

We suppose that soluble RANKL detected on osteoclast surfaces was produced by the close residing mesenchymal cells (osteoblasts, fibroblasts, marrow stromal cells and fibroblasts replacing annulocytes) and was either secreted into the extracellular space or solubilized from cell surface to subsequently diffuse and bind to RANK receptors of prefusion macrophages and osteoclasts. However, other possible sources of RANKL have been suggested. Kartsogiannis *et al.* (22) found moderate levels of RANKL messenger RNA in some osteoclasts, in particular in areas of bone resorption, although most osteoclastic cells did not express RANKL messenger RNA. Immunohistochemical analysis performed by these authors revealed a similar localization pattern of RANKL indicating that some of the osteoclast RANKL might have been synthesize by themselves and was acting back in an auto- and/or paracrine fashion. However, as most of the RANKL+ cells clearly belonged to mesenchymal lineage and probably most of the RANKL driving osteoclasts were in our study derived from them. Lynch *et al.* (11) suggested that MMP-7, an enzyme known to directly degrade the extracellular disc matrix, cleaves RANKL from the osteoblast cell surface. This cleavage releases a soluble active form of RANKL that could activate the distant macrophages and osteoclasts.

The second aim of the study was to check if a local discus injury is reflected in RANKL expression at location remote from the injury site. To maintain strength, the vertebrate skeleton needs to adapt to the prevailing mechanical needs of the organism. The effector cells for this bone remodelling process are the osteoclasts. How cells are precisely targeted to where resorption is needed is poorly understood. It however seems that starting point could be mechanical forces stress. The osteogenic effects of

loading and of load engendered strains have been evident for some time (28, 29). Bone morphology adapts to suit functional loading patterns by responding to the size and distribution of strains that loading engenders in the bone tissue (30-32). This involves local modification of the rates of bone formation and resorption (33-35). We have recently shown that osteoblast stretching *in vitro* regulates their orientation and matrix metalloproteinase synthesis (36). Our present observation shows that such bone remodelling also occurs in experimental lumbar intervertebral disc degeneration and shows an active RANKL-driven role for osteoclasts in this process. This effect was seen also at sites remote from the local endplate and disc injury. As in metabolic bone diseases, the effects of mechanical derangements caused by and/or associated with intervertebral disc degeneration were most prominent in trabecular spongy rather than cortical compact bone. RANKL-positive osteoclasts were clearly more frequent on the surface of medullary bone trabeculae than below the periosteum on bone surface. To us this induction of RANKL remote from the local site of injury suggests that such local lesions have secondary effects not only locally, but on the whole spine.

Although full-blown degenerative changes are seen in the disc and paradiscal structures in this model, this does not imply that the observed tissue changes are equal to changes that develop during slowly developing disc degeneration disease. Pathogenic pathway leading to human degenerative disc disease is certainly different from the one activated in the experimental model described in this study. In disc degeneration endplate permeability is impaired whereas in the present study a perforation of the endplate ensues. Changes in the endplate in our experiment follow as a result of two consecutive and partially overlapping causes: primarily, acute aseptic posttraumatic healing changes and secondarily, the adaptive functional unspecific fibrosis which replaced damaged intervertebral disc as a sign of healing by scarring. This fibrotic tissue response resembles the process seen in naturally slowly developing intervertebral disc degeneration. These differences form one of the possible limitations of this experimental model.

In conclusion, in response to disc damage and degeneration, RANKL system seems to be activated as was shown by an increased RANKL expression in diseased compared to control samples (a summary figure of the process is presented as Figure 4). RANKL was apparently produced by osteoblasts, fibroblasts and mesenchymal bone marrow cells but also by fibroblasts replacing annulocytes of the degenerated fibrotic discs. Part of it was apparently produced in soluble form and/or was solubilized from its membrane-bound form and had been bound by some macrophage-like cells and osteoclasts probably driving their differentiation, survival and activity. The effects of the activated RANKL system were much more evident in the fast metabolized and rapidly adaptable spongy trabecular rather than the cortical compact bone. This work demonstrates that disc degeneration and fibrosis also involves peridiscal bone which adapts to altered loading pattern and biomechanics by active remodelling both in injured and contiguous vertebrae.

References

1. KITAZAWA S, KAJIMOTO K, KONDO T, KITAZAWA R: Vitamin D3 supports osteoclastogenesis via functional vitamin D response element of human RANKL gene promoter. *J Cell Biochem* 2003; 89: 771-7.
2. SUDA T, TAKAHASHI N, MARTIN TJ: Modulation of osteoclast differentiation. *Endocr Rev* 1992; 13: 66-80.
3. BURGESS TL, QIAN Y, KAUFMAN S *et al*.: The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts. *J Cell Biol* 1999; 145: 527-38.
4. KONG YY, YOSHIDA H, SAROSI I *et al*.: OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* 1999; 397: 315-23.
5. HSU H, LACEY DL, DUNSTAN CR *et al*.: Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proc Natl Acad Sci USA* 1999; 96: 3540-5.
6. LACEY DL, TIMMS E, TAN HL *et al*.: Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 1998; 93: 165-76.
7. LIU XH, KIRSCHENBAUM A, YAO S, LEVINE AC: Interactive effect of interleukine-6 and prostaglandin E2 on osteoclastogenesis via the OPG/RANKL/RANK system. *Ann N Y Acad Sci* 2006; 1068: 225-33.
8. MYERS DE, COLLIER FM, MINKIN C *et al*.: Expression of functional RANK on the mature rat and human osteoclasts. *FEBS Lett* 1999; 463: 295-300.
9. HOFBAUER LC, HEUFELDER A: Role of receptor activator of nuclear factor-kappaB ligand and osteoprotegerin in bone cell biology. *J Mol Med* 2001; 79: 243-53.
10. YASUDA H, SHIMA N, NAKAGAWA N *et al*.: Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci USA* 1998; 95: 3597-602.
11. LYNCH CC, HIKOSAKA A, ACUFF HB *et al*.: MMP-7 promotes prostate cancer-induced osteolysis via the solubilization of RANKL. *Cancer Cell* 2005; 7: 485-96.
12. SALO J, LEHENKARI P, MULARI M, METSIKKO K, VAANANEN HK: Removal of osteoclast bone resorption products by transcytosis. *Science* 1997; 276: 270-3.
13. KOSHIHARA Y, SUEMATSU A, FENG D, OKAWARA R, ISHIBASHI H, YAMAMOTO S: Osteoclastogenetic potential of bone marrow cells increase with age in elderly women with fracture. *Mech Ageing Dev* 2002; 123: 1321-31.
14. KRANENBARG S, VAN CLEYNENBREUGEL T, SCHIPPER H, VAN LEEUWEN J: Adaptive bone formation in acellular vertebrae of sea bass (Dicentrarchus labrax L). *J Exp Biol* 2005; 208: 3493-502.
15. HOLM S, HOLM AK, EKSTROM L, KARLADANI A, HANSSON T: Experimental disc degeneration due to endplate injury. *J Spinal Disord Tech* 2004; 17: 64-71.
16. KONTTINEN YT, GRÖNBLAD M, ANTTIPOIKA I *et al*.: Neuroimmunohistochemical analysis of peridiscal nociceptive neural elements. *Spine* 1990; 15: 383-6.
17. LAM J, NELSON CA, ROSS FP, TEITELBAUM SL, FREMONT DH: Crystal structure of the TRANCE/RANKL cytokine reveals determinants of receptor-ligand specificity. *J Clin Invest* 2001; 108: 971-9.
18. LUM L, WONG BR, JOSIEN R *et al*.: Evidence for a role of a tumor necrosis factor-alpha (TNF-alpha)- converting enzyme-like protease in shedding of TRANCE, a TNF family member involved in osteoclastogenesis and dendritic cell survival. *J Biol Chem* 1999; 274: 13613-8.
19. MIYAMOTO N, HIGUCHI Y, MORI K *et al*.: Human osteosarcoma-derived cell lines produce soluble factor(s) that induces differentiation of blood monocytes to osteoclast-like cells. *Int Immunopharmacol* 2002; 2: 25-38.
20. NAGAI M, KYAKUMOTO S, SATO N: Cancer cells responsible for humoral hypercalcemia express mRNA encoding a secreted form of ODF/TRANCE that induces osteoclast formation. *Biochem Biophys Res Commun* 2002; 269: 532-6.
21. JIMI E, AKIYAMA S, TSURUKAI T *et al*.: Osteoclast differentiation factor acts as a multifunctional regulator in murine osteoclast differentiation and function. *J Immunol* 1999; 163: 434-42.

22. KARTSOGIANNIS V, ZHOU H, HORWOOD NJ *et al.*: Loalization of RANKL (receptor activator of NFκB ligand) mRNA and protein in skeletal and extraskeletal tissues. *Bone* 1999; 25: 525-34.
23. SHALHOUB V, FAUST J, BOYLE WJ *et al.*: Osteoprotegerin and osteoprotegerin ligand effects on osteoclast formation from human peripheral blood mononuclear cell precursors. *J Cell Biochem* 1999; 72: 251-61.
24. UDAGAWA N, TAKAHASHI N, JIMI E *et al.*: Osteoblasts/stromal cells stimulate osteoclast activation through expression of osteoclast differentiation factor/RANKL but not macrophage colony-stimulating factor: receptor activator of NF-kappaB ligand. *Bone* 1999; 25: 517-23.
25 IKEDA I, KASAI M, UTSUYAMA M, HIROKAWA: Determination of three isoforms of the receptor activator of nuclear factor-kappaB ligand and their differential expression in bone and thymus. *Endocrinology* 2001; 142: 1419-26.
26. KWAN TAT S, PELLETIER JP, LAJEUNESSE D, FAHMI H, LAVIGNE M, MARTEL-PELLETIER J: The differential expression of osteoprotegerin (OPG) and receptor activator of nuclear factor kappaB ligand (RANKL) in human osteoarthritic subchondral bone osteoblasts is an indicator of the metabolic state of these disease cells. *Clin Exp Rheumatol* 2008; 26: 295-304.
27. MANDELIN J, LI TF, LILJESTROM M *et al.*: Imbalance of RANKL/RANK/OPG system in interface tissue in loosening of total hip replacement. *J Bone Joint Surg Br* 2003; 85:1196-201.
28. BURGER EH, KLEIN-NULEND J: Mechanotransduction in bone-role of the lacuno-canalicular network. *FASEB J* 1999; 13 (Suppl.): S101-S112.
29. GROSS TS, EDWARDS JL, MCLEOD KJ, RUBIN CT: Strain gradients correlate with sites of periosteal bone formation. *J Bone Miner Res* 1997; 12: 982-8.
30. FROST HM: From Wolff's law to the mechanostat: a new "face" of physiology. *J Orthop Sci* 1998; 3: 282-6.
31. LANYON LE: Functional strain in bone tissue as an objective, and controlling stimulus for adaptive bone remodelling. *J Biomech* 1987; 20: 1083-93.
32. LANYON, LE: Control of bone architecture by functional load bearing. *J Bone Miner Res* 1992; 7 (Suppl. 2): S369-S375
33. BRONCKERS AL, GOEI W, LUO G *et al.*: DNA fragmentation during bone formation in neonatal rodents assessed by transferase-mediated end labeling. *J Bone Miner Res* 1996; 11: 1281-91.
34. NOBLE BS, PEET N, STEVENS HY *et al.*: Mechanical loading: biphasic osteocyte survival and targeting of osteoclasts for bone destruction in rat cortical bone. *Am J Physiol Cell Physiol* 2003; 284: C934-C943.
35. NOBLE BS, STEVENS H, LOVERIDGE N, REEVE J: Identification of apoptotic changes in osteocytes in normal and pathological human bone. *Bone* 1997; 20: 273-82.
36. SASAKI K, TAKAGI M, KONTTINEN YT *et al.*: Upregulation of matrix metalloproteinase (MMP)-1 and its activator MMP-3 of human osteoblast by uniaxial cyclic stimulation. *J Biomed Mater Res B Appl Biomater* 2007; 80: 491-8.