RANK (Receptor Activator of Nuclear Factor kappa B) and RANK Ligand Are Expressed in Giant Cell Tumors of Bone

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Abstract

In giant cell tumors of bone (GCTBs), the mesenchymal stromal cells are the neoplastic cells and induce recruitment and formation of osteoclasts (OCs). Studies on recently discovered members of the tumor necrosis factor receptor-ligand family have demonstrated a crucial role of RANKL (receptor activator of nuclear factor kappa B [RANK] ligand) expressed by osteoblast/stromal cells and of its receptor RANK expressed by OCs during OC differentiation and activation. OCs typically are present in large numbers in GCTBs, suggesting that these tumors may contain cells expressing factors that stimulate OC precursor recruitment and differentiation. We used immunohistochemical analysis to study RANKL and RANK expression in 5 GCTBs. Multinucleated cells and some mononuclear cells showed strong positive staining with anti-RANK antibodies; RANKL was present in a subset of mononuclear cells that did not express the hematopoietic lineage cell marker CD45, a feature that identified them as mesenchymal tumor cells. Our results suggest that RANKL expression may have a role in the pathogenesis of GCTBs and in the formation of the large OC population present in these tumors.

Bone cells responsible for bone remodeling include osteoclasts (OCs), which are bone-resorbing cells derived from hematopoietic cells of the monocyte-macrophage lineage, and osteoblasts, which are bone-forming cells of mesenchymal origin. Mesenchyme-derived stromal cells have a critical role in supporting and stimulating OC differentiation.[1] The discovery of new members of the tumor necrosis factor (TNF) receptor-ligand family has shown that RANK (receptor activator of nuclear factor kappa B) and its ligand RANKL have a crucial role in OC differentiation and activation.[2,3] RANKL is a membrane-bound TNF-related factor expressed by osteoblast/stromal cells. OC precursors and mature OCs express RANK, a membrane-bound TNF receptor that recognizes RANKL through direct cell-to-cell interaction with osteoblast/stromal cells.[4] Finally, osteoprotegerin (OPG), a secreted decoy receptor that belongs to the TNF receptor family and lacks the transmembrane domain of RANK, recognizes RANKL, thereby blocking the RANK-RANKL interaction and inhibiting OC differentiation and activation.[5]

Human giant cell tumor of bone (GCTB) is an uncommon osteolytic primary bone neoplasm occurring in young adults and characterized by the presence of numerous OCs. The critical role of the RANKL-RANK system in OC differentiation prompted us to hypothesize that this system may be involved in producing the large OC population typical of GCTBs. This has been suggested in previous studies showing RANKL and RANK messenger RNA (mRNA) expression in GCTB using reverse transcription–polymerase chain reaction and in situ hybridization.[6,7] RANKL also was detected by immunofluorescence staining in cultured cells from GCTBs.[8] Moreover, in in vitro experiments, addition of exogenous OPG to cultured

GCTB inhibited bone resorption and osteoclast formation.[8] The aim of our study was to evaluate RANKL and RANK expression at a protein level and in situ within the tumor. We used immunohistochemical analysis to evaluate this expression in hematopoietic and mesenchymal cells from GCTBs.

Materials and Methods

Giant Cell Tumors of Bone

Tissue specimens from 5 GCTBs were obtained from surgical biopsies of lytic bone lesions. Samples of these tumors were fixed in 4% paraformaldehyde or in Bouin solution and embedded in paraffin for histologic study.

Cell Transfection Studies

Cos7 cells were transiently transfected with a plasmid vector expressing either human RANKL or human RANK complementary DNA (cDNA; provided by AMGEN, Thousand Oaks, CA),[3] using cationic lipid reagents (Lipofectamine, Life Technologies, Gaithersburg, MD). Plasmid DNA (0.1 µg/µL) in serum-free medium (SFM; Opti-MEM, Life Technologies) and lipofectamine solution (6% in SFM) were mixed together and incubated at room temperature for 30 minutes. Cos7 cells cultured in plastic, 4-well chamber/slides (Lab-Tek, NUNC, Naperville, IL) were washed in SFM, and 500 µL of the transfection mixture (previously diluted 5-fold) were added to each well. After overnight incubation, the transfection mixture was removed and replaced with 10% fetal calf serum–supplemented medium. Forty-eight hours after transfection initiation, the cells were washed in phosphate-buffered saline (PBS), fixed in cold methanol, and stored at –80°C. Nontransfected Cos7 cells were used as a negative control for the immunocytochemical experiments.

Immunohistochemical Analysis

Immunohistochemical analysis was performed using a biotin-streptavidin-peroxidase kit (LSAB2 kit, DAKO, Carpinteria, CA) to stain paraffin-embedded tumor sections and transfected cultured cells. Tissue sections were deparaffinized in 100% xylene, then rehydrated by serial incubations in ethanol, followed by PBS. To detect RANK and RANKL, we used rabbit polyclonal antibodies (Chemicon, Temecula, CA). Anti-RANK antibody was directed against the intracellular domain of the RANK transmembrane protein and anti-RANKL antibody against the RANKL extracellular domain. Detection of CD45, a membrane antigen expressed by cells of the hematopoietic lineage, was by a mouse monoclonal antibody (DAKO). Tissue sections were pretreated by microwave heating in citrate buffer, pH 7.3, for 10 minutes. The specimens were incubated with the primary antibody diluted 1:800 or with the same concentration of normal rabbit serum (DAKO) overnight at 4°C in a humid chamber. For immunocytochemical analysis performed on cultured cells, we used primary antibody diluted 1:3,200. In some experiments, primary antibody was omitted. Specimens were treated with 3% hydrogen peroxide in PBS for 5 minutes to inactivate endogenous peroxidase activity. Sequential incubations then were performed with biotinylated secondary antibody and peroxidase-labeled streptavidin (DAKO). Staining was revealed with AEC chromogen (DAKO). The slides were counterstained and mounted in Glycergel (DAKO).

For immunofluorescent double-labeling, we used a goat antirabbit antibody labeled with the fluorescent marker Alexa 488 (Interchim, Montluçon, France) and a goat antimouse antibody labeled with the fluorescent marker Alexa 546 (Interchim).

Results

Histologic Features of Human GCTB Tissue Sections

Cellular density was high, with many prominent multinucleated cells (MNCs). The mononuclear cell component typically consisted of spindle-shaped fibroblast-like cells admixed with smaller round macrophage-like cells. In 2 samples, trabecular bone was seen adjacent to the tumor.

Specificity of RANK and RANKL Immunocytochemical Detection

As a positive control for the anti-RANK and anti-RANKL antibodies, we used Cos7 cells transiently transfected with a vector expressing either human RANK or human RANKL cDNA. Of the RANK-transfected cells, 14% were positive with the anti-RANK stain **Image 1A**, and 15% of the RANKL-transfected cells were positive with the anti-RANKL stain **Image 1B**. Wild-type cells did not stain with either anti-RANK or anti-RANKL antibodies **Image 1C**. None of the cells stained in experiments omitting primary antibodies or conducted with normal rabbit serum instead of the primary antibody.

In some biopsy specimens, normal trabecular bone was present adjacent to the tumor. Multinucleated cells that were near the bone surface and corresponded to OCs were strongly stained by anti-RANK antibody **Image 1D** but not by anti-RANKL antibody. Anti-RANKL antibody stained a subset of mononuclear cells that were either in the stroma or located near the bone surface and belonged to the osteoblast lineage **Image 1E**. No cells were stained in experiments

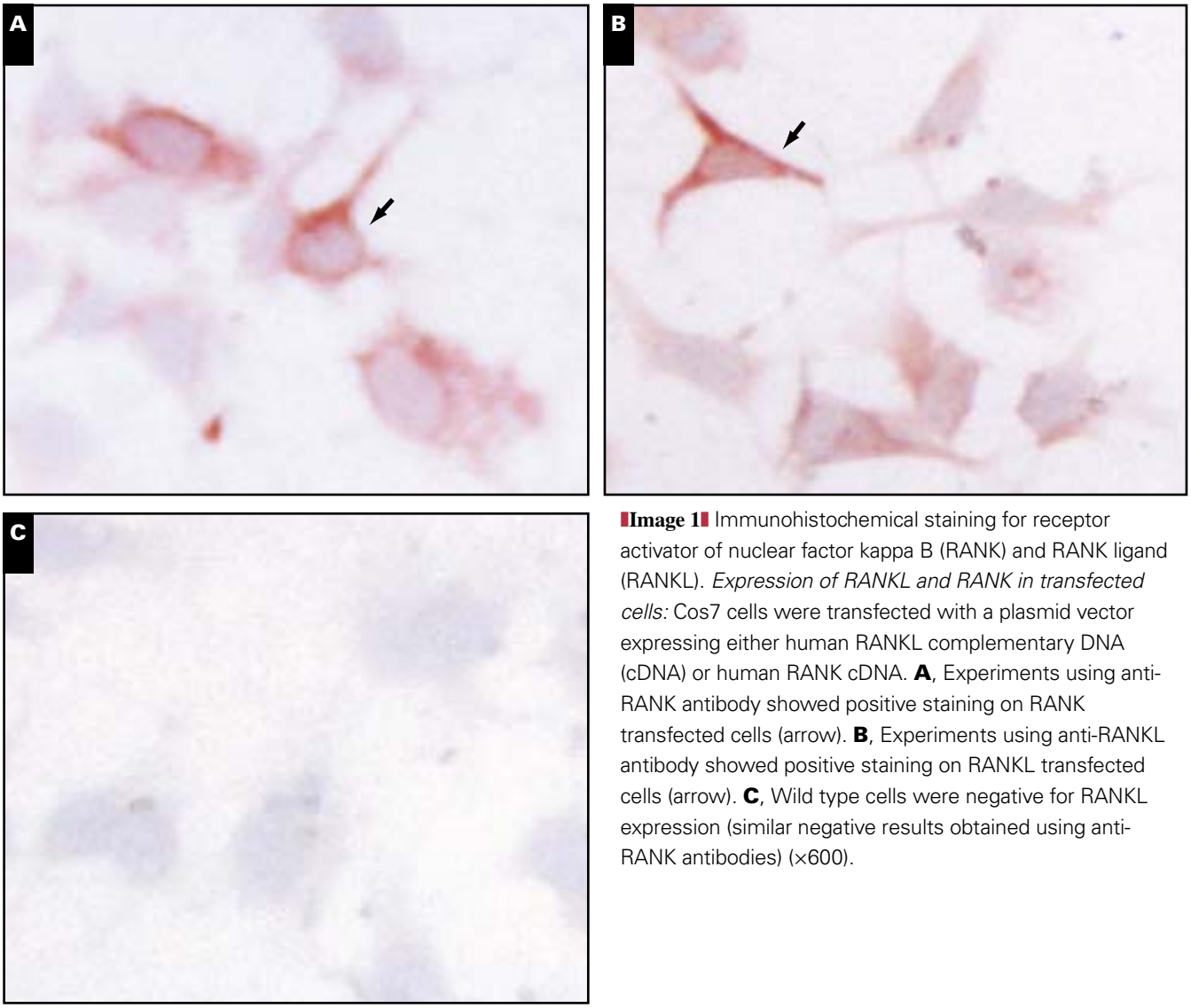

Image 1 Immunohistochemical staining for receptor activator of nuclear factor kappa B (RANK) and RANK ligand (RANKL). *Expression of RANKL and RANK in transfected cells:* Cos7 cells were transfected with a plasmid vector expressing either human RANKL complementary DNA (cDNA) or human RANK cDNA. **A**, Experiments using anti-RANK antibody showed positive staining on RANK transfected cells (arrow). **B**, Experiments using anti-RANKL antibody showed positive staining on RANKL transfected cells (arrow). **C**, Wild type cells were negative for RANKL expression (similar negative results obtained using anti-RANK antibodies) (×600).

omitting primary antibody or conducted with normal rabbit serum instead of the primary antibody **Image 1F**.

Expression of RANK and RANKL in GCTBs

All MNCs and many mononuclear cells produced a diffuse stain of variable intensity when anti-RANK antibody was applied to GCTB sections **Image 1G**. No cells were stained in the negative control experiments conducted with no primary antibody or with normal rabbit serum instead of the primary antibody **Image 1H**.

RANKL expression was detected in the mononuclear cells of the GCTBs. Among the MNCs, most were not stained, and fewer than 5% were weakly stained **Image 1I**. The staining pattern was diffuse, often with a more intense rim. To identify the RANKL-expressing cells, we performed double labeling using an indirect immunofluorescence method. The MNCs stained positive with anti-CD45 antibody, as did many of the mononuclear cells, confirming that the MNCs were derived from hematopoietic cells **Image 1J**. However, cells that were RANKL+ did not stain with anti-CD45 **Image 1K** and **Image 1L**, indicating that they were not of hematopoietic lineage. Thus, RANKL+ cells probably represent the mesenchymal component of GCTBs.

Discussion

GCTBs typically contain large numbers of MNCs, in addition to many mesenchyme-derived and macrophage-like cells. These MNCs are highly active OCs: they exhibit the characteristic markers of the OC phenotype, such as tartrate-resistant acid phosphatase, vitronectin receptor, and calcitonin receptor; and they can resorb bone.[9,10] The neoplastic component of GCTBs consists of mesenchymal cells and is

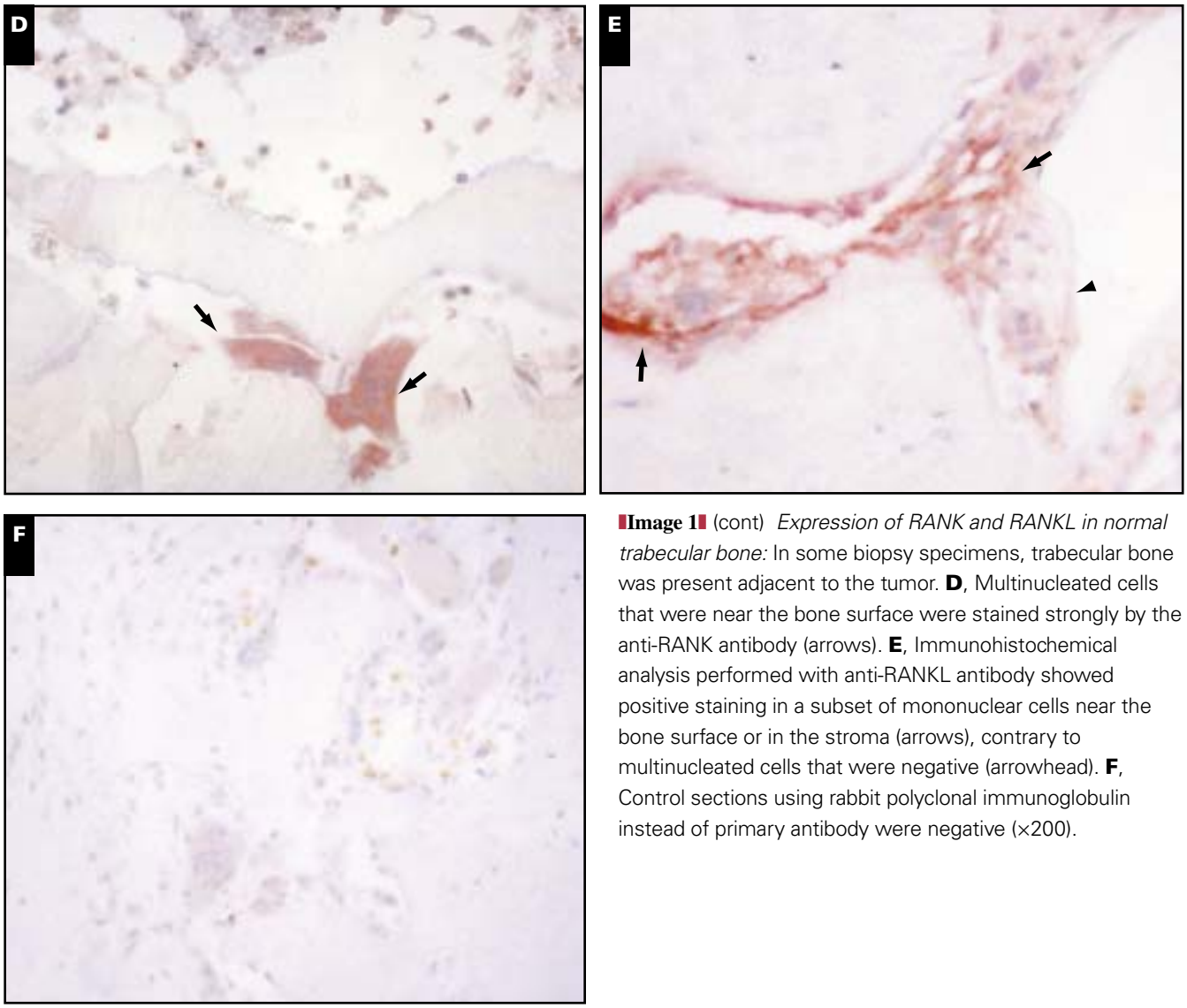

Image 1 (cont) *Expression of RANK and RANKL in normal trabecular bone:* In some biopsy specimens, trabecular bone was present adjacent to the tumor. **D**, Multinucleated cells that were near the bone surface were stained strongly by the anti-RANK antibody (arrows). **E**, Immunohistochemical analysis performed with anti-RANKL antibody showed positive staining in a subset of mononuclear cells near the bone surface or in the stroma (arrows), contrary to multinucleated cells that were negative (arrowhead). **F**, Control sections using rabbit polyclonal immunoglobulin instead of primary antibody were negative (×200).

responsible for the formation of numerous OCs. This large OC population indicates that OC differentiation occurs at an abnormally high rate in these tumors, and Roux et al[9] have previously shown that GCTBs release soluble factors that induce recruitment and differentiation of OC precursors.

In the present work, we used immunohistochemical analysis to evaluate RANK and RANKL expression. In normal trabecular bone adjacent to the tumor, we found that OCs were strongly stained with anti-RANK antibodies and that mononuclear cells along the endosteal surface were stained with anti-RANKL antibodies. These results are in keeping with previous evidence that OCs express RANK and that osteoblast/stromal cells express RANKL.[2] They provided evidence that labeling was specific with the anti-RANK and anti-RANKL antibodies used to stain our tissue sections. In GCTBs, we observed that MNCs expressed RANK throughout these tumors, a feature they shared with many mononuclear cells. This is in accordance with the OC phenotype of MNCs in GCTBs. RANKL, in contrast, was expressed by many mononuclear cells but not by MNCs as a rule, although a few MNCs stained with anti-RANKL antibody.

The mesenchymal stromal cells of GCTBs exhibit the phenotype of undifferentiated mesenchymal cells rather than of osteoblasts.[11] No marker capable of detecting these cells in situ is available. Thus, to identify cells that expressed RANKL, we used double labeling with anti-RANKL and anti-CD45 antibodies. Anti-CD45 antibodies are specific for cells of hematopoietic lineage. The mononuclear cells that expressed RANKL were CD45–. Given the cellular composition of GCTBs, these results indicate that RANKL+ cells were not of hematopoietic origin and represented the mesenchymal neoplastic component.

RANKL has been described as a membrane-bound protein. However, a soluble form of RANKL has been

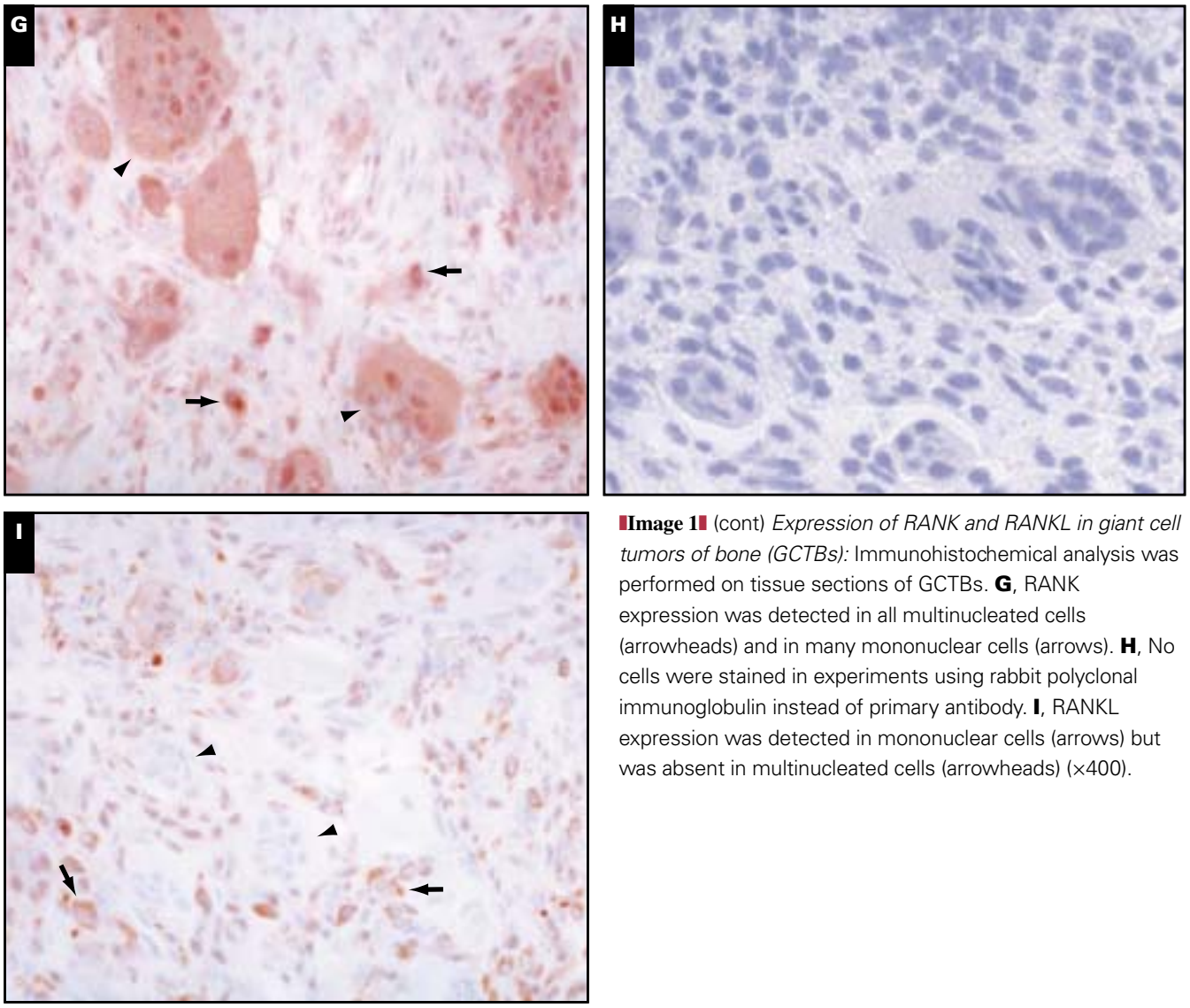

Image 1 (cont) *Expression of RANK and RANKL in giant cell tumors of bone (GCTBs):* Immunohistochemical analysis was performed on tissue sections of GCTBs. **G**, RANK expression was detected in all multinucleated cells (arrowheads) and in many mononuclear cells (arrows). **H**, No cells were stained in experiments using rabbit polyclonal immunoglobulin instead of primary antibody. **I**, RANKL expression was detected in mononuclear cells (arrows) but was absent in multinucleated cells (arrowheads) (×400).

shown to be produced by human fibroblasts transfected with an expression vector for RANKL and by activated murine T cells in vitro.[3,12] It is unclear whether this soluble form has a role in vivo in normal bone homeostasis or in abnormal processes characterized by increased bone resorption. Nevertheless, 2 surprising results suggest that a soluble form of RANKL may be produced in GCTBs. First, Roux et al[9] previously showed that a soluble factor produced in cultured GCTB-conditioned medium induced OC differentiation of cord blood monocytes. Second, in the present study, we observed that a small minority of OCs were positive for RANKL, a finding that is consistent with findings in an earlier study[13] and that may reflect the presence of RANKL bound to its receptor on these OCs.

In previous studies, RANKL and RANK mRNA were detected in GCTBs using reverse transcription–polymerase chain reaction and in situ hybridization.[6,7] These highly sensitive techniques do not permit definitive conclusions about the levels of RANK and RANKL protein production in GCTBs and, therefore, fail to shed light on the potential role of RANK and RANKL in the pathogenesis of these tumors. In addition, RANKL also has been detected by immunofluorescence in cultured cells derived from GCTBs.[8] However, RANKL expression in cultured stromal GCTB cells may not reflect RANKL expression in situ, as suggested by the previous finding of phenotypic instability of these cells in culture.[9]

We found immunohistochemical evidence that RANK is expressed by OC-like cells and RANKL by mononuclear cells forming the mesenchymal component of GCTBs. These results are consistent with involvement of RANKL and RANK expression in the pathogenesis of GCTBs. Overexpression of RANKL by the tumor stromal cells may be responsible for the formation of numerous OCs and for the

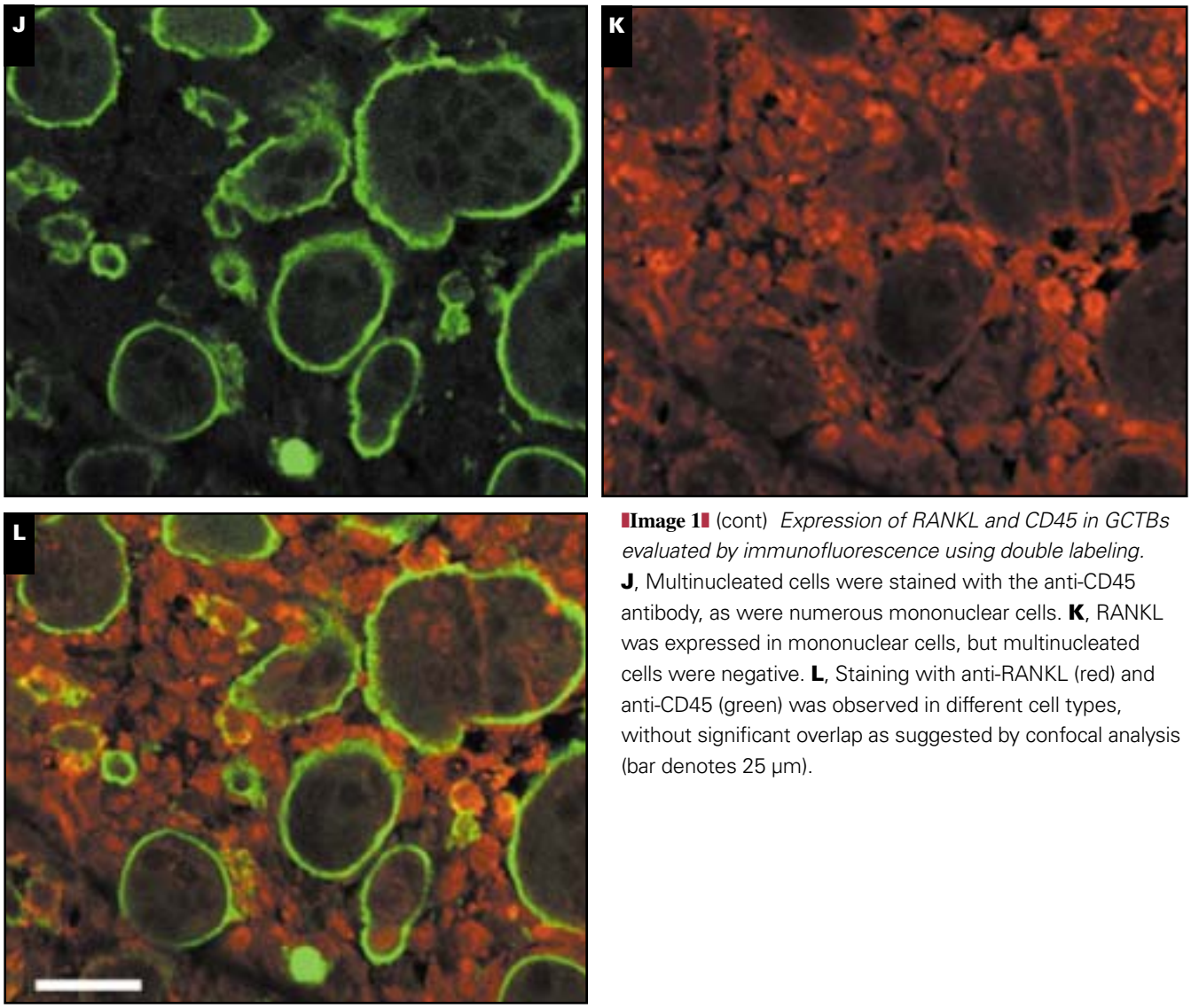

Image 1 (cont) *Expression of RANKL and CD45 in GCTBs evaluated by immunofluorescence using double labeling.* **J**, Multinucleated cells were stained with the anti-CD45 antibody, as were numerous mononuclear cells. **K**, RANKL was expressed in mononuclear cells, but multinucleated cells were negative. **L**, Staining with anti-RANKL (red) and anti-CD45 (green) was observed in different cell types, without significant overlap as suggested by confocal analysis (bar denotes 25 μm).

osteolytic behavior of these cells in GCTBs. This overexpression may be constitutive or induced by other factors present in GCTBs. Numerous cytokines are expressed in GCTBs,[9] and bone resorbing factors such as interleukin-6 may be involved in both the OC formation and the bone lysis seen in GCTBs.[14] These osteotropic factors may act on bone resorption via a common final pathway involving increased production of RANKL,[2] and they may contribute to increase RANKL expression in GCTBs. Thus, our findings may have clinical implications: the specific RANKL inhibitor OPG might prove useful for inhibiting the bone resorption that is a major source of morbidity in patients with GCTB.

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References

1. Takahashi N, Akatsu T, Udagawa N, et al. Osteoblastic cells are involved in osteoclast formation. *Endocrinology*. 1988;123:2600-2602.
2. Hofbauer LC, Khosla S, Dunstan CR, et al. The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption. *J Bone Miner Res*. 2000;15:2-12.

3. Lacey DL, Timms E, Tan HL, et al. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell*. 1998;93:165-176.
4. Nakagawa N, Kinosaki M, Yamaguchi K, et al. RANK is the essential signaling receptor for osteoclast differentiation factor in osteoclastogenesis. *Biochem Biophys Res Commun*. 1998;253:395-400.
5. Simonet WS, Lacey DL, Dunstan CR, et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell*. 1997;89:309-319.
6. Atkins GJ, Haynes DR, Graves SE, et al. Expression of osteoclast differentiation signals by stromal elements of giant cell tumors. *J Bone Miner Res*. 2000;15:640-649.
7. Huang L, Xu J, Wood DJ, et al. Gene expression of osteoprotegerin ligand, osteoprotegerin, and receptor activator of NF-kappa B in giant cell tumor of bone: possible involvement in tumor cell–induced osteoclast-like cell formation. *Am J Pathol*. 2000;156:761-767.
8. Atkins GJ, Bouralexis S, Haynes DR, et al. Osteoprotegerin inhibits osteoclast formation and bone resorbing activity in giant cell tumors of bone. *Bone*. 2001;28:370-377.
9. Roux S, Quinn J, Pichaud F, et al. Human cord blood monocytes undergo terminal osteoclast differentiation in vitro in the presence of culture medium conditioned by giant cell tumor of bone. *J Cell Physiol*. 1996;168:489-498.
10. Goldring SR, Roelke MS, Petrison KK, et al. Human giant cell tumors of bone: identification and characterization of cell types. *J Clin Invest*. 1987;79:483-491.
11. Oreffo RO, Marshall GJ, Kirchen M, et al. Characterization of a cell line derived from a human giant cell tumor that stimulates osteoclastic bone resorption. *Clin Orthop*. 1993;229-241.
12. Kong YY, Feige U, Sarosi I, et al. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature*. 1999;402:304-309.
13. Kartsogiannis V, Zhou H, Horwood NJ, et al. Localization of RANKL (receptor activator of NF kappa B ligand) mRNA and protein in skeletal and extraskeletal tissues. *Bone*. 1999;25:525-534.
14. Ohsaki Y, Takahashi S, Scarcez T, et al. Evidence for an autocrine/paracrine role for interleukin-6 in bone resorption by giant cells from giant cell tumors of bone. *Endocrinology*. 1992;131:2229-2234.

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