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Cellular Localization of RANKL and RANK Production in Giant Cell Tumors of Bone and Tendon Sheath by Immunohistochemistry

Ha Rin Cheong · Hanna Kang · Dong Eun Song · Min-Sun Cho

Department of Pathology, Ewha Womans University College of Medicine

= 국문초록 =

골 및 건초 거대세포종에서 RANKL 및 RANK 발현 세포에 대한 면역조직화학적 연구

이화여자대학교 이화의학전문대학원 병리학교실

정하린 · 강한나 · 송동은 · 조민선

목 적 : Receptor activator of NF-κB ligand(RANKL)과 수용체인 receptor activator of NF-κB (RANK)에 작용하여 파골세포형성을 유발하는 싸이토카인이다. RANKL/RANK/osteoprotegerin 체계는 뼈의 정상적인 파골세포 형성 과정뿐만 아니라 종양과 같은 병적 상황에서도 파골양 거대세포 형성에 관여한다. 그러나 골 거대세포종양에서 이들 싸이토카인을 생산하는 세포에는 이견이 있어서 파골양 거대세포가 분비한 RANKL이 autocrine 기전으로 작용한다는 연구결과가 있는 반면, 주변 기질 단핵세포가 분비한 RANKL이 파골양 거대세포를 형성을 유발한다는 보고가 있다. 이에 따라 파골양 거대세포를 많이 함유하는 골 거대세포종양 및 건초 거대세포종양에서 RANKL 및 RANK의 생산 세포를 규명하고자 하였다.

방 법 : 골거대세포종양 5예 및 건초 거대세포종양 8예의 포르말린 고정 파라핀 포매조직을 이용하여 RANKL, RANK, osteonectin, TRAP 및 CD68에 대한 면역조직화학염색을 시행하였다.

결 과 : 두 종양 모두에서 RANK는 기질단핵세포 및 파골양 거대세포양 거대세포가 발현하였다. RANKL은 기질단핵세포만이 발현하였는데 골거대세포종양에서는 거의 모든 단핵세포가 발현한 반면 건초 거대세포종양에서는 일부 단핵세포가 발현하였다. 이들 세포는 osteonectin에도 양성반응, TRAP 및 CD68에는 음성반응을 보였다. 파골양 거대세포는 RANKL을 발현하지 않았다.

결 론 : 골거대세포종양 및 건초 거대세포종양의 기질 단핵세포가 RANKL을 발현하여 paracrine기전으로 파골양 거대세포 형성을 유도함을 알 수 있었다.

중심 단어 : 골거대세종양 · 건초수막염 · 파골세포 · Receptor activator of nuclear factor-kappa B · RANK Ligand.

Address for correspondence: Min-Sun Cho, MD
Department of Pathology, Ewha Womans University School of Medicine, 911-1 Mok-dong Yangcheon-gu, Seoul 158-710, Korea
Tel : (02) 2650-5822 · 전송 : (02) 2650-2879 · E-mail : mcho1124@ewha.ac.kr

Introduction

Osteoclasts are multinucleated giant cells (GCs) that are responsible for bone resorption and participates in the constant process of bone remodeling in conjunction with osteoblasts. The osteoclasts are generated from the monocyte/macrophage hematopoietic precursor cells, which commit to either the osteoclast or the monocyte-macrophage lineage, depending upon the stimuli received from the immediate environment[1)2)]. A receptor activator of NF-κB ligand (RANKL) interacts via its receptor called the receptor activator of NF-κB (RANK) to activate osteoclastogenesis from its precursors, and increases the activity of mature osteoclasts and inhibits the apoptosis of osteoclasts[3)4)]. Osteoprotegerin (OPG) is also called osteoclastogenesis inhibitory factor, and it operates as a decoy receptor by blocking RANKL from binding to its cellular receptor RANK. OPG has been shown to neutralize and interrupt stromal cell-derived RANKL signals, resulting in reduced osteoclastogenesis[5)6)]. It is known that the RANKL/RANK/OPG system is a major regulator of osteoclastogenesis[1)7)].

Many ostotic and nonostotic disorders including benign and malignant tumors contain osteoclasts or osteoclast-like GCs as reactive components. Giant cell tumor of bone (GCTB), a so-called osteoclastoma, is a typical tumor that contains a large number of osteoclasts. It also contains mononuclear stromal cells (MSCs) as a neoplastic component. Many studies have shown that the RANKL/RANK/OPG system is involved the generation of osteoclasts from monocyte/macrophage precursors in GCTB. GCTB exhibits increased RANKL production but unchanged or decreased OPG, which explains the abundant osteoclastogenesis[8-11)]. However, studies for *in situ* localization to define the RANKL-producing cells using mRNA *in situ* hybridization (ISH) or immunohistochemistry with cultured MSCs and/or paraffin-embedded tissue have produced contradictory results. Some investigators have demonstrated RANKL expression in MSCs[10)12)] whereas others found it in osteoclast-like GCs[8)13)]. RANKL expression at MSCs has been shown in other disorders involving osteoclast-like GCs-containing disorders, such as giant cell granulomas of the jaw[14)15)]. The expression of RANKL was localized in MSCs as well as in osteoclast-like GCs in pigmented villonodular synovitis showed that RANKL was expressed in[16)]. Therefore, there remains some controversy about which cells produce RANKL in lesions involving osteoclast-like GCs.

In this study, we used immunohistochemistry to evaluate RANKL and RANK in GCTB and giant cell tumor of tendon sheath (GCTTS), which also contains osteoclast-like GCs but exhibits different histogenetic and biological behavior compared with GCTB. The expression of osteonectin, CD68, and tartrate-resistant acid phosphatase (TRAP) was also evaluated. The purpose of this study was to clarify the RANKL-producing cells in GCTB and GCTTS in order to explain the mechanism underlying osteoclastogenesis and to contribute to the understanding of the mechanisms underlying the clinical behavior of these lesions.

Materials and Methods

Five cases of GCTB and eight cases of GCTTS that appeared between 1993 and 2004 from the Department of Pathology, University Hospital were selected. Four micron-thick sections were cut from formalin-fixed, paraffin-embedded tissue blocks and subjected to immunohistochemistry with primary antibodies for RANK (MAB 683, R&D Systems, Minneapolis, MN, USA), RANKL (MAB626, R&D Systems), Osteonectin (15G12, Novocastra™, Leica Microsystems, Newcastle, UK), TRAP (26E5, Leica Microsystems), CD68 (NCL-CD68-KP1, Leica Microsystems). Briefly, the slides were deparaffinized twice in xylene for 5minutes and rehydrated through a graded series of ethanol solutions. Antigen retrieval was done by heating the sections in a microwave oven 10mM sodium citrate buffer (pH 6.0) for RANK, osteonectin, TRAP and CD68 and 10mM Tris/1mM EDTA (pH 9.0) for RANKL. Immunohistochemical staining was then performed using an automated device (Bond™ Automated Immunohistochemistry, Vision Biosystems, Mount Waverley, VIC, Australia) and a bond polymer detection system with counterstaining (Vision Biosystems). The procedure included blocking the endogenous peroxidase with 3% hydrogen peroxide for 5minutes, incubation with primary antibodies for 15minutes at room temperature,

the use of polymeric horseradish-peroxidase-linker antibody conjugates as the secondary antibody and counterstaining with 3,3'-diaminobenzidine.

Results

1. Clinicopathologic findings

The age at the time of surgical resection ranged from 30 to 62years for the patients with GCTB and from 22 to 65 for those with GCTTS. All but one GCTB patient and all GCTTS patients were females. All GCTBs presented around the knee joint(tibia, two cases : femur, three cases), and all GCTTSs presented in the hand. GCTB comprised variable populations of MSCs and osteoclast-like GCs. The GCTTS comprised mixed populations of plump and spindle-shaped MSCs and multinucleated GCs(the foreign body type and the osteoclast-like type) with varying amounts of xanthomatous macrophages, siderophages and fibrosis.

2. Immunohistochemical analysis

RANK was expressed weakly in the cytoplasm of most MSCs and osteoclast-like GCs in both GCTB and GCTTS(Fig. 1). RANKL was expressed in the cytoplasm of most MSCs in GCTB(Fig 2A). In GCTTS, most of the MSCs were negative for RANKL, but a few(less than 5%) singly scattered plump MSCs that were strongly positive for RANKL were located in the cytoplasm of osteoclast-like GCs or close proximity to them(Fig. 2B). Osteoclast-like GCs in both GCTB and GCTTS did not express RANKL(Fig. 2). The RANKL-positive MSCs in both GCTB and GCTTS were also positive for osteonectin(Fig. 3A, D). Osteonectin was expressed weakly in the cytoplasm of most of the osteoclast-like GCs in both GCTB and GCTTS(Fig. 3A, D). About 30% of MSCs(including RANKL-positive ones) were also positive for osteonectin in GCTTS(Fig. 3D) In GCTB, most osteoclast-like GCs and 5-10% of MSCs were positive for TRAP and CD68(Fig 3B, C). In GCTTS, most os-

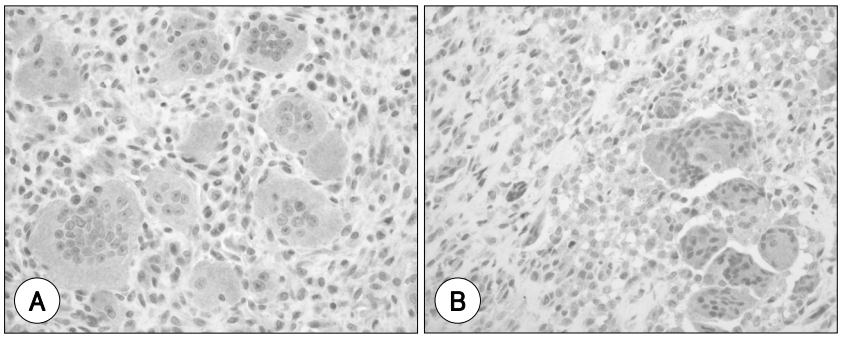

Fig. 1. Immunohistochemical stain for RANK shows weak expression at mononuclear stromal cells and osteoclast-like giant cells in giant cell tumor of bone(A) and tendon sheath(B).

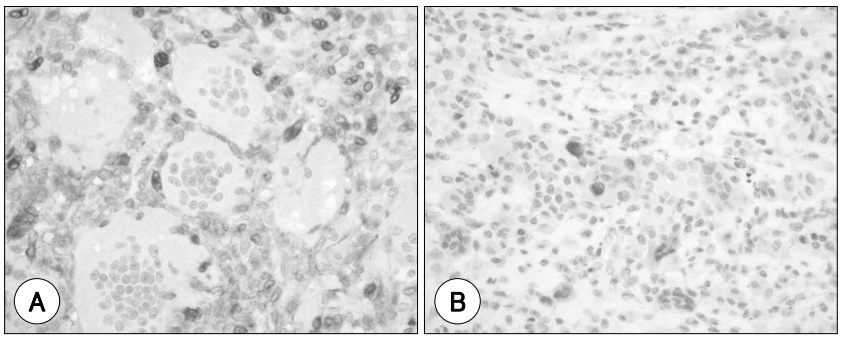

Fig. 2. RANKL is expressed in most mononuclear stromal cells in giant cell tumor of bone(A) and a few mononuclear stromal cells located in the cytoplasm of osteoclast-like GCs or adjacent to them in giant cell tumor of tendon sheath(B).

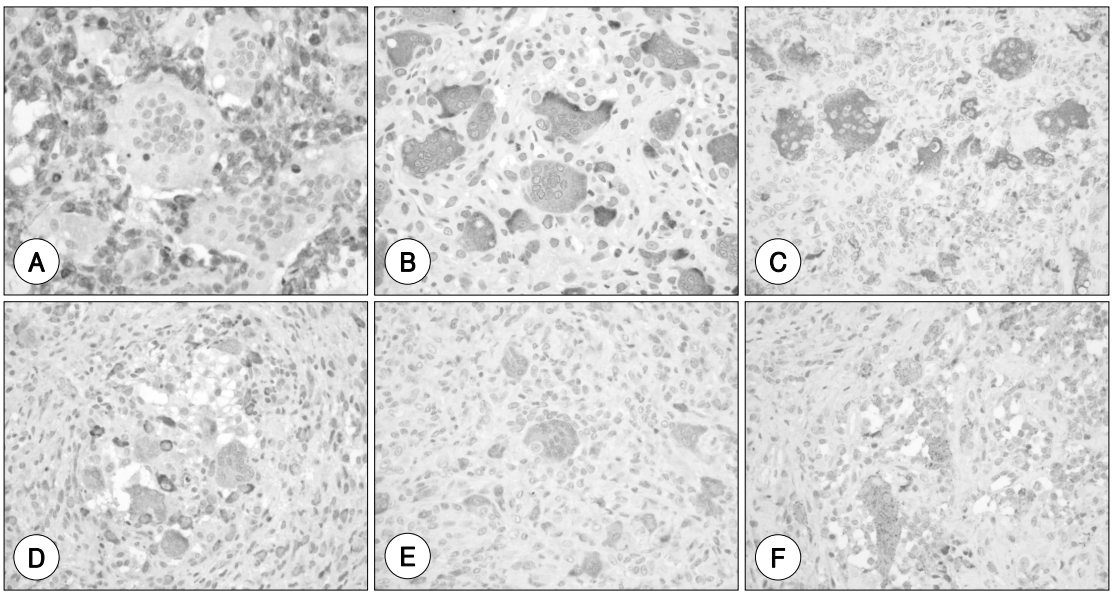

Fig. 3. Most of mononuclear stromal cells in giant cell tumor of bone express diffuse osteonectin positivity (A) but focal TRAP (B) and CD68 expression (C). About 30% of mononuclear stromal cells including the RANKL-positive cells in giant cell tumor of tendon sheath express osteonectin (D). TRAP and CD68 is expressed in osteoclast-like giant cells of giant cell tumor of bone (B, C) and tendon sheath (E, F).

teoclast-like GCs and less than 10% of MSCs were positive for TRAP (Fig. 3E). In GCTTS, more than 60% of MSCs, foreign-body-type GCs, xanthomatous macrophages, and siderophages were positive for CD68 in GCTTS (Fig. 3F).

Discussion

We have investigated the cellular localization of RANK and RANKL production in GCTB and GCTTS by performing immunohistochemistry with paraffin-embedded tissue blocks and commercially available antibodies. RANK was expressed in both multinucleated osteoclast-like GCs and MSCs., whereas RANKL (the osteoclastogenic cytokine) was expressed in MSCs throughout both lesions, but not in osteoclast-like GCs. These MSCs also expressed osteonectin suggesting a preosteoblastic lineage.

The deranged RANK/RANKL/OPG system in GCTB is responsible for the generation of large numbers of non-neoplastic osteoclast-like GCs as a reactive component. A GCTB produces a greater amount of RANKL than OPG resulting in high RANKL/OPG ratio that induces osteoclastogenesis from monocytic precursors[9-11]. The amount of RANKL mRNA is greater for GCTB than for normal bone and other types of bone tumor containing less osteoclast-like GCs, such as osteosarcoma and soft tissue sarcomas[10)11)]. However, studies have produced some discrepancies about the cellular source of this increased RANKL production. *In vitro* culture studies using separated MSCs and osteoclast-like GCs obtained from GCTB, the RANKL mRNA was detected in the cultured MSCs fraction but not in the osteoclast-like GCs fraction[9-11)]. They also demonstrated the formation of osteoclast from Raw 264.7cells (a monocytic cell line) and peripheral blood monocytes when MSCs of GCTB were cocultured with them[9)10)17)]. Because cell separation from tissue for *in vitro* culture can be incomplete and inadequate, confirmation at the *in situ* level has been performed simultaneously and also by other investigators. Fluorescence ISH with RANKL cDNA revealed the expression of RANKL transcript in cultured MSCs but not in osteoclast-like GCs[10)]. Immunohistochemistry revealed the expression of RANKL protein in MSCs using paraffin-embedded tissue[12)]. However, Kartsogiannis *et al.* demonstrated RANKL expression in MSCs as well as osteoclast-like GCs at the mRNA and protein levels using ISH and

immunohistochemistry[13]. In addition, Morgan *et al.* reported a high expression of RANKL in the osteoclast-like GCs but not in the MSCs using cDNA microarray and RT-PCR with fractionated MSCs and osteoclast-like GCs, and at the *in situ* level using immunohistochemistry with paraffin-embedded tissue[8]. They suggested that either unknown molecules other than RANKL from the MSCs were responsible for osteoclast formation or autocrine signaling stimulated the osteoclastogenesis.

In the present work, we detected the strong expression of RANKL protein in MSCs in both GCTB and GCTTS. This result differs from those of Kartsogiannis *et al.* and Morgan *et al.,* which might be due to the use of different tissue-processing method and/or different primary antibodies for RANKL in the immunohistochemistry. Morgan *et al.,* used the same method as in the present work, immunohistochemistry on paraffin-embedded tissue sections with the same RANKL antibody (MAB626, R&D Systems). This makes it more difficult to explain discrepancy in the results between the present study and that of Morgan *et al.* ; however the use of this antibody to localize RANKL production in other disorders containing osteoclast-like GCs has produced results that are consistent with ours. In central giant cell granuloma of jaw, cultured spindle-shaped MSCs express RANKL[14], and CD1a-positive dendritic cells around osteoclast-like GCs express RANKL protein when applying immunohistochemistry to Langerhans cell histiocytosis[18].

We also found that RANKL-positive MSCs were located in the cytoplasm of osteoclast-like GCs or in close proximity to them in both lesions. The direct cell-to-cell interaction between RANKL-producing cells and monocyte/macrophage precursors is important for the generation of osteoclasts[2)19], although osteoclastogenesis can occur without cell-to-cell contact[17]. We demonstrated that the production of RANKL from cells in the vicinity of its precursor cells is important to osteoclastogenesis.

Immunohistochemical staining for osteonectin (which is one of the preosteoblastic markers) revealed that RANKL-positive MSCs were also positive for osteonectin in both GCTB and GCTTS. In GCTB, osteonectin expression indicates preosteoblastic differentiation of MSCs and this is compatible with the results of other studies demonstrating the expression of many osteoblastic markers[20)21]. In GCTTS, there were few RANKL-positive MSCs and they were located around osteoclast-like GCs but not around foreign-body-type GCs, which constitute a large proportion of the multinucleated GCs. These MSCs were negative for CD68 and TRAP but positive for osteonectin suggesting an osteoblastic lineage. The synovium contains multipotent mesenchymal stromal cells that can differentiate into various mesodermal tissues, such as osteoblasts, chondrocytes and adipocytes[22]. There were also many plump and spindle-shaped MSCs showing positivity for osteonectin but not for RANKL. Osteonectin is a noncollagenous bone matrix protein produced in large amounts from osteoblastic cells in developing bones and teeth[23]. It is a biologically active glycoprotein involved in the regulation of cell adhesion, proliferation and extracellular matrix synthesis/turnover. Its expression is restricted both spatially and temporally. Although its production is restricted to low-to-undetectable levels in normal quiescent tissue, high levels of osteonectin mRNA and protein synthesis are present in various tissues undergoing remodeling during normal development or in response to injury, and in neoplastic tissue[24)25]. Therefore, the osteonectin expression of RANKL-negative MSCs in GCTTS suggests the presence of active fibroblastic activity.

In summary, we have used immunohistochemistry to demonstrate that MSCs are the cells that produce RANKL for osteoclastogenesis in both GCTB and GCTTS.

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