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Expression of RANKL and OPG in Middle Ear Cholesteatoma Tissue

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Objective: **The objective of this study was to investigate how the expression of the RANK-RANKL-OPG system mediates the formation and differentiation of osteoclasts and causes bone resorption in cholesteatoma.** ***Methods:*** **An immunohistochemical analysis was carried out in 22 cholesteatoma tissues obtained during middle ear surgery and 15 normal postauricular skin tissues to examine the expression of RANKL and OPG.** ***Results:*** **All 22 cases of cholesteatoma and the 15 cases of normal postauricular skin expressed RANKL and OPG. The count and rate of RANKL-positive cells in cholesteatoma was significantly higher than in normal postauricular skin. The count and rate of OPG-positive cells in normal postauricular skin was significantly higher than in cholesteatoma. The ratio of the positive expression rates of RANKL and OPG in cholesteatoma was statistically higher than in normal postauricular skin.** ***Conclusions:*** **We provide evidence suggesting that RANKL, which activates osteoclasts, plays a significant role in the mechanism of bone destruction in cholesteatoma, and that the ratio of RANKL to OPG may be a reliable indicator of bone destruction in cholesteatoma.** ***Key Words:*** **Cholesteatoma, RANKL, osteoprotegerin, immunohistochemistry.**

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INTRODUCTION

Cholesteatoma of the middle ear leads to the bone resorption of localized areas adjacent to the mass by desquamation and excessive keratinization. Bone resorption results in various symptoms and complications, which not only cause the destruction of the middle ear, but also of the inner ear.[1] Previous reports conclude that these serious complications of bone resorption are a result of a pressure effect, of numerous enzymes, or various cytokines.[2–4] It has been argued that the correlation of such effects leads to the localized activation of osteoclasts leading to bone resorption.[5]

Recently, it has been demonstrated that the balance between osteoblasts and osteoclasts plays a major role in bone formation and absorption, and that the receptor activator of NF-κB (RANK), the receptor activator of NF-κB ligand (RANKL), and osteoprotegerin (OPG), known together as the RANK-RANKL-OPG system, which is part of the ligand-receptor system[6,7] most effectively controls the balance between osteoblasts and osteoclasts. It is thought that the binding of RANK and RANKL activates the formation and maturation of osteoclasts.[8] OPG functions as a decoy receptor of RANKL and blocks the activation of RANK by preferentially binding itself to RANKL.[6] Research on the RANK-RANKL-OPG system related to osteoclast activation has been carried out in numerous studies based on bone diseases; however, our understanding of its effect on bone resorption in middle ear cholesteatoma is still insufficient.[7] Therefore, the authors want to investigate the effect of the RANK-RANKL-OPG system on bone resorption in middle ear cholesteatoma by semiquantitatively analyzing the manifestation of RANKL and OPG using immunohistochemistry.

MATERIALS AND METHODS

Cholesteatoma specimens were obtained from 22 patients who had undergone surgery for cholesteatoma-induced otitis media (Department of Otolaryngology, Hanyang University, Seoul, Korea). Fifteen cases of normal meatal skin specimens were used as the control group. Specimens for immunohistochemistry were immediately fixed in formalin and embedded in paraffin.

Immunohistochemistry

The tissue samples were cut into 4-μm thick sections using a paraffin block cutter (Sahndon, U.K.). Hematoxylin–eosin (HE) stain was then applied to confirm whether the samples included connective tissues of the epithelium and subepithelium. To prevent the omission of tissue samples, the cuts were mounted on slides (Polysine microslide). After undergoing deparaffinization and rehydration, the slides were treated in an autoclave at 121°C for 10 minutes in a 10 mmol/L citric acid buffer (pH 6.0) for antigen retrieval. Endogenous peroxidase activity was blocked by incubation in 10% H_2O_2 methanol for 10 minutes. Sections were

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stained with a monoclonal antibody of OPG (rhOsteoprotegerin, goat polyclonal 1:20; R&D) and an antibody of RANKL (rhRANKL, Clone 70525 mouse monoclonal 1:40; R&D) for 2 hours at room temperature. An ABC reaction was carried out for 30 minutes at room temperature using the Histone Kit (Nichirei, Japan) for OPG and the $LSAB_2$ kit (DAKO) for RANKL. The immunohistochemical reactions were then developed in freshly prepared 3,3′-diaminobenzidine tetrahydrochloride (DAB). The slides were then counterstained in Harris hematoxylin and mounted with Canada balsam. The stained slides were examined with an Olympus light microscope at 100× and 400× magnification. Digital photographs were taken for analysis. The number of total stained cells and OPG, RANKL-positive cells in the subepithelial perimatrix of the cholesteatoma and normal skin tissues were counted semiquantitatively. The total number of stained cells and the number of positive cells were counted under 400× magnification in the fixed domain, so we obtained the number of total stained cells, the number of RANKL-positive cells, the positive RANKL expression rate (number of positive expression cells/total number of stained cells), the number of OPG-positive cells, the positive OPG expression rate (number of positive expression cells/total number of stained cells), and the ratio of positive RANKL/OPG expression rates (positive RANKL expression rate/positive OPG expression rate).

Analysis of Clinical Aspects

A retrospective study was carried out to track the patients' duration of disease, degree of hearing loss (HL), degree of invasion, and degree of bone destruction. The semiquantitative analytical numbers obtained from the immunohistochemistry were used for comparison. The duration of disease was calculated from the time that the patient recognized the existence of otitis media up to the time of surgery. The degree of conductive HL was observed by measuring the air–bone gap. The degree of invasion of cholesteatoma was studied by observing the degree of invasion of the epitympanum, mesotympanum, aditus ad antrum, and mastoid antrum according to surgical findings. The degree of invasion was classified into four grades, in which grade 1 involves one area; grade 2, two areas; grade 3, three areas; and grade 4, four areas. The classification of the degree of bone destruction based on surgical findings is as follows: mild destruction, imperceptible erosion of the scutum and ossicle; moderate destruction, tegmen destruction and damage of most of the ossicle; and severe destruction, complete destruction of the ossicle and the destruction of the bony labyrinth, posterior wall of the external ear, and facial canal.

Statistics and Analysis

The Mann-Whitney *U* test was used to determine the differences between the number of RANKL and OPG-positive expression cells, RANKL and OPG-positive expression rates, and the ratios of the expression rates of RANKL and OPG based on immunohistochemistry. The Pearson correlation test was used to calculate the correlation between the RANKL and OPG-positive expression rates and the ratio of positive expression rates of RANKL and OPG with each clinical finding of the cholesteatoma tissues.

Statistical evaluation was carried out by SPSS program version 11 (SPSS Inc., Chicago, IL). Statistical significance was defined as a P value less than .05.

RESULTS

The following results were obtained by immunohistochemical analysis of 22 cholesteatoma tissues and 15 normal auditory meatal skin tissues (Figs. 1 and 2). RANKL and OPG were expressed in all samples of both cholesteatoma and normal skin tissues. Positive expression was detected in cells of the subepithelium, which is adjacent to the epithelial cells and bone. RANKL was stained, mostly in the nuclei, in all tissues of the epithe-

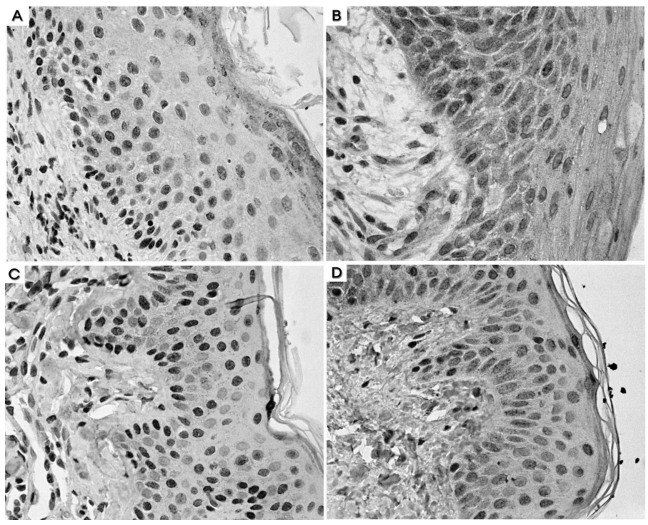

Fig. 1. Immunohistochemical demonstration of RANKL and OPG in the epithelium of cholesteatoma and normal postauricular skin. (**A**) RANKL in cholesteatoma; (**B**) OPG in cholesteatoma; (**C**) RANKL in normal postauricular skin; (**D**) OPG in normal postauricular skin (magnification 400×). The expression of RANKL and OPG is noted at the epithelium of cholesteatoma and normal postauricular skin, especially at the basal layer.

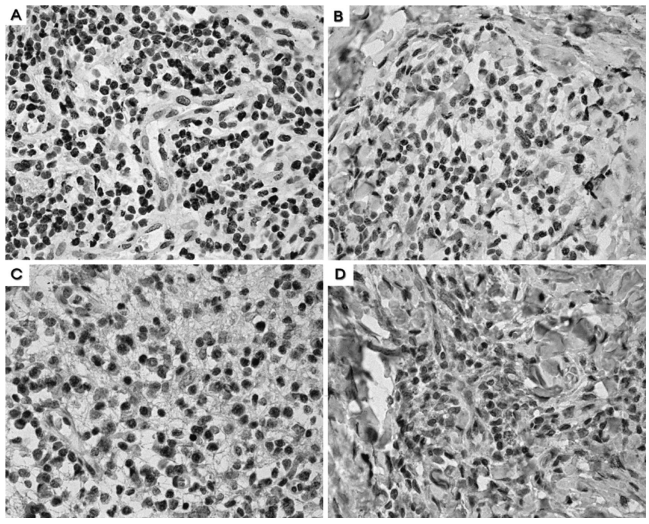

Fig. 2. Immunohistochemical demonstration of RANKL in the subepithelial connective tissue. (**A**) Cholesteatoma; (**B**) normal postauricular skin and immunohistochemical demonstration of OPG in the subepithelial connective tissue; (**C**) cholesteatoma; (**D**) normal postauricular skin (magnification 400×). The expression of RANKL (brown-colored) was higher in the cholesteatoma (A) than in the normal postauricular skin (B). Counterstained cells appear blue. RANKL is mainly stained in the nucleus. The expression of OPG (brown-colored) was higher in the normal postauricular skin (D) than in the cholesteatoma (C). Counterstained cells appear blue. OPG is mainly stained in the cytoplasm.

lium and subepithelium. OPG was mostly stained in the cytoplasm (Figs. 1 and 2).

Number of Positive Cells and Expression Rate

In the case of RANKL, the total number of stained cells under 400× magnification was 99.0 ± 18.0 in the cholesteatoma tissues and 52.2 ± 19.1 in the normal skin tissues. For OPG, the total number of stained cells under 400× magnification was 98.0 ± 23.1 in the cholesteatoma tissues and 48.7 ± 18.7 in the normal skin tissues. The total number of stained cells includes the positive expression cells and counterstained cells.

The number of RANKL-positive cells was 87.4 ± 19.3 in cholesteatoma tissues and 27.3 ± 12.9 in normal skin tissues. Cholesteatoma tissues had a significantly higher number of RANKL-positive cells ($P = .001$). The number of OPG-positive cells was 7.0 ± 2.9 in cholesteatoma tissues and 9.9 ± 2.6 in normal skin tissues. The normal skin tissues had a significantly higher number of OPG-positive cells ($P = .008$). The RANKL-positive expression rate (positive expression cells/total number of stained cells) in the cholesteatoma tissues was 87.9 ± 6.0% compared with 51.1 ± 8.6% in normal skin tissues. The positive expression cell rate of RANKL was significantly higher in the cholesteatoma tissues than in normal postauricular skin ($P = .001$; Fig. 3). The OPG-positive expression rate (positive expression cells/total number of stained cells) was 7.1 ± 2.8% in the cholesteatoma tissues and 21.9 ± 5.6% in normal skin tissues. The rate of positive expression cells of OPG was significantly higher in the normal postauricular skin than in the cholesteatomas ($P = .001$; Fig. 3). The ratio of expression rates for RANKL and OPG (positive expression cell rate of RANKL/positive expression cell rate of OPG) was 14.5 ± 6.7 in cholesteatoma tissues and 2.6 ± 1.1 in normal skin tissues. The cholesteatoma tissues showed a significantly higher ratio ($P = .001$; Fig. 4).

Analysis of Clinical Aspects

In this study, clinical aspects and the expression rates of the cholesteatoma tissues were correlated. The

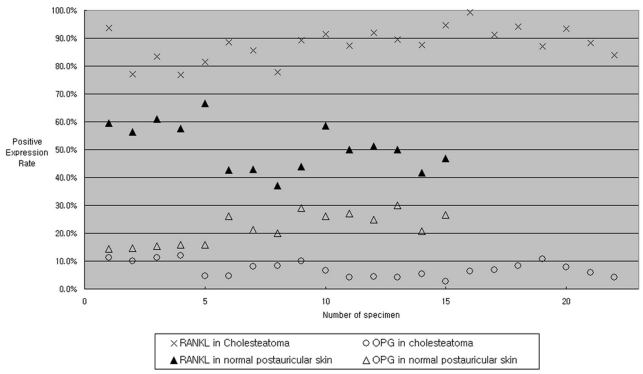

Fig. 3. Distribution of immunohistochemical stains of cholesteatoma and normal postauricular skin for RANKL and OPG. The number of positive expression cells for RANKL was significantly higher in cholesteatoma tissue than in normal postauricular skin. The rate of positive expression cells for OPG was significantly higher in normal postauricular skin than in cholesteatoma.

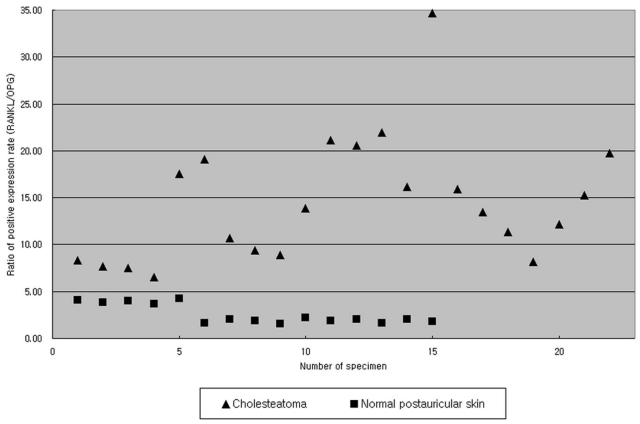

Fig. 4. Distribution of ratios of RANKL/OPG for positive expression rates of immunohistochemical stains of cholesteatoma and normal postauricular skin for RANKL and OPG. The ratio of positive expression rates of RANKL and OPG (positive expression cell rate of RANKL/positive expression cell rate of OPG) was significantly higher in cholesteatoma tissue than in the normal postauricular skin.

average duration of disease was 14.5 ± 14.3 years with a duration period of 2 months to 40 years. There was no significant relationship between the duration of sickness and the RANKL expression rate ($P = .412$), OPG expression rate ($P = .133$), or ratio of expression rates for RANKL and OPG ($P = .169$). The degree of HL on air conduction was from 19 dB to deafness, with an average of 57.9 ± 21.5 dB, and from 11 dB to deafness on bone conduction, with an average of 25.0 ± 13.6 dB. The air–bone gap was 11 dB to 46 dB with an average of 32.9 ± 10.5 dB. There was no significant relationship between the air–bone gap and the expression rate of RANKL ($P = .139$), expression rate of OPG ($P = .226$), or ratio of expression rates for RANKL and OPG ($P = .309$). The range of invasion was grade 1 in eight cases, grade 2 in four cases, grade 3 in nine cases, and grade 4 in one case. There was no significant relationship between the range of invasion and the expression rate of RANKL ($P = .296$), the expression rate of OPG ($P = .487$), or the ratio of expression rates for RANKL and OPG ($P = .203$). The degree of bone destruction was mild in seven cases, moderate in five cases, and severe in 10 cases. There was no significant relationship between the degree of bone destruction and the expression rate of RANKL ($P = .189$), the expression rate of OPG ($P = .769$), or the ratio of expression rates for RANKL and OPG ($P = .919$).

DISCUSSION

The most distinct clinical finding of cholesteatoma is the bone resorption of local areas adjacent to the perimatrix of the mass. Chloe reported that osteoclasts were always detected in areas where bone resorption and the diameter of osteoclasts and the size of the bone resorption area concord with one another microscopically; osteoclasts have been acknowledged as the main bone resorption cell.[5]

The role of osteoclasts in cholesteatoma has been widely acknowledged in other studies as well. Osteoclasts, the bone-resorbing cells in cholesteatoma, are known to be derived from hematopoietic precursor cells of the mono-

cyte/macrophage lineage.[9] The results of the immunohistochemical staining of cell markers demonstrate a higher concentration of the osteoclast progenitor cell lineage and of macrophages in cholesteatoma compared with normal auditory canal skin.[1]

The differentiation and activation of osteoclasts occur simultaneously with that of osteoblasts, and this signaling system has been studied by many different researchers. It has been recognized that macrophage-colony stimulating factor (M-CSF) and the activator, RANKL, are necessary for osteoclast differentiation and activation. OPG is the decoy receptor for RANKL.[6,7] The signaling process between these cells is controlled by the RANK-RANKL-OPG system, and it controls the formation and activation of osteoclasts.[10]

RANK is a receptor that is expressed in dendritic cells, chondrocytes, osteoclast precursors, and mature osteoclasts. It is mostly manifested in hematopoietic osteoclast progenitors during osteoclast differentiation.[7]

RANKL, the essential ligand for osteoclastogenesis, is a member of the TNF family of cytokines and is anchored to the stromal cell membrane (osteoblasts). RANKL is also known as OPGL (osteoprotegerin ligand), TRANCE (tumor necrosis factor-related activation-induced cytokine), and ODF (osteoclast differentiation factor).[8,10,11] Thus, the expression of the receptor activator of RANKL, a ligand, on the surfaces of osteoblast precursor cells, and the activation of its cognate receptor RANK on the surfaces of osteoclast precursor cells, are mandatory for the formation and maturation of osteoclasts.[8] In addition to the crossreaction of RANK and RANKL, M-CSF is also necessary. It has been reported that osteoclastogenesis can occur with only these three substances.[10] Mice that are defective for RANKL cannot produce osteoclasts, whereas soluble RANKL enables osteoclast precursors to differentiate in the presence of M-CSF, even in the absence of osteoblast/stromal cells.[7] Also, NF-κB activated by RANKL-RANK linkage stimulates cytokine synthesis and secretion.[11]

OPG, a soluble protein derived from osteoblasts, retards osteoclast ontogeny and activation by inhibiting the formation of the RANKL/RANK ligand/receptor complex, primarily by attaching itself to RANKL and thus blocking the activation of RANK. The inhibitory effect of OPG on osteoclastogenesis has already been verified in an experiment using transgenic mice. The overexpression of OPG in transgenic mice leads to profound osteopetrosis secondary and to a near total lack of osteoclasts. Conversely, the ablation of the OPG gene causes severe osteoporosis in mice.[12]

In summary, the binding of RANK and RANKL in the RANK-RANKL-OPG system increases the formation and activation of osteoclasts. OPG recognizes RANKL, and this decoy receptor blocks the interaction between RANK and RANKL, leading to an inhibition of osteoclast differentiation and activation.[10]

Hamzei et al.[13] report that the immunoreactivity of RANKL was higher than that of OPG in cholesteatoma, compared with normal auditory meatal skin, in an immunohistochemical study. Its manifestation was also confirmed with reverse transcription–polymerase chain reaction. The manifestation of M-CSF, a necessary molecule along with the RANK-RANKL lineage for the activation of osteoclasts, has also been confirmed. However, the authors did not carry out a statistical or semiquantitative analysis of RANKL and OPG expression.

This study, on the other hand, used semiquantitative and statistical analysis, and thus is a significant advance over the other study. In the study, a positive immunoreactivity was observed in epithelial cells and subepithelial cells under the epithelium scattered near the bone. RANKL staining was localized mostly in the nucleus and OPG mostly in the cytoplasm. Both RANKL and OPG staining were not only present in the subepithelium, but in the epithelium as well. Staining was stronger primarily in the basal layer. Strong staining in the subepithelial tissues was noticeable primarily in areas near the bone. We believe that such results have been acquired because this area contains stromal cells, osteoblast precursor cells, and osteoclast precursor cells, along with numerous inflammatory cells. RANKL and OPG expression has also been observed in inflammatory cells such as T-lymphocytes. We believe that further research using specific markers for each cell must be carried out for additional clarification of which cells RANKL and OPG are expressed in. We also believe that because inflammatory reactions occur as often as bone resorption in cholesteatoma, positive immunoreactions could be manifested more often in cholesteatoma than in normal auditory meatal skin. However, this apperception should be confirmed using cell-specific double-labeling immunohistochemistry testing in the near future.

The total number of cells in each fixed domain was higher in the cholesteatomas than in the normal auditory meatal skin. We believe that this reflects a more active cell proliferation ability and inflammatory reaction in cholesteatoma.

The absolute number of RANKL-immunoreactive cells and the positive expression rate with respect to the total number of cells were all statistically significant. OPG was statistically significant in normal auditory meatal skin. As mentioned before, RANKL and OPG play a similar role in cholesteatoma like in other bone disorders. This reflects the fact that RANKL and OPG have a mutual antagonist effect.

The existence and sufficient absolute quantity of RANKL does not always lead to bone resorption. In this study, RANKL was detected in all of the normal auditory meatal skin tissues investigated. It has been confirmed that RANKL exists in various organs such as the skin, liver, and heart, which have no relationship to bone destruction. In such cases, it is assumed that RANKL participates in cell survival and is related to mechanisms initiating programmed cell death.[14] Therefore, it is assumed that the balance between RANKL and OPG, which have a mutual antagonist effect, is the key regulator of osteoclastogenesis and bone resorption by osteoclasts. Thus, the RANKL/OPG ratio, rather than the absolute values of RANKL and OPG,[8,9] is the most informative statistic. Because the RANKL/OPG ratio was also significantly higher in the cholesteatomas in this study, it can be suggested that the RANKL/OPG ratio is also a key

indicator of bone resorption through osteoclast formation and activation in cholesteatoma.

However, the correlations of RANKL and OPG expression rates and the RANKL/OPG ratio with clinical findings such as duration of disease, degree of HL, extent of invasion, and degree of bone resorption were not statistically significant. We believe that this is probably because osteoclast formation and activation had already progressed at the time of surgery. For this reason, one cannot say that the degree of osteoclast formation and activation are at their highest at the time of surgery.

CONCLUSION

Until now, various cytokines have been suggested to play a role in bone resorption in middle ear cholesteatoma. However, the RANK-RANKL-OPG system, which has been suggested to play a key role in bone metabolism disorders in recent studies, has also been confirmed as a key regulator of bone resorption in middle ear cholesteatoma.

Based on the results of this study, we conclude that RANKL, an osteoclast activator, plays a key role in bone resorption in middle ear cholesteatoma. We particularly believe that an increased RANKL/OPG expression ratio plays an important role in osteoclast activation.

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