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Expression of RANKL and Proliferation Abilities of Cultured Human Middle Ear Cholesteatoma Epithelial Cells

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ABSTRACT

One of the most distinct characteristics of middle ear cholesteatomas is their capacity for bone destruction during the growth process. In this study, we examined the relationship between inflammatory mechanisms and both bone absorption and the proliferation of epithelial cholesteatoma cells. Cultured cholesteatoma epithelial cells were stimulated by lipopolysaccharide (LPS) and dexamethasone (Dex). We found that the expression of receptor activator of NF-κB ligand (RANKL) and Ki-67 in cultured cholesteatoma cells was increased by LPS stimulation, indicating that LPS promotes not only bone destruction but also the proliferative activities of these cells. The constitutive expression of RANKL and Ki-67 and the production of IL-6 and IL-8 were significantly inhibited by Dex treatment. Further, Dex significantly suppressed the stimulatory effects of LPS on RANKL and Ki-67 expression and on IL-6 and IL-8 production. Based on results so far, Dex likely exerts a beneficial action against acute inflammation. However, further studies might be required to assess its clinical features.

Key words: *Cholesteatoma, Lipopolysaccharide, RANKL, Dexamethasone*

One of the most distinct characteristics of middle ear cholesteatomas is their capacity for bone destruction during the growth process. It is clinically acknowledged that infectious inflammatory conditions facilitate the growth of middle ear cholesteatomas with bone absorption. Debates regarding the mechanisms of this bone absorption as well as of cholesteatoma cell proliferation have adopted numerous viewpoints, including inflammatory cytokine expression and production[1,9,15]. Pathologically, human middle ear cholesteatomas possess a matrix structure with stratified squamous epithelium and subepithelial tissue. It is crucial to remove all of this matrix when middle ear cholesteatoma is treated by surgery. Receptor activator of NF-κB (RANK) is expressed on the cell membrane of osetoclasts. Receptor activator of NF-κB ligand (RANKL) is a cell membrane-combined cytokine expressed on T lymphocytes, osteoblasts and the like. When RANK is followed by RANKL, the differentiation of osteoclasts is accelerated.

In this study, we examined the relationship between inflammatory mechanisms and bone absorption and proliferation by epithelial cholesteatoma cells.

Epithelial cholesteatoma cells were taken from surgically removed middle ear cholesteatomas, then stimulated by lipopolysaccharide (LPS) and dexamethasone (Dex), and evaluated by immunostaining and ELISA. Both RANKL and Ki-67 expressions were increased by LPS and decreased by Dex, and production of IL-6 and IL-8 was inhibited by Dex. These results showed that bone absorption and the proliferation of epithelial cholesteatomas are accelerated by bacterial infection and inhibited by a steroid, and that inflammation is also inhibited by the steroid.

We consider the present study to be valuable not only in elucidating the role of inflammatory mediators involved in the proliferative activities of cholesteatoma, but also in understanding the clinical features of human cholesteatoma development and growth.

MATERIALS AND METHODS

Cholesteatoma tissues were obtained from 6

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patients who had undergone surgery for middle ear cholesteatoma at Hiroshima University Hospital.

Tissue preparation and cell culture

Middle ear cholesteatoma tissues were obtained by surgical removal and stored in sterilized PBS containing antibiotics overnight. Adherent blood, mucus, and keratinized debris were carefully removed from the cholesteatoma matrix using tweezers. The matrix cells were dispersed in 20 ml of 0.2% collagenase solution in a thermostat bath at 37.5°C for 1 hr. The content was then poured into a dish and the tissues were gently rubbed with a cell scraper for further dispersion of epithelial cells. The cell suspension solution was centrifuged and rinsed with a volume of Dulbecco's Modified Eagle Medium three times. The cell supernatant was suspended in 40 ml of serum-free culture medium (Defined-Keratinocyte-SFM, Invitrogen, CA) with attached supplement and antibiotics. The cell suspension fluid was then harvested into 75㎠ collagen-coated flasks (BD Biosciences, NJ). The cell culture was maintained in a humidified atmosphere with 5% CO_2 at 37°C. Cell morphology was checked by phase-contrast microscopy, and the medium was replaced every 2 or 3 days. A subculture of the cells was performed using 0.1% trypsin-0.038% EDTA-PBS when they became confluent; the third passage was used for the experiment.

Cell culture condition and assessment for in vitro assay

We designed bacterial infection and inflammatory suppression models using the cultured cholesteatoma cells. The third-passage cells were resuspended at a density of 1-2 × 10^5 cells/㎠ and were seeded onto type I collagen-coated 35-mm dishes (IWAKI, Tokyo). At the confluent stage, the cells were incubated with LPS (10 ng/ml), Dex (10^{-6} M), or LPS (10 ng/ml) and Dex (10^{-6} M) for 48 hr, as shown in Fig. 1.

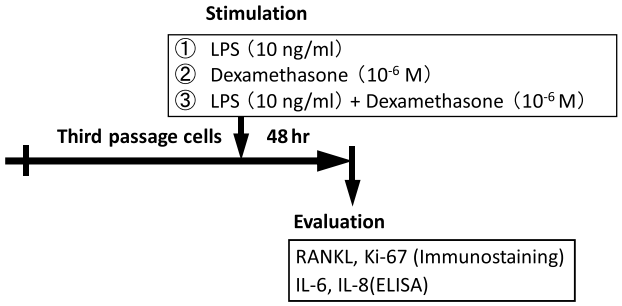

Fig. 1. Time-course schema of *in vitro* assay.

The degrees of RANKL and Ki-67 expression in cultured cholesteatoma cells were evaluated semi-quantitatively by immunocytochemistry. The culture dishes were fixed in 4% paraformaldehyde solution for 30 min. The primary antibodies used here were mouse monoclonal anti-human TRANCE/RANK L/TNFSF11 MAb (Clone 70525, R&D, MN) for RANKL and mouse monoclonal anti-human Ki-67 antigen (Clone MIB-1, Dako, DK) for Ki-67. After being rinsed in PBS, they were coated with PBS containing 0.1% bovine serum albumin and stored overnight at 4°C in a humid chamber. As the secondary antibody, the LSAB™+ system (Dako) was employed. Color development was performed with 3,3-diaminobenzidine tetrahydrochloride chromogen. The stained cells were observed with a Nikon light microscope and digital photomicrographs were taken for analysis. The percentages of RANKL and Ki-67-positive cells were counted within each microscopic field of 5 randomly selected areas at a magnification of × 200 and the degree of positivity was assessed (Fig. 2). The concentrations of IL-6 and IL-8 from the supernatant of cultured cells were measured quantitatively by the ELISA method (D6050, D8000C, R&D), according to the instructions supplied by the manufacturer.

For between-group analysis, the unpaired Student's t-test was used to determine statistical significance. A p value < .05 was considered significant.

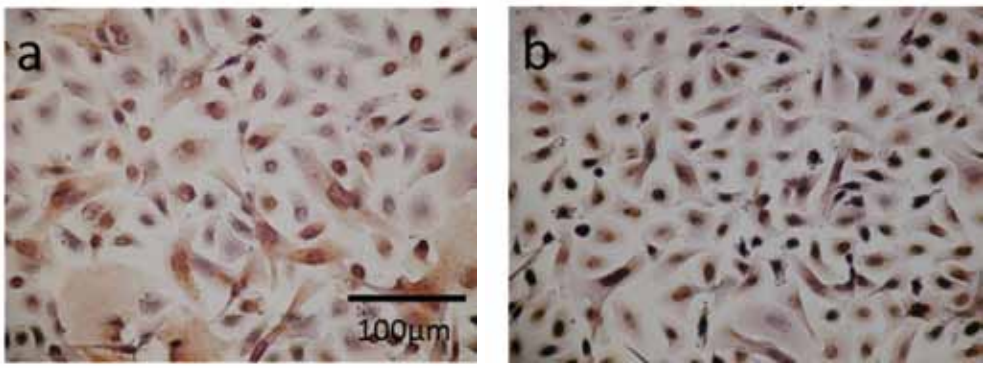

Fig. 2. Representative photomicrographs of **a**: RANKL (positive staining in cytoplasm) and **b**: Ki-67 (positive staining in cell nuclei) immunocytochemistry. Bar = 100 μm.

RESULTS

Immunocytochemistry of cultured cholesteatoma cells

The use of Defined Keratinocyte-SFM and type-I collagen-coated dishes demonstrated superior cell growth of primary cholesteatoma while maintaining epithelial morphology and biological markers. Immunocytochemical expression of RANKL was detected mainly in the perinuclear cytoplasm of the cultured cells.

The average proportions of RANKL-positive cells were 20.0% in the control group, 30.0% in the LPS group, 8.3% in the Dex group, and 10.5% in the LPS and Dex group (Fig. 3, 4). There were significant differences between the control group and the LPS group ($p < .05$), the control group and the Dex group ($p < .01$), and the LPS group and the LPS and Dex group ($p < .01$). The results clearly indicated that RANKL expression is increased by LPS and decreased by Dex.

Immunocytochemical expression of the Ki-67 antigen was observed in the nuclei of the cultured cells. Cultured cells were judged to be positive for Ki-67 immunostaining only when the nuclear staining was obvious. The mean expression rates of Ki-67 positive cells were 16.7% in the control group, 28.1% in the LPS group, 8.3% in the Dex group, and 11.7% in the LPS and Dex group (Fig. 5, 6). Statistical analysis showed that there were significant differences in the Ki-67-positive rate between the control group and the LPS group ($p < .05$) and between the LPS group and the LPS and Dex group ($p < .01$). In addition, there was a tendency for fewer cells to be Ki-67-positive in the Dex group than in the control group. The results

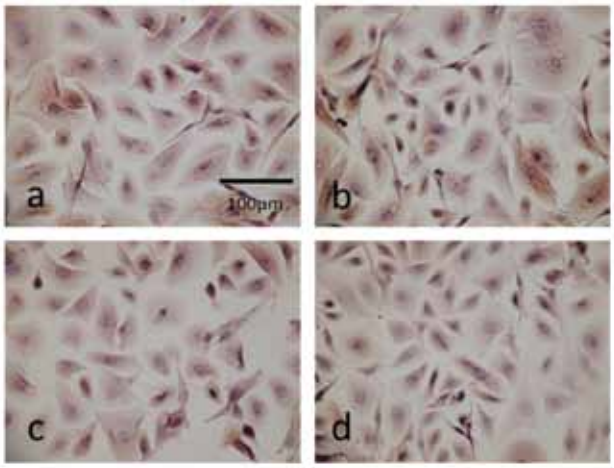

Fig. 3. Immunocytochemical photomicrographs of RANKL expression in the cultured cholesteatoma cells. **a**: control, **b**: LPS 10 ng/ml, **c**: dexamethasone 10^{-6} M, **d**: LPS and dexamethasone. Bar = 100 μm.

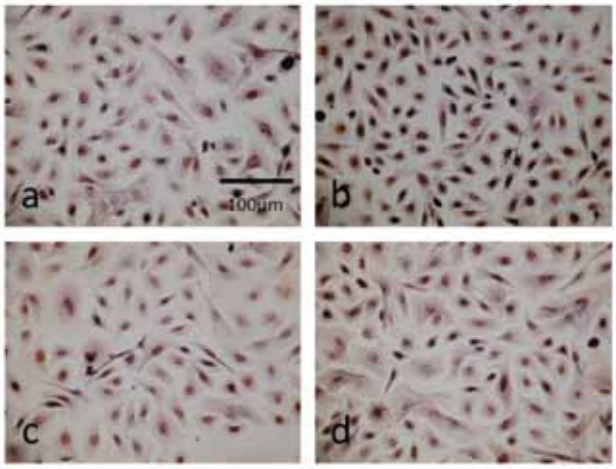

Fig. 5. Immunocytochemical photomicrographs of Ki-67 expression in the cultured cholesteatoma cells. **a**: control, **b**: LPS 10 ng/ml, **c**: dexamethasone 10^{-6} M, **d**:LPS and dexamethasone. Bar = 100 μm.

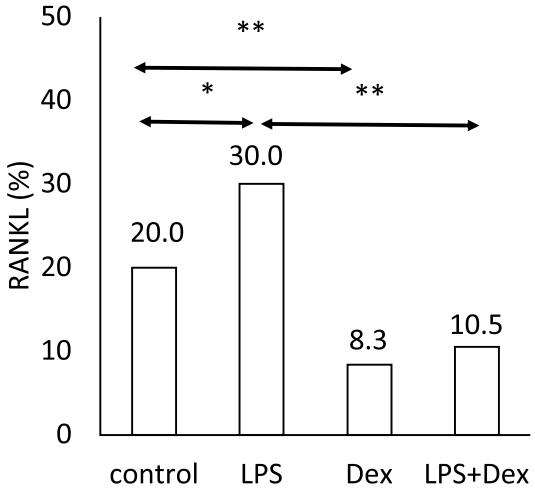

Fig. 4. Comparison of the proportion of RANKL-positive cells in each group. *p < .05, **p < .01, Dex: dexamethasone.

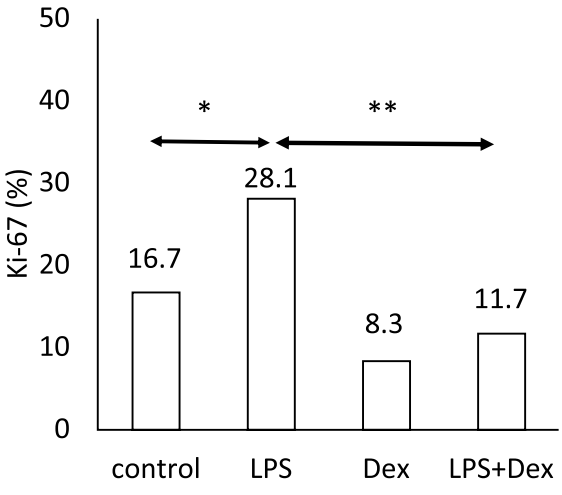

Fig. 6. Comparison of the expression rates of Ki-67 positive cells in each group. *p < .05, **p < .01, Dex: dexamethasone.

indicated that the proliferating ability of cultured cholesteatoma cells was promoted by LPS stimulation and inhibited by Dex treatment.

Cytokine production by cultured cholesteatoma cells

A comparative study was carried out to examine the effects of LPS and Dex on the production of IL-6 and IL-8 by cultured cholesteatoma cells. We found relatively high levels of constitutive production of both IL-6 and IL-8 in the control group (Fig. 7). Addition of LPS to the medium at a concentration of 10 ng/ml resulted in almost no difference in IL-6 and IL-8 production by the cultured cells as compared with the control. On the other hand, addition of Dex had a remarkable inhibitory effect on the production of these cytokines. There were significant differences in IL-6 and IL-8 production between the control group and the Dex group ($p < .01$) and between the LPS group and the LPS and Dex group ($p < .01$).

DISCUSSION

It is commonly known that RANKL plays an important role in the mechanism of bone destruction in many kinds of diseases, including chronic articular rheumatism and metastatic bone cancer. Recognition of RANKL by RANK expressed on the cell membranes of osteoclasts induces the differentiation of osteoclasts and also facilitates bone destruction. Various cytokines and growth factors, including TNF-α, IL-6, IL-8, and TGF-α, are involved in the differentiation process of osteoclasts[1,9,15]. RANKL expression has been detected in both epithelial and subepithelial tissue[3]. In this experiment, we proposed the hypothesis that chronic inflammation related to cholesteatoma induces bone resorption by the release of RANKL from epithelial and stromal cells, along with numerous inflammatory cells. The *in vitro* cell culture model has the advantage of allowing keratinocyte cytokine production to be examined in the absence of *de novo* inflammatory cell recruitment. The obtained results suggest that bone destruction mechanisms may be promoted by the coexistence of bacterial infection, and that Dex may have an inhibitory role in RANKL production.

Osteoclasts were observed in epithelial cholesteatoma tissue adjacent to a bone resorbing area with an electron microscope or by immmunohistological identification[2,4]. But osteoclasts are not always visible in histologic sections for several reasons. 1) Osteoclasts are sometimes difficult to discern in whole temporal bones processed by conventional techniques (embedded in selloidin and sectioned at 20 μm). 2) The osteoclast is a transient cell with a relatively short life[11] and may not be present at the moment of fixation of the tissue. 3) Active bone resorption sites are not usually seen in surgical specimens because surgery is normally performed after inflammation has been controlled[2]. Therefore, to evaluate the bone destruction activity of cholesteatoma, it could be simpler and more reliable to use factors like RANKL than to try to identify the osteoclasts directly.

A relationship between bone resorption mechanisms and subepithelial tissue has been suggested in the literature. Inflammatory cell infiltration and fibroblasts were observed in subepithelial tissue, and the effect on bone absorption and proliferation of cholesteatoma was pointed out[5,14]. Expression of RANKL was found in subepithelial infiltrated lymphocytes, and had a tendency to increase near the middle ear bone[16].

LPS is a component of the outer membrane of gram-negative bacterial cells such as *Pseudomonas aeruginosa*, and has the nature of an endotoxin. LPS stimulates the formation of osteoclasts through the activation of toll like receptor-4[10].

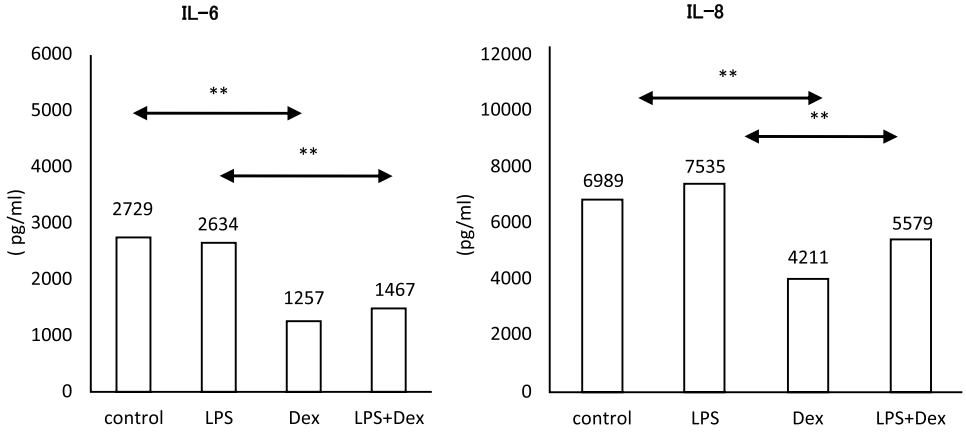

Fig. 7. Comparison of the production of IL-6 and IL-8 in each group.
*p < .05, **p < .01, Dex: dexamethasone.

Nason et al[12] reported that osteoclast precursor cells differentiated into osteoclasts with a higher bone-absorbing capacity after stimulation by LPS derived from *Pseudomonas aeruginosa* under a certain concentration of RANKL. Under LPS stimulation, osteoclast precursor cells released many kinds of cytokines including TNF-α, IL-1, and IL-6 through an autocrine/paracrine pathway, and this promoted the bone destruction process. In human cholesteatoma, Peek et al found that the LPS concentration of cholesteatoma tissue with severe bone destruction was higher than that without bone destruction, suggesting that LPS is one of the important factors of bone destruction in cholesteatomas[13]. We also found that expression of RANKL and Ki-67 in cultured cholesteatoma cells was increased by LPS stimulation, indicating that LPS promotes not only the bone destructive capacity but also the proliferative activity of these cells. We further speculate that these phenomena, which mimic clinical environments as a result of bacterial inflammation, may be crucial for regulating cell proliferation in the cholesteatoma epithelium in a paracrine manner.

The anti-inflammatory role of steroid treatment in human cholesteatoma in relation to the mechanisms behind proliferation of the cholesteatoma matrix is still controversial. It is well known that steroids have potent anti-inflammatory effects at the cellular level. Steroids exert suppressive effects on T lymphocytes, monocytes, and macrophages, and inhibit the secretion of various inflammatory cytokines such as IL-1, IL-2, IL-6, IL-8, and TNF-α. Kaygusuz et al[7] reported that patients with middle ear cholesteatoma with acute bacterial infection showed a quicker recovery when treated with both antibiotics and Dex compared with antibiotics alone. In the present study, the degrees of constitutive expression of RANKL and production of IL-6 and IL-8 were significantly inhibited by Dex treatment. Further, Dex significantly suppressed the stimulatory effects of LPS in RANKL, Ki-67 expression and IL-6 and IL-8 production. Based on the results so far, Dex likely has beneficial effects against acute inflammatory exacerbation. However, further studies might be required to assess its clinical features.

IL-6 is one of the proinflammatory cytokines produced by keratinocytes, lymphocytes, fibroblasts, and macrophages and plays an important role in the inflammatory response. An immunohistochemical study of epithelial tissue in cholesteatomas revealed that IL-6 expression was present in all epithelial layers at a more pronounced degree in the external auditory canal skin[1]. The concentration of IL-6 in a cholesteatoma extract measured by ELISA was also increased in the same specimen. In another study, relatively high levels of NF-κB DNA-binding activity and a high expression level of IL-6 mRNA were observed in excised cholesteatoma tissues as compared with normal external auditory canal skin[17]. These facts suggest that overexpression of IL-6 in middle ear cholesteatoma is partly responsible for the clinical aspects of hyperproliferation and bone resorption activities.

IL-8 is also considered to have an important role in the pathological changes of cholesteatomas. The production and mRNA expression of IL-8 was reported to be elevated in the cholesteatoma epithelium[8]. Chung et al[3] measured cytokine production in human cultured middle ear cholesteatoma cells and external auditory skin cells obtained by surgical excision. The quantities of IL-1α and IL-8 in the culture supernatant were higher in the cholesteatoma than in the skin cells. They suggested that both IL-1α and IL-8 are responsible for bone destruction and that certain substances from the subepithelial granulation tissue may stimulate cytokine release from the cholesteatoma epithelium. We found similar results using the epithelial culture system. These findings, including our own, validate the evolving concept that activated keratinocytes in cholesteatomas lead to the secretion of various cytokines and growth factors responsible for their clinical features.

CONCLUSION

LPS promoted not only the bone destruction capacity but also the proliferative activities of the cultured epithelial choesteatoma cells. Dex suppressed bone destruction and cell proliferation, and also the production of the pro-inflammatory cytokines IL-6 and IL-8.

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Conflict of interest: None of the authors have any conflict of interest to declare in relation to this study.
The study protocol was approved by the Institutional Review Board at the Hiroshima University School of Medicine.

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