



Immune cell profile in invasive cholesteatomas: Preliminary findings

Mahmoud Rezk Abdelwahed Hussein^{a,*}, Ramadan H. Sayed^b, Eman E. Abu-Dief^c

^a Pathology Department, Assuit University Hospitals, Sohag University, Egypt

^b ENT Department, Sohag Faculty of Medicine, Sohag University, Egypt

^c Histology Department, Sohag Faculty of Medicine, Sohag University, Egypt

ARTICLE INFO

Article history:

Received 26 March 2009

and in revised form 4 December 2009

Available online 4 January 2010

Keywords:

Immunity

Immune cells

Cholesteatoma

ABSTRACT

Background: Cholesteatoma consists of keratinizing squamous epithelium, granulation tissue and keratin plugs. The pathogenesis of cholesteatoma may be related to alterations in the stromal immune cell infiltrate. **Objective:** To examine the immunophenotypic characteristics of the immune cell infiltrate in invasive cholesteatomas.

Materials and methods: This study included 12 patients with invasive cholesteatomas causing wide bone erosion of the mastoid, middle ear structures, and the bony plates of middle ear cleft. Diagnosis of invasiveness was based on the clinical, radiological and intraoperative findings. Canal wall-down surgical approach was done in all cases to control the disease process. We used the cholesteatomatous tissue specimens to perform immunohistochemical stains for B cells (CD20), T cells (CD3), histiocytes (CD68) and Langerhans' cells (CD1a). Mouse monoclonal antibodies and immunoperoxidase staining methods were used. The results of immunohistology were scored as mean values of positively stained immune cells. The data were compared with findings in 10 specimens of external ear skin (control group).

Results: Immunohistochemistry showed highly significant ($p < 0.00$) counts of immune cells in invasive cholesteatomas (CD3: 4.7 ± 0.4 , CD68: 4.6 ± 0.5 , CD20: 0.8 ± 0.1 and CD1a: 0.8 ± 0.1) compared to those in external canal skin (control group: CD3: 0.8 ± 0.3 , CD68: 1.0 ± 0.4 , CD20: 0.2 ± 0.1 and CD1a: 0.1 ± 0.1). In cholesteatomas, the predominant of CD3⁺ T lymphocytes and CD68⁺ cells (histiocytes). Rare CD20⁺ cells and CD1a⁺ cells (Langerhans' cells) were also observed.

Conclusions: This preliminary study describes the profile of the immune cell infiltrate in invasive cholesteatomas. The numeric dominance of CD3⁺ cells and CD68⁺ cells suggests that cell-mediated immunity has important role in the development of cholesteatoma and in its autodestructive properties. Further studies are recommended to categorize the T cell subsets in different stages of cholesteatomas.

© 2009 Elsevier Inc. All rights reserved.

Introduction

Cholesteatoma (keratoma) represents the presence of a non-neoplastic accumulation of keratinizing stratified squamous epithelium (skin) and desquamated keratin debris in the tympanic cavity and/or mastoid. This accumulation may occur along with underlying subepithelial fibroconnective tissue; granulation tissues as well as chronic inflammatory cell infiltrate (Quaranta et al., 1995). There are at least three types of cholesteatomas that arise in the middle ear: one resulting from invagination (retraction's pocket), another from migration and the last one from congenital inclusion (Gersdorff et al., 2006). The development of cholesteatomas needs three successive inflammatory phases. The first one leads to formation of a retraction pocket. The intermediate phase is associated with pathologic changes

in the epidermis and in the floor of the external auditory canal. The final stage is characterized by invasion and auto-destruction of the middle ear with bone resorption (Gersdorff et al., 2006). Bone resorption/ destruction of the tympanic cavity and/or mastoid areas may occur as a consequence of expansion of the cholesteatomatous mass and/or elaboration of collagenase and other proteolytic enzymes and inflammatory mediators (Gersdorff et al., 2006 and Abramson, M). This phase is complex with several factors play different roles including: matrix metalloproteinases (Banerjee et al., 2001; Kobayashi et al., 2005; Mehta et al., 2007; Schmidt et al., 2001; Suchozebrska-Jesioneck et al., 2008), osteoclasts, proinflammatory cytokines (Akimoto et al., 2000; Bujia et al., 1996a,b; Ottaviani et al., 1999; Sastry et al., 1999; Szczepanski et al., 2006), nitric oxide, interleukins (IL-1), prostaglandins, fibroblast (Laeq and Faust, 2007), bacteria and their biofilms (Aberg et al., 1993; Gersdorff et al., 2006). Once the squamous epithelium reaches the tympanic cavity and/or mastoid areas, from its origin in the external auditory canal or tympanic membrane, a locally invasive process commences. Bone resorption/ destruction may occur as a consequence of expansion of

* Corresponding author. Department of Pathology, Faculty of Medicine, Assuit University Hospitals, Assuit, Egypt. Fax: +2 088 333327.

E-mail addresses: mrcpath17@gmail.com, mrh17@gawab.com (M.R.A. Hussein).

the cholesteatomatous mass and/or elaboration of collagenase and other proteolytic enzymes and inflammatory mediators (Abramson and Huang, 1977; Gersdorff et al., 2006).

Immune cell infiltrate is a constant feature in invasive cholesteatomas (Bujia et al., 1993). Several studies suggest that the immune cells play roles in the development of these lesions. Schilling et al. examined the immune cells in the stroma of acquired aural cholesteatomas using immunohistochemical staining methods. The vast majority of the infiltrating cells were CD45⁺ cells, with dominance of CD3⁺ T lymphocytes and CD6⁺ cells beneath the squamous epithelium of cholesteatoma. Occasional CD19⁺ and CD22⁺ B lymphocytes were also detected (Schilling et al., 1991). Expression of HLA-DR was almost as high as that of CD45⁺, whereas CD25⁺ cells were detected in lower amounts. The authors inferred that the majority of T cells and macrophages in the stroma of cholesteatomas are in an immunologically activated state (Schilling et al., 1991). Van Dijk et al found both Langerhan's cells and T-lymphocytes in the human cholesteatomatous matrices using immunostaining methods (van Dijk et al., 1986). Ahn et al. observed the localization of interleukin 1 (proinflammatory cytokines) in the epithelial layer of human cholesteatomas. This cytokine can stimulate fibroblasts and macrophages to produce collagenases and prostaglandins leading to bone resorption. Also, interleukin 1 was found in bone cells and monocytes in the region of active bone destruction (Ahn et al., 1990a,b). Taken together, these previous studies suggest a role for the immune cells in the development of invasive cholesteatomas. To date, our knowledge about the histological profile of immune cells in invasive cholesteatomas is limited. In the current study, we examined the immune cells in invasive cholesteatomas compared to external canal skin (control specimens) to elucidate this issue.

Materials and methods

Clinical characteristics of the study group

This investigation was carried out at the Pathology, ENT and Histology Departments, Faculty of Medicine, Sohag University Hospitals, Sohag, Egypt. The study was approved by the Institutional Ethics and Review Committee of the Faculty of Medicine, Sohag University Hospitals, Sohag, Egypt. The study included 12 cases of stage IV invasive cholesteatomas (Aberg et al., 1993 and Potsic et al., 2002) with wide bone erosion of mastoid, middle ear structures, and bony plates (tegmen and sinus plates). The diagnosis was based on clinical, radiological and intraoperative findings. Canal wall down approach was done in all cases to control the disease process of extensive cholesteatoma. Biopsy specimens including cholesteatomatous matrix, perimatrix and contents were obtained from all cases and fixed in formalin. In addition, tiny biopsy obtained specimens from the external canal skin of the deep meatus, in 10 patients undergoing myringoplasty for dry perforation, served as a control group. Initially, Hematoxylin and eosin-stained sections were used for immunohistochemical assay and examined (M.R. Hussein and E.S. Abu-Deif) to evaluate the mononuclear cell infiltrate.

Histological evaluation of the mononuclear cell infiltrate

We evaluated the mononuclear cell infiltrate following Hussein and Hussein et al. (Hussein and Ahmed, 2005; Hussein et al., 2006a and Hussein, 2008). The cells were counted in serial sections in at least 10 different fields (at X400 magnification) in until 100 cells had been counted.

Immunohistochemical analysis of immune cells in cholesteatomas

Immunostaining was carried out as previously described (Hussein, 2008; Hussein et al., 2006a). Formalin fixed paraffin-embedded

sections were stained with immunoperoxidase staining techniques. Four-micron sections were cut and placed in on glass slides (Silanized slides, CE: S3003, Dako Inc, Carpinteria, CA, USA). The sections were deparaffinized in xylene, hydrated in graded ethanol and immersed in 0.3% hydrogen peroxide to block endogenous peroxidase activity. The slides were then washed twice in 0.05% mol/L phosphate-buffered saline, pH 7.4 and subsequently transferred to plastic Coplin jars filled with retrieval buffer (10-mM sodium citrate buffer, pH 6.0) and heated in a microwave oven (at a power of 750 watts) for 4 cycles of 5 min duration, each. The slides were left to cool at room temperature for 20 min. Microwave-antigen retrieval was used for CD20, CD68 and Cd1a staining whereas enzymatic digestion (trypsin) method was used for CD3 staining. The slides were treated with 10% normal goat serum for 10 min to inhibit nonspecific protein binding to antisera. Sections were then incubated with mouse monoclonal antibodies for 30 min at room temperature (PG-M1, L26, PC3/188A for CD68, CD20 and CD3, respectively, Dako Inc, Carpinteria, CA, USA). The monoclonal antibody used in this study labels CD3^ε T cells. CD3 consists of at least four different components (γ , δ , ϵ , ζ) of 20–28 kDa. On the lymphocyte cell surface, CD3 is non-covalently associated with the T-cell receptor. It is believed that the CD3 components of the TCR/CD3 complex mediate signal transduction upon antigen recognition by T-cell receptor (Jacobs, 1997). A monoclonal anti-CD1a antibody (Anti-CD1a monoclonal, 010, Novocastro, Newcastle, UK) was used. The working dilution was 1/50 dilution and the antibody was incubated with the tissue sections for 30 min at room temperature. CD1a was chosen, as it is more sensitive than other markers (S-100) for Langerhans' cells. Sections were next treated with Peroxidase-labeled Streptavidin (LSABTM2, CE, K0673, HRP, Rabbit /Mouse, Liquid DAB, Dako Inc, Carpinteria, CA, USA) for 30 min at room temperature. The enzyme was developed with 14-diaminobenzidine and 0.06% H₂O₂ for 5 min. After three washes in tap water, the slides were counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene and cover-slipped. The immunostaining of the different antibodies was contrasted by the positive and negative controls.

Positive and negative controls

Positive control slides consisted of lymph nodes with reactive lymphoid hyperplasia. Positive staining for CD68 (histiocytes in the sinuses), CD20 (B-lymphocytes in mantle zone), and CD3 (T lymphocytes in the paracortex) was seen. Specimens diagnosed as eosinophilic granulomas were used as positive controls for CD1a (Hussein, 2008). Negative control slides received the same immunohistologic treatment with substitution of the primary antibody with phosphate-buffered saline (Hussein, 2008; Hussein et al., 2006a).

Evaluation of the immunostaining results

CD3 and CD20 signals were identified as membranous brown rim around the basophilic nucleus. Signals for CD68 and CD1a appeared as diffuse and granular cytoplasmic signals. The slides were independently evaluated by counting cells (at $\times 400$ magnification) in at least 10 different fields (until 100 cells had been counted) (Hussein, 2008; Hussein et al., 2006a).

Statistical analysis

The results were expressed as mean and standard error of mean of the positively stained cells relative to other cell population. The results were statistically analyzed and computed on IBM PC microprocessor using statistical package for Social Sciences SPSS for windows. Fisher Exact Test and ANOVA (Analysis of Variance) were used. The probability value (*p* value) less than 0.05 was considered significant.

Table 1
Clinical and operative features of invasive cholesteatomas.

No	Age	Sex	Clinical presentation	Operative findings
1	30	F	Left chronic suppurative otitis media with sagging of bony meatal wall and mastoid abscess	Cholesteatoma causing extensive bone erosion of the mastoid, meatomastoid and lateral semicircular canal fistulae and no ossicles
2	28	M	Right chronic suppurative otitis media with subtotal perforation and middle ear cholesteatoma	Cholesteatoma causing extensive bone erosion of the mastoid, meatomastoid fistulae, sinus plate erosion and no ossicles
3	25	M	Right chronic suppurative otitis media with posterosuperior perforation and cholesteatoma	Cholesteatoma causing extensive bone erosion with mastoid fistula and extension into soft tissues, attic, middle ear with absent incus and stapes
4	18	M	Right chronic suppurative otitis media with posterosuperior perforation, cholesteatoma flakes and granulation tissue	Cholesteatoma causing extensive bone erosion with meatomastoid, tympano-mastoid fistulae, erosion of VII cranial nerve canal and absent incus and stapes
5	10	F	Left chronic suppurative otitis media with aural polyp	Cholesteatoma causing extensive bone erosion with meatomastoid fistula and no ossicles.
6	16	M	Right recurrent attic cholesteatoma with aural polyp	Cholesteatoma causing wide bone erosion of mastoid and sinus plate with perisinus abscess and no ossicles
7	10	F	Right chronic suppurative otitis media with posterosuperior perforation and granulation tissue	Cholesteatoma and granulation tissue with wide bone erosion of the mastoid and absent incus, malleus, and stapes superstructures.
8	18	F	Right chronic suppurative otitis media with attic cholesteatoma and mastoid fistula with overlying granulation tissue	Cholesteatoma and granulation tissue causing wide bone erosion with mastoid, meatomastoid fistulae and erosion of the tegmental plate and ossicles
9	13	M	Right chronic suppurative otitis media with aural polyp	Cholesteatoma and granulation tissue causing wide bone erosion of mastoid and ossicles
10	18	F	Right chronic suppurative otitis media with posterosuperior perforation and cholesteatoma	Cholesteatoma causing wide bone erosion of mastoid, incus long process and stapes superstructures
11	17	F	Left chronic suppurative otitis media with recurrent cholesteatoma	Cholesteatoma causing wide bone erosion of mastoid and ossicles
12	16	M	Right chronic suppurative otitis media with posterosuperior perforation, granulation tissue and labyrinthitis	Cholesteatoma and granulation tissue causing wide bone erosion of mastoid, incus, and stapes

Results

Clinical and operative findings

Twelve patients with invasive cholesteatomas were enrolled in this study. The age range of the studied group was 10 to 30 years with a mean age of 18.3 years and equal male to female ratio. Clinical and operative findings are shown in Table 1 and Fig. 1.

Immunohistological findings

Examination of the mononuclear cell infiltrate in invasive cholesteatoma showed a statistically significant increase in the total cell counts compared to those of the external canal skin ($p < 0.00$). The infiltrate consists of lymphocytes, histiocytes, and plasma cells with the lymphocytes being the most predominant cell population, followed by histiocytes. Some osteoclasts like giant cells were also

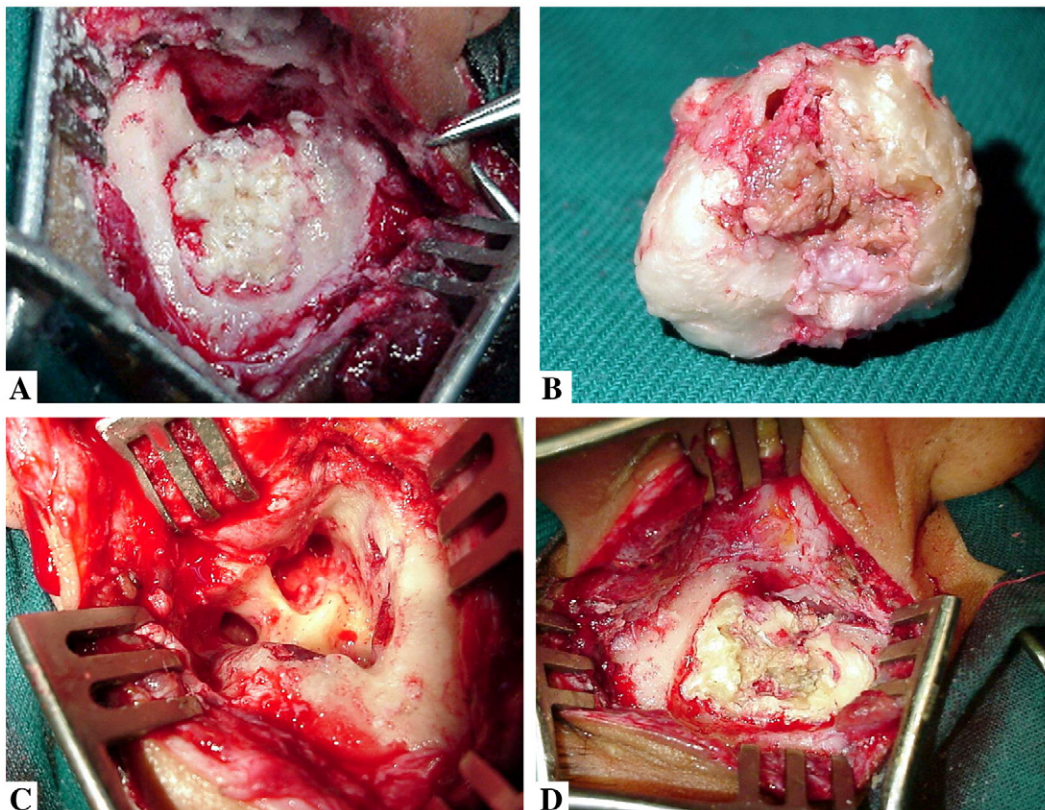


Fig. 1. Operative photographs of cholesteatomas to A: right extensive cholesteatoma creating meatomastoid fistulae; B: removed cholesteatomatous ball; C: left canal wall-down mastoidectomy; and D: right extensive cholesteatomas creating meatomastoid fistulae.

Table 2

Mean counts of the immune cells in external ear skin and cholesteatomas.

Immune cells	External ear skin	Invasive cholesteatoma	p value
Lymphocytes	1.0 ± 0.6	4.4 ± 0.4	<0.000
Histiocytes	0.8 ± 0.4	4.8 ± 0.5	<0.000
Plasma cells	0.7 ± 0.5	3.0 ± 0.2	<0.000
CD3 ⁺ cells	0.8 ± 0.3	4.7 ± 0.4	<0.000
CD68 ⁺ cells	1.0 ± 0.4	4.6 ± 0.5	<0.000
CD20 ⁺ cells	0.2 ± 0.1	0.8 ± 0.1	<0.001
CD1a ⁺ cells	0.1 ± 0.1	0.8 ± 0.1	<0.008

The slides were evaluated by counting cells (at ×400 magnification) in at least 10 different fields (until 100 cells had been counted). The results were expressed as mean and standard error of mean of the positively stained cells relative to other cell population (%). The counts of the mononuclear and immune cells in the invasive cholesteatomas were statistically significantly high compared to those in the external ear skin ($p < 0.00$).

observed. A summary of these results is shown in Table 2 and Figs. 2–3. Further immunohistological evaluation revealed highly significant counts of immune cells (CD3⁺ and CD68⁺ cells) in invasive cholesteatomas compared to the external canal skin ($p < 0.00$). In both of them, the immune cell infiltrate consisted predominantly of CD3⁺ cells and CD68⁺ (histiocytes). Rare CD20⁺ B-lymphocytes and CD1a⁺ Langerhans' cells were observed in the lesions. A summary of these finding is shown in Table 2 and Figs. 3–6.

Discussion

Cholesteatoma of the middle ear is an inflammatory process characterized by presence of a keratinized squamous epithelium, keratin and inflammatory cells. It may be associated with bone resorption (destruction) that can be mediated by various factors (cytokines) produced by an activated macrophages (granulation tissue) and

keratinocytes (Aumente et al., 1996). Here, we report the immunohistological profile of the immune cell infiltrate in invasive cholesteatomas. Our data clearly demonstrate a significantly high immune cell counts in invasive cholesteatomas compared to external canal skin. Dendritic cells (CD1a⁺ cells) were also observed among the squamous epithelial cells of cholesteatomas. These findings support the notion that the immune response in cholesteatomas is essentially a cell mediated one.

The numeric dominance of CD3⁺ cells and macrophages (CD68⁺ cells) in our series concurs with previous studies and suggests a role for the cell mediated immune response in the development of the autodestructive properties of cholesteatomas (Aberg et al., 1993; Bujia et al., 1993, 1996a, b; Liu et al., 1995; Makiishi-Shimobayashi et al., 2004; Szczepanski et al., 2006). Szczepanski et al. (2006) examined the surgical specimens of human acquired cholesteatoma and normal external auditory canal using antibodies targeting CD3⁺ T cells and CD11c⁺, HLA-DR⁺ macrophages, and found numerous T cells and relatively few macrophages in the perimatrix (15). Aberg et al. addressed the morphologic events of cholesteatoma progression 8 months after ligation of the ear canal in 14 Mongolian gerbils. They recognized four stages of morphologic development during the cholesteatomatous process: stage I, formation of an orthokeratotic plug in the ear canal; stage II, partial retraction of the tympanic membrane; stage III, a buildup of granulation tissue with prominent macrophage infiltration; and stage IV, bone destruction. The authors suggested that the development of the granulation tissue with activated macrophages is critical for the bone destruction. Bujia et al. (1993, 1996a,b) found that the vast majority of cells infiltrating the stroma of cholesteatomas consist of immunologically activated T-cells and macrophages. Makiishi-Shimobayashi et al. (2004) examined middle ear tissues including cholesteatoma obtained from 32 cases who underwent tympanoplasty and found a considerable numbers of CD68⁺ cells. It would be interesting for further studies to categorize T cell subsets in the different stages of cholesteatomas.

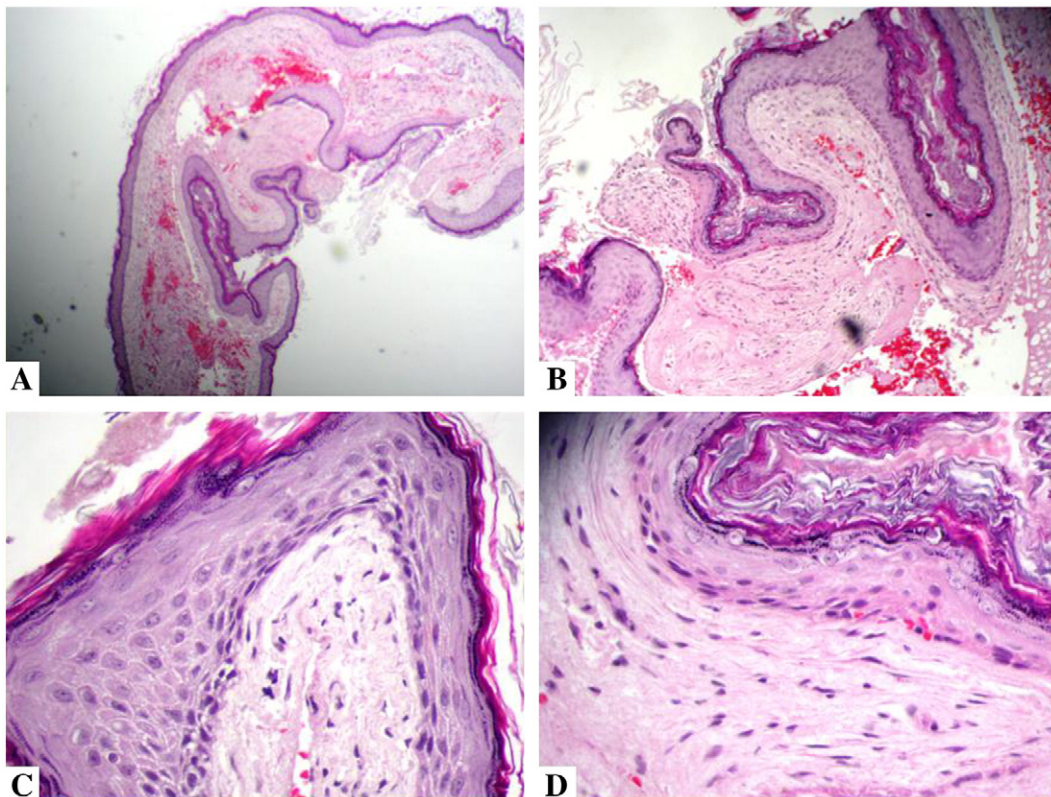


Fig. 2. Histological features of the external ear skin. The skin is composed of both epidermis and dermis. Subepithelial few mononuclear cell infiltrate (lymphocytes and histiocytes), fibroblasts, loose connective tissue stroma, and microhemorrhage are seen. No plasma cells or granulation tissue seen (A: ×100, B: ×200, C: ×400 and D: ×400).

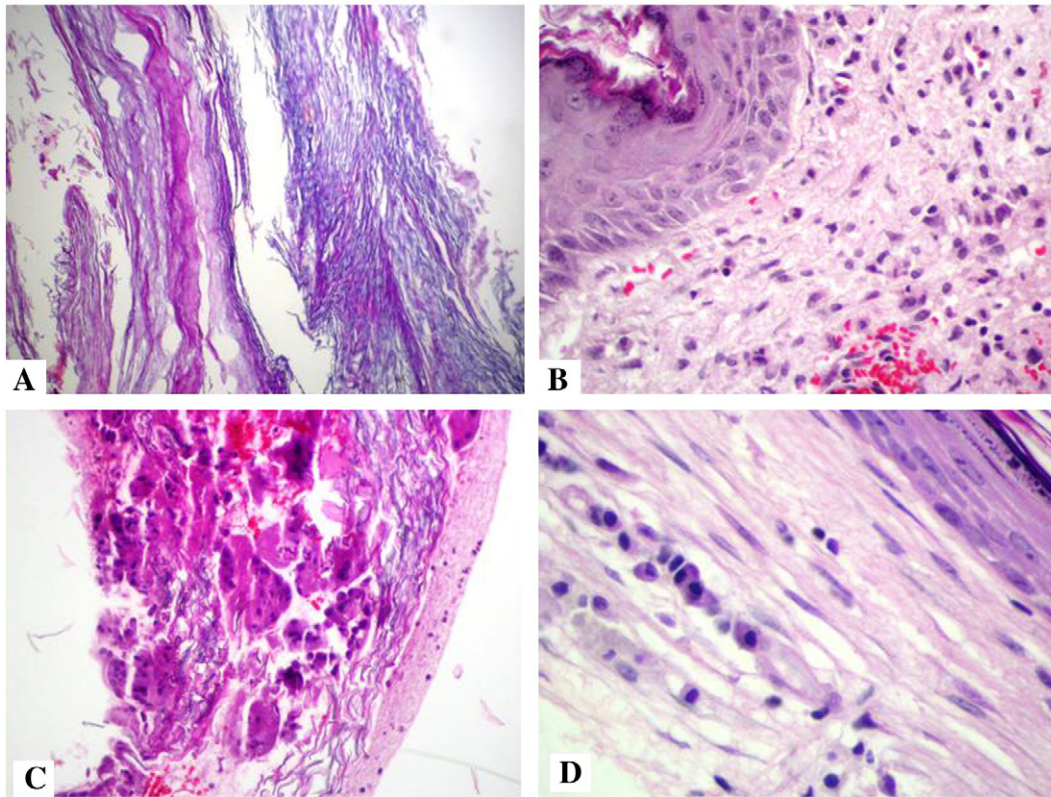


Fig. 3. Histological features of cholesteatomas. Keratinized stratified squamous epithelium with keratin debris (A: $\times 200$), subepithelial immune cells and extravasation of RBCs (B: $\times 400$), occasional multinucleated giant cells (C: $\times 400$) and aggregates of plasma cells (D: $\times 400$).

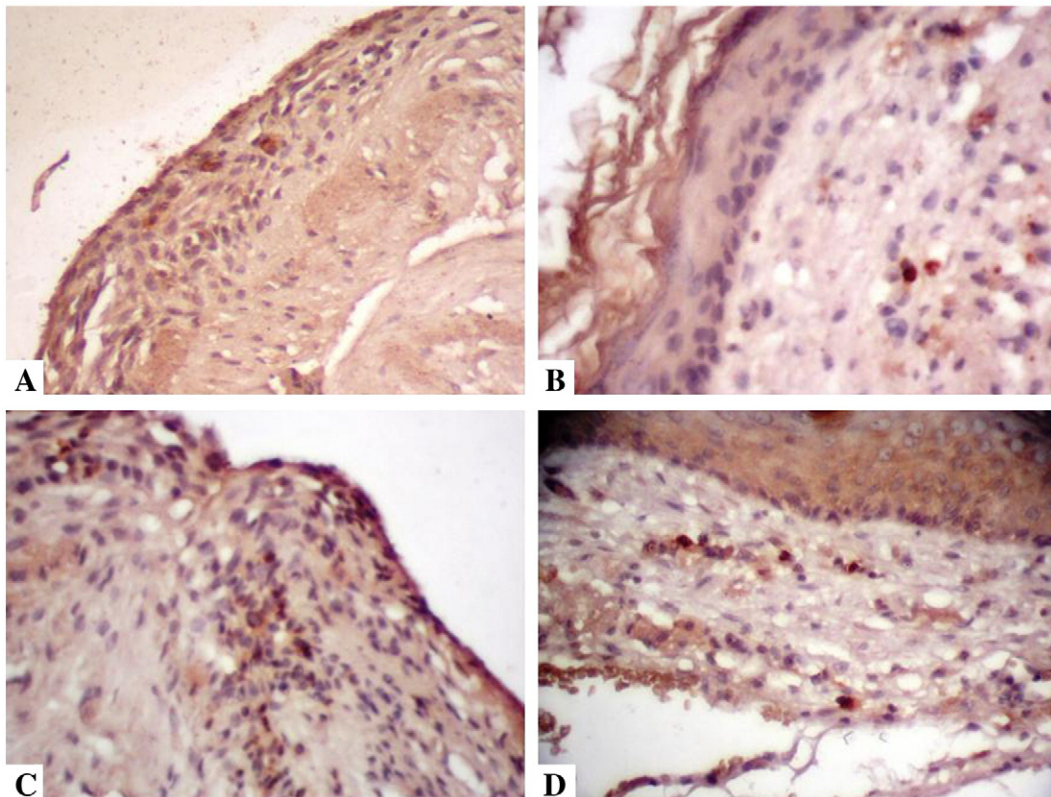


Fig. 4. Immunohistological features of mononuclear cell infiltrate in external ear skin. (A) CD1a (Langerhans' cells, $\times 400$), (B) CD20+ B lymphocytes ($\times 400$), CD3+ T lymphocytes ($\times 400$) and CD68+ histiocytes ($\times 400$).

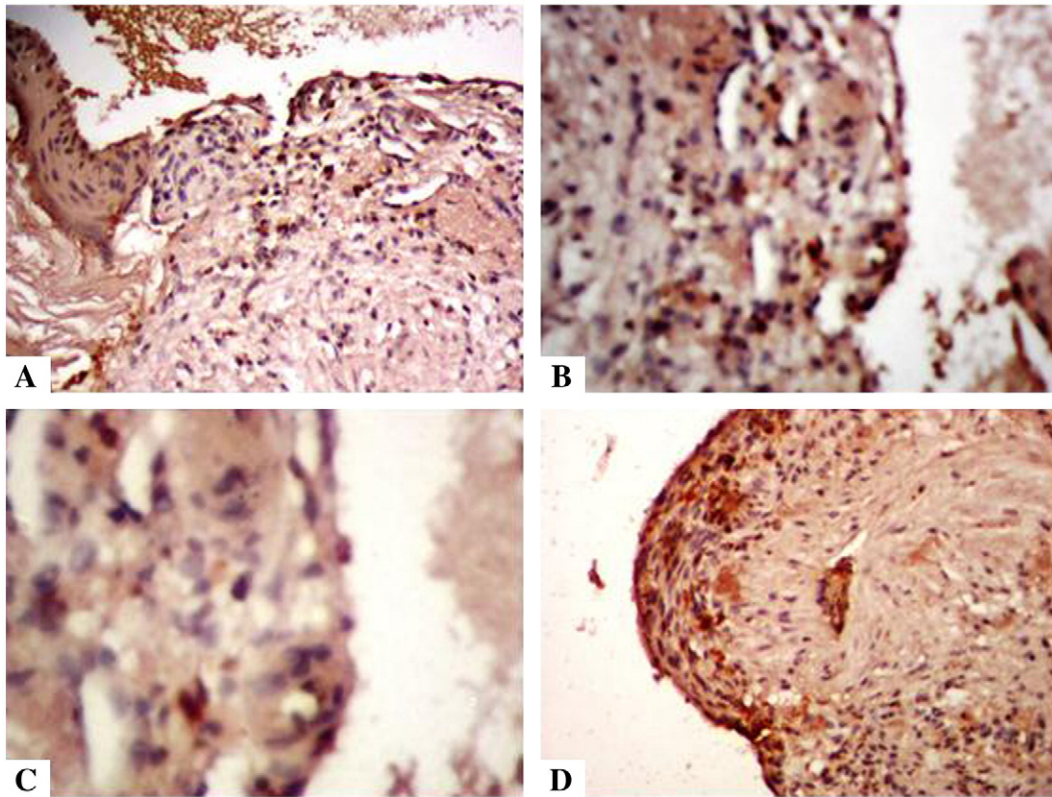


Fig. 5. Immunohistological features of mononuclear cell infiltrate (CD3 positive T-cells) in invasive cholesteatomas. The reactivity for CD3 appears as membranous staining (A–B, D: $\times 200$ and C: $\times 400$).

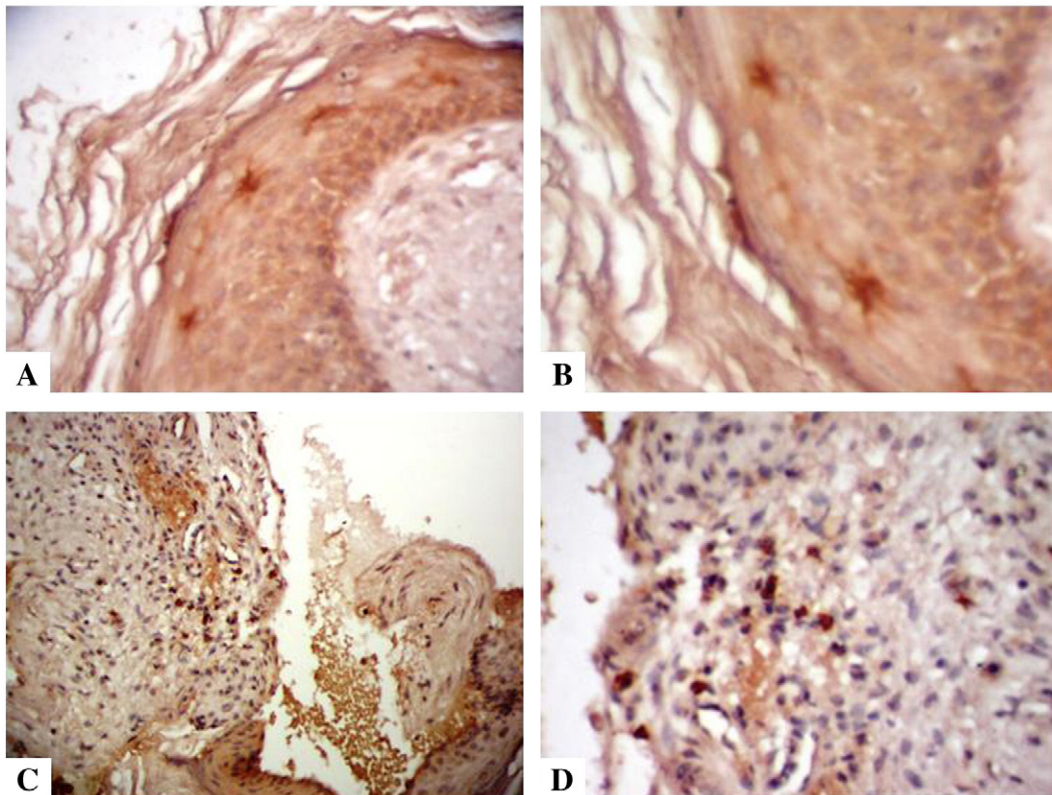


Fig. 6. Immunohistological features of mononuclear cell infiltrate (CD1a and CD68) in invasive cholesteatomas. Cd1a⁺ cells (A–B: $\times 400$) and CD68⁺ cells (C–D: $\times 400$) are shown. Cd1a⁺ cells are seen amid the squamous epithelial cells. CD68⁺ cells are observed in the subepithelial stroma.

In this investigation, we observed high counts of CD68⁺ cells in invasive cholesteatomas. This can be reasoned to the chemotactant effects of the cholesteatoma debris and some of its constituents (especially alpha-keratin). The latter can induce significant migration of the peripheral blood monocytes (Iino and Toriyama, 1992). Iino and his colleagues examined the effect of human cholesteatoma debris on mouse peritoneal macrophages *in vivo*. The number of macrophages and lymphocytes increased 5 days after injection of the debris into the peritoneal cavity. A similar increase in peritoneal cells was observed when a urea-extracted fraction of the cholesteatomatous debris or alpha-keratin, a major component of the debris, was injected (Iino et al., 1990). These findings indicate that cholesteatomatous debris acts on monocytes/macrophages as a strong chemotactant, a potent priming factor, and as an inducer of production of tumor necrosis factor. The latter is a bone-resorbing cytokine. It is conceivable that macrophages induced by the cholesteatomatous debris are involved in destructive properties of the invasive cholesteatomas (Iino and Toriyama, 1992). Interestingly, we observed some osteoclast like cells in the invasive cholesteatomas. This finding is in agreement with previous reports (Ahn et al., 1990a,b). Although the histogenesis of these cells is uncertain, it is possible that interleukin 1 (in the epithelial layer of human cholesteatomas) can promote the multinucleation of the monocytes leading to the formation of osteoclast like giant cells. Also interleukin 1 can stimulate the production of collagenases which share to the process of osteoclastic bone destruction in cholesteatomas (Ahn et al., 1990b). In cholesteatomas, there are alterations in the levels of some inflammatory cytokines that are related to tissue destruction (Akimoto et al., 2000; Bujia et al., 1996a,b; Sastry et al., 1999; Szczepanski et al., 2006). Proinflammatory cytokines such as interleukin-1 alpha (IL-1 alpha) and tumor necrosis factor-alpha (TNF-alpha) can induce bone resorption, *in vitro* (Akimoto et al., 2000). Akimoto et al. used reverse transcriptase-polymerase chain reaction, immunohistochemistry, and enzyme-linked immunosorbent assay to examine the expression of these molecules in cholesteatomas. Increased levels of IL-1 and TNF-alpha were detected in cholesteatomas as compared to normal skin. The authors suggested that TNF-alpha may play a crucial role in the pathogenesis of both acquired and congenital cholesteatomas by regulating bone resorption and cell infiltration (Akimoto et al., 2000). Bujia et al. used immunostaining methods to examine the distribution of IL-6 in the cholesteatomatous tissue. Levels of this cytokine were quantified in tissue extracts using an enzyme-linked immunosorbent assay. Also, the presence of biologically active IL-6 was analyzed in the murine cell line 7TD1. Immunohistologically, a strong IL-6 protein expression was observed in cholesteatomatous tissue compared with moderate expression in normal skin (control tissue). The over-expression of IL-6 in middle ear cholesteatoma suggests a participation of this cytokine in epithelial hyperproliferation and bone resorption (Aumente et al., 1996). Serum TNF-alpha levels in patients with cholesteatomas were significantly higher than in controls. These levels were high in patients with bone destruction compared to those without bone destruction. The TNF-alpha was localized in various layers of cholesteatomatous epithelium using indirect immunoperoxidase staining (Sastry et al., 1999).

The presence of Langerhans' cells in our series is in agreement with other studies (Ma, 1990; Park, 1994; van Dijk et al., 1986). Langerhans' cells are distinct class of leukocytes (Hussein, 2008). They are competent and professional antigen-presenting cells. They are also characterized by their ability to freely migrate through tissues where they can engulf, process, and present antigens. Langerhans' cells closely exert their custodial functions in collaboration with the T-lymphocytes, i.e. Langerhans' cells can interact with, stimulate, and direct T-lymphocyte responses. As such, they are capable of triggering a primary T cell response (Hussein, 2005 and Hussein et al., 2006b). Using monoclonal antibodies against Langerhans' cells and T-lymphocyte membrane receptors, these cell populations were found in the cholesteatomatous

matrices (van Dijk et al., 1986). It is possible that some Langerhans' cells-T cell interplay in cholesteatomas may share to the production of prostaglandins and lymphokines, which may play an important role in the bone destruction (Liu et al., 1995; Ma, 1990).

Several molecules are involved in the pathogenesis of invasive cholesteatomas such as matrix metalloproteinases, oncogenes and apoptosis inhibitors (Banerjee et al., 2001; Kobayashi et al., 2005; Mehta et al., 2007; Park et al., 2009; Schmidt et al., 2001; Suchozebrska-Jesionek et al., 2008). Matrix metalloproteinases are enzymes capable of a proteolytic degradation of the extracellular matrix and therefore are important during tissue destruction and invasion (Shokry et al., 2009). Previous studies indicated that cholesteatomatous tissue express several matrix metalloproteinases (Matrix metalloproteinases: MMP-1, MMP-2, MMP-3 MMP-8, and MMP-9) (Banerjee et al., 2001; Kobayashi et al., 2005; Mehta et al., 2007; Schmidt et al., 2001; Suchozebrska-Jesionek et al., 2008). Matrix metalloproteinase 1 is over-expressed by the stromal fibroblasts present in cholesteatomas as compared with deep meatal skin (Banerjee et al., 2001). Matrix metalloproteinase 2 and 9 derive from different genes, but they have similar structure and substrate specificity. These two proteinases are synthesized as latent proenzymes and must be activated in order to show their proteolytic activities and degrade various components of extracellular matrix including type IV, V, VII, and X collagens, fibronectin and gelatin (Shokry et al., 2009). Morales and his colleagues examined the expression pattern of matrix metalloproteinase-2 (MMP-2) in invasive (causing complications) compared to latent cholesteatomas (not causing complications). A strong matrix metalloproteinase 2 expression was observed in the majority (7 out of 8 cases) of invasive cholesteatomas. In contrast, few cases (3 out of 11 cases) of latent cholesteatomas showed strong matrix metalloproteinase 2 expression (Morales et al., 2007). Juhasz et al. examined cholesteatomatous tissue samples immunohistochemically. Tissue samples were arranged on the basis of bone destruction for the expression pattern of matrix metalloproteinase 9. A prominent matrix metalloproteinase 9 staining was detected in invasive cholesteatomas compared with non-invasive ones. Therefore, these molecules seem to play pivotal roles in bone destruction during cholesteatoma progression (Juhasz et al., 2009). Taken together, it is tempting to hypothesize that "tissue invasion during the development of cholesteatoma is partly regulated by matrix-metalloproteinases and therefore aberrations in metalloproteinases-expression may play important roles in the development of cholesteatomas". Further studies are required to test this hypothesis. Keratinocytes in cholesteatomas show uncoordinated hyperproliferation, migration, and invasion properties (Park et al., 2009). p63 is a p53 homologue and a marker expressed in replicating keratinocytes. It is expressed in certain epithelial cells at high levels under normal conditions. Over-expression of specific p63 splice variants is observed in squamous carcinomas suggesting that p63 may act as an oncogene. Survivin, also called baculoviral inhibitor of apoptosis repeat-containing 5 is expressed in most solid and hematologic malignancies. Survivin is a member of the inhibitor of apoptosis family. It functions to inhibit caspase activation therefore leading to negative regulation of apoptosis. Park et al. examined the expression of these molecules in 40 human middle ear cholesteatomas epithelium and 5 skin tissues obtained from patients during ear surgery. P63 expression was diffusely observed in entire samples of cholesteatomas, especially in acquired cholesteatomas, compared with the control group. Primary and recurrent cholesteatomas showed no significant difference in p63 expression. Survivin protein expression was seen in the majority of the cholesteatomatous specimens. Acquired cholesteatomas showed prominent survivin expression compared with congenital cases. The authors suggested that these molecules are involved in the pathogenesis of cholesteatomas (Park et al., 2009).

To conclude, here we report the profile of the immune cells in invasive cholesteatomas. The presence of these cells (especially CD68⁺

cells and CD3⁺ cells) in the subepithelial tissue suggests that they share to the autoaggressive bone resorption properties associated with cholesteatomatous process. Further analysis of the types of T cell subsets and the expression of proinflammatory cytokines, certain oncogenes (such as p53, p63), apoptosis inhibitor genes (such as survivin) and matrix metalloproteinases in the different developmental stages of cholesteatomas is open for further investigations.

References

- Aberg, B., Edstrom, S., Bagger-Sjoberg, D., Kindblom, L.G., 1993. Morphologic development of experimental cholesteatoma. *Arch. Otolaryngol. Head Neck Surg.* 119 (3), 272–275.
- Abramson, M., Huang, C.C., 1977. Localization of collagenase in human middle ear cholesteatoma. *Laryngoscope* 87, 771–791.
- Ahn, J.M., Huang, C.C., Abramson, M., 1990a. Localization of interleukin-1 in human cholesteatoma. *Am. J. Otolaryngol.* 11, 71–77.
- Ahn, J.M., Huang, C.C., Abramson, M., 1990b. Third place–Resident Basic Science Award 1990. Interleukin 1 causing bone destruction in middle ear cholesteatoma. *Otolaryngol. Head Neck Surg.* 103 (4), 527–536.
- Akimoto, R., Pawankar, R., Yagi, T., Baba, S., 2000. Acquired and congenital cholesteatoma: determination of tumor necrosis factor- α , intercellular adhesion molecule-1, interleukin-1- α and lymphocyte functional antigen-1 in the inflammatory process. *ORL J. Otorhinolaryngol. Relat. Spec.* 62, 257–265.
- Aumente, P.O., Bujia, J., Kim, C., Jimenez Gimenez, J., Lopez Villarejo, P., 1996. A quantitative study of the presence of interleukin-1 and interleukin-6 in cholesteatoma of the middle ear. *Acta Otorrinolaryngol. Esp.* 47 (4), 259–262.
- Banerjee, A.R., Jones, J.L., Birchall, J.P., Powe, D.G., 2001. Localization of matrix metalloproteinase 1 in cholesteatoma and deep meatal skin. *Otol. Neurotol.* 22, 579–581.
- Bujia, J., Holly, A., Kim, C., Schilling, V., Kastenbauer, E., 1993. New aspects on the pathogenesis of cholesteatoma: the possible role of immune cell-induced keratinocyte hyperproliferation. *Laryngorhinootologie* 72, 279–283.
- Bujia, J., Holly, A., Stammberger, M., Sudhoff, H., 1996a. Angiogenesis in cholesteatoma of the middle ear. *Acta Otorrinolaryngol. Esp.* 47, 187–192.
- Bujia, J., Kim, C., Ostos, P., Kastenbauer, E., Hultner, L., 1996b. Role of interleukin 6 in epithelial hyperproliferation and bone resorption in middle ear cholesteatomas. *Eur. Arch. Otorhinolaryngol.* 253, 152–157.
- Gersdorff, M.C., Debaty, M.E., Tomasi, J.P., 2006. Pathophysiology of cholesteatoma. *Rev. Laryngol. Otol. Rhinol. (Bord)* 127, 115–119.
- Hussein, M.R., 2005. Dendritic cells and melanoma tumorigenesis: an insight. *Cancer Biol. Ther.* 4 (5), 501–505.
- Hussein, M.R., 2008. Evaluation of Langerhans' cells in normal and eczematous dermatitis skin by CD1a protein immunohistochemistry: preliminary findings. *J. Cutan. Pathol.* 35 (6), 554–558.
- Hussein, M.R., Ahmed, R.A., 2005. Analysis of the mononuclear inflammatory cell infiltrate in the non-tumorigenic, pre-tumorigenic and tumorigenic keratinocytic hyperproliferative lesions of the skin. *Cancer Biol. Ther.* 4 (8), 819–821.
- Hussein, M.R., Elhers, D.A., Fadel, S.A., Omar, A.E., 2006a. Immunohistological characterisation of tumour infiltrating lymphocytes in melanocytic skin lesions. *J. Clin. Pathol.* 59 (3), 316–324.
- Hussein, M.R., Hamed, S.A., Mostafa, M.G., Abu-Dief, E.E., Kamel, N.F., Kandil, M.R., 2006b. The effects of glucocorticoid therapy on the inflammatory and dendritic cells in muscular dystrophies. *Int. J. Exp. Pathol.* 87 (6), 451–461.
- Iino, Y., Toriyama, M., 1992. Human monocytes show chemotaxis in response to cholesteatoma debris. *Nippon Jibiinkoka Gakkai Kaiho* 95, 25–31.
- Iino, Y., Toriyama, M., Ohmi, S., Kanegasaki, S., 1990. Activation of peritoneal macrophages with human cholesteatoma debris and alpha-keratin. *Acta Otolaryngol.* 109, 444–449.
- Jacobs, H., 1997. Pre-TCR/CD3 and TCR/CD3 complexes: decamers with differential signalling properties? *Immunol. Today* 18, 565–569.
- Juhasz, A., Sziklai, I., Rakosy, Z., Ecsedi, S., Adany, R., Balazs, M., 2009. Elevated level of tenascin and matrix metalloproteinase 9 correlates with the bone destruction capacity of cholesteatomas. *Otol. Neurotol.* 30, 559–565.
- Kobayashi, H., Asano, K., Kanai, K., Suzuki, H., 2005. Suppressive activity of vitamin D3 on matrix metalloproteinase production from cholesteatoma keratinocytes in vitro. *Mediators Inflamm.* 2005, 210–215.
- Laeq, S., Faust, R., 2007. Modeling the cholesteatoma microenvironment: coculture of HaCaT keratinocytes with WS1 fibroblasts induces MMP-2 activation, invasive phenotype, and proteolysis of the extracellular matrix. *Laryngoscope* 117, 313–318.
- Liu, L., Li, Z., Hu, M., 1995. Langerhans cells and human aural cholesteatoma. *Zhonghua Er Bi Yan Hou Ke Za Zhi* 30, 33–36.
- Ma, W.L., 1990. Research on the mechanism of epidermal Langerhans cells on bone destruction in cholesteatoma. *Zhonghua Er Bi Yan Hou Ke Za Zhi* 25, 345–347.
- Makiishi-Shimobayashi, C., Tsujimura, T., Iwasaki, T., Kakihana, M., Shimano, K., Terada, N., Sakagami, M., 2004. Localization of osteopontin at calcification sites of cholesteatoma: possible role as a regulator of deposition of calcium phosphate in the middle ear. *Auris, Nasus, Larynx* 31, 3–9.
- Mehta, D., Daudia, A., Birchall, J.P., Banerjee, A.R., 2007. The localization of matrix metalloproteinases-8 and -13 in cholesteatoma, deep-meatal and post-auricular skin: a comparative analysis. *Acta Otolaryngol.* 127, 138–142.
- Morales, D.S., Penido Nde, O., da Silva, I.D., Stavale, J.N., Guilherme, A., Fukuda, Y., 2007. Matrix metalloproteinase 2: an important genetic marker for cholesteatomas. *Braz. J. Otorhinolaryngol.* 73, 51–57.
- Ottaviani, F., Neglia, C.B., Berti, E., 1999. Cytokines and adhesion molecules in middle ear cholesteatoma. A role in epithelial growth. *Acta Otolaryngol.* 119, 462–467.
- Park, H.R., Min, S.K., Min, K., Jun, S.Y., Seo, J., Kim, H.J., 2009. Increased expression of p63 and survivin in cholesteatomas. *Acta Otolaryngol.* 129, 268–272.
- Park, K., 1994. Significance of Langerhans' cells in middle ear cholesteatoma. *Yonsei Med. J.* 35, 438–445.
- Potsic, W.P., Samadi, D.S., Marsh, R.R., Wetmore, R.F., 2002. A staging system for congenital cholesteatoma. *Arch. Otolaryngol. Head Neck Surg.* 128 (9), 1009–1012.
- Quaranta, A., Bartoli, R., Lozupone, E., Resta, L., Iurato, S., 1995. Cholesteatoma in children: histopathologic findings in middle ear ossicles. *ORL J. Otorhinolaryngol. Relat. Spec.* 57, 296–298.
- Sastry, K.V., Sharma, S.C., Mann, S.B., Ganguly, N.K., Panda, N.K., 1999. Aural cholesteatoma: role of tumor necrosis factor- α in bone destruction. *Am. J. Otol.* 20, 158–161.
- Schilling, V., Bujia, J., Negri, B., Schulz, P., Kastenbauer, E., 1991. Immunologically activated cells in aural cholesteatoma. *Am. J. Otolaryngol.* 12, 249–253.
- Schmidt, M., Grunsfelder, P., Hoppe, F., 2001. Up-regulation of matrix metalloproteinase-9 in middle ear cholesteatoma—correlations with growth factor expression in vivo? *Eur. Arch. Otorhinolaryngol.* 258, 472–476.
- Shokry, M., Omran, O.M., Hassan, H.I., Elsedfy, G.O., Hussein, M.R., 2009. Expression of matrix metalloproteinases 2 and 9 in human trophoblasts of normal and preeclamptic placentas: preliminary findings. *Exp. Mol. Pathol.* 87, 219–225.
- Suchozebrska-Jesionek, D., Szymanski, M., Kurzepa, J., Golabek, W., Stryjecka-Zimmer, M., 2008. Gelatinolytic activity of matrix metalloproteinases 2 and 9 in middle ear cholesteatoma. *J. Otolaryngol. Head Neck Surg.* 37, 628–632.
- Szczepanski, M., Szyfter, W., Jenek, R., Wrobel, M., Lisewska, I.M., Zeromski, J., 2006. Toll-like receptors 2, 3 and 4 (TLR-2, TLR-3 and TLR-4) are expressed in the microenvironment of human acquired cholesteatoma. *Eur. Arch. Otorhinolaryngol.* 263, 603–607.
- van Dijk, C.M., Visser, C.E., Veldman, J.E., 1986. Spatial distribution of Langerhans' cells and T-lymphocyte subpopulations in human tympanic membrane and aural cholesteatoma. *Virchows Arch., B Cell Pathol. Incl. Mol. Pathol.* 52, 143–152.