Cellular signaling in human cholesteatoma

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Chapter 3

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Cholesteatoma epithelium is characterized by increased expression of Ki-67, p53 and p21,with minimal apoptosis

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Abstract

Objective- to investigate differences in cell proliferation, cell cycle arrest and apoptosis between cholesteatoma and control skin.

Materials and Methods- Immunohistochemical sections of 15 cholesteatoma and 15 paired control retro-auricular skin samples were examined for Ki-67, p53, p21 and active caspase 3 using image analysis, as well as for DNA fragmentation.

Results- The retro-auricular skin samples contained 5.7± 3.6% of Ki-67-positive cells and showed a normal expression pattern. In the cholesteatoma epithelium 11.7± 9.5% of the cells were Ki-67-positive and these cells were dominantly expressed in the basal and parabasal cell layers. Retro-auricular skin contained 5.8± 5.4% p53-positive cells and 1.0± 0.9% p21-positive cells. In the cholesteatoma epithelia 17.8± 12.3% of the cells were p53-positive and 14.3± 11.6% were p21-positive. The expression of Ki-67, p53 and p21 differed significantly between the two groups ($p<0.05$). In the cholesteatoma epithelium a positive correlation was found between p53 and p21 expression ($p= 0.016$). Active caspase 3 positivity and DNA fragmentation were rarely seen in the cholesteatoma epithelium.

Conclusion- Our results indicate that increased cell proliferation in cholesteatoma epithelium is accompanied by an increase in p53 and p21 protein levels whilst apoptosis is minimal.

Keywords: *active caspase 3, apoptosis, cholesteatoma, immunohistochemistry, Ki-67, p21, p53, terminal deoxynucleotide transferase-mediated dUTP nick-end labeling.*

Introduction

Cholesteatoma is a benign, but destructive middle ear tumor, characterized by a hyperproliferative epithelium with progressive accumulation of keratin. Homeostatic growth of keratinocytes depends on the controlled coordination of cell proliferation and programmed cell death. However, in cholesteatoma this process appears to be unbalanced. It has been shown that proliferating cells in the cholesteatoma epithelium are dislocated[1] and that the level of expression of proliferation markers is aberrant[2]. Cell viability or cell death is determined by the activity of a complex intertwined gene family of cell death stimulators and inhibitors. One of the most important members of this family is the p53 protein. It can activate a cascade of programmed cell death executioner cysteine proteases, known as caspases[3,4]. Among these, activated caspase 3 is considered to be an important marker of ongoing apoptosis[5]. Besides initiating apoptosis, p53 can also perform other functions such as controlling the initiation of mitosis[6]. One part of the mechanism by which p53 blocks cells at the G1 checkpoint involves upregulation of p21, a cyclin-dependent kinase inhibitor[7]. This dual function, the regulation of either apoptosis or cell cycle arrest, makes the role of p53 in the formation and development of cholesteatoma of particular interest. In the literature, however, there is controversy regarding the level of expression of p53[2,8,9] and the degree of apoptosis in the cholesteatoma.[9-11]. There is also a difference of opinion concerning

the degree of cell proliferation in cholesteatoma with respect to the expression of p53[2,8,9]. Furthermore, in cholesteatoma, studies of cell cycle arrest by means of p21 expression have not previously been published. The present study was instituted in order to compare the degree of cell proliferation, cell cycle arrest and apoptosis in the cholesteatoma epithelium. For this purpose we have determined the expression of: p53, the proliferation marker Ki-67, p21 and activated caspase 3. As a second measure of apoptosis we determined DNA fragmentation by means of the terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) technique and calculated the percentage of apoptotic cells in the tissue samples. The results obtained in cholesteatoma tissue were compared to those in paired control samples from retro-auricular skin.

Materials and methods

Clinical and histopathological data

Cholesteatoma specimens from the pars flaccida and biopsies of retro-auricular skin were obtained from fifteen patients, and immediately placed in phosphate buffered saline. The Committee of Medical Ethics of the Leiden University Medical Center approved the protocol. The specimens were prepared for histological examination by fixation in 4% buffered formaldehyde for 20 h. and dehydration in ethanol and were then embedded in paraffin wax.

Immunohistochemistry

Serial sections (4μm) were taken from each tissue block. The first and the last sections were stained with hematoxylin- eosin (HE). Subsequent sections were immunostained with either p53, p21, Ki-67 or active caspase 3 antibodies. The penultimate section was used for in situ detection of fragmented DNA using the TUNEL technique. Sections from the same tissue served as negative controls, i.e. the primary antibody was omitted. The expressions of p53, p21, Ki-67 and active caspase 3 were determined using an indirect immunoperoxidase method. Anti-p53 (DO7), anti-p21(WAF1) and anti- Ki-67(MIB-1) were monoclonal antibodies purchased from NeoMarkers Inc (Fremont, CA), Oncogene Research Products (Cambridge, UK) and Immunotech (Marseilles, France), respectively. The dilutions used were 1: 1000, 1:200 and 1: 400, respectively. The active caspase 3 (3p20) polyclonal antibody was purchased from Promega (Madison, WI) and diluted 1: 400. Sections from coloncarcinoma (p53), sigmoid colon (p21), tonsil (Ki-67), thymus and castrated rat prostate (active caspase 3) were used as positive controls. To inactivate endogenous peroxidase, the deparaffinized sections were treated with methanol containing 3% H_2O_2 for 20 minutes. After rehydration, the sections were subjected to microwave antigen retrieval in citrate buffer (0.01M, pH 6.0) for 12 min[12]. The sections assigned for p21 and active caspase 3 antibody treatment were also subjected to pepsin antigen retrieval (4% pepsin in 3mM HCl) for 5 min at room temperature (RT).[13] All sections were incubated with the primary antibody overnight at RT and then washed in PBS. The specimens were incubated with appropriate biotinylated secondary antibodies for 30 min at RT, washed and

subsequently incubated with peroxidase-conjugated streptavidin at RT for 30 min. They were then treated with 3,3' di- aminobenzidine (DAB) chromogen containing 0.02% H_2O_2 and counterstained with HE for 1 min.

TUNEL staining

Fragmented DNA was monitored by means of an adapted fluorochrome/ enzyme immunoassay, a modification of the method of Negoescu et al[14]. We used samples of thymus and castrated rat prostate as positive controls. After deparaffination and rehydration, the sections were pretreated with microwave irradiation (750W, 0.1M citrate buffer) for 1min and rapidly cooled. After washing with Tris-buffered saline (TBS), they were incubated with blocking buffer (0.1 M Tris-HCl, 3% bovine serum albumin and 20% newborn calf serum) for 15 min at RT. The slides were rinsed with TBS and the specimens were incubated with labeling mixture in a humid atmosphere at 37ºC for 60 min. The labeling mixture contained terminal deoxynucleotidyl transferase and fluorescein-d uridine triphosphate [In Situ Cell Death (ISCD) detection kit; Roche Diagnostics, Mannheim, Germany]. Transferring the slides to stop buffer (300mM sodiumchloride, 30 mM sodiumcitrate) for 15 min at RT terminated the reaction. After washing with TBS, incubation with blocking buffer was repeated and the slides were then rinsed again in TBS. Fluorescein was labeled with peroxidase-conjugated rabbit anti-fluorescein isothiocyanate (DAKO, Glostrup, Denmark), diluted 1:50 with blocking buffer for 30 min at RT. This modification was used because pilot studies with the ISCD kit converter revealed false positives. The sections were stained with DAB chromogen for 1 min and counterstained with HE.

Morphometric analysis of immunohistochemical data

For each of the immunohistochemical markers studied, DAB positive staining was quantified using an image analysis system (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK). The microscope was a Leica DMLB with a Leica DC 200 digital camera. The computer-assisted system used to determine the immunohistochemical positive staining has been described elsewhere[15]. For each section, images from at least five different areas were stored as digitized images. For cell counting the same areas of the sections, but with various stains, were used. The epithelial compartment was delineated on the screen and positive and negative cells were counted automatically. In each section >1000 cells were counted and the percentage of positive cells was determined.

Data analysis

Data are expressed as means ± SD. In order to compare the means of paired variables, the paired samples t-test was used, with a level of significance of $p < 0.05$ was performed. The Pearson two-tailed correlation test was used to calculate possible correlations. Correlation was considered significant at the 0.05 level. The SPSS 10 software package (SPSS, Chicago, IL) was used for the calculations.

Results

Histopathological findings

In 10/15 cholesteatoma samples we found inflammatory cells and newly formed blood vessels in the connective tissue. In one tissue sample there was insufficient perimatrix for analysis. In the retro-auricular skin sections there was no evidence of inflammation.

Immunohistochemical staining of Ki-67

In the retro-auricular skin, the proportion of Ki-67-positive cells in the upper basal layer ranged from 2.0% to 13.0%. The cholesteatoma epithelium showed positive Ki-67 staining in cells of the upper basal layer and to a lesser extend in the suprabasal layers (Fig1B). In the connective tissue some Ki-67-positive cells were present. The proportion of Ki-67-positive nuclei in the cholesteatoma epithelium showed large individual variations, ranging from 1.7% to 35.6%. On average, however, the cholesteatoma samples showed a significantly increased percentage of Ki-67-positive cells, compared to retro-auricular skin ($p=0.031$). The average percentages of Ki-67-positive cells in retro-auricular skin and cholesteatoma epithelium are summarized in Table1. The increased cell proliferation in the cholesteatoma epithelium was not related to the presence of inflammation, as non-inflamed cholesteatoma tissues also showed an increased Ki-67 expression.

Tissue	Ki-67	p53	p21
Retro-auricular skin	5.7 ± 3.6	5.8 ± 5.4	1.0 ± 0.9
Cholesteatoma epithelium	11.7 ± 9.5*	17.8 ± 12.3**	14.3 ± 11.6**

Table 1. *Percentages of Ki-67-, P53- and P21-positive cells in control skin and cholesteatoma epithelium.**p< 0.05; **p< 0.01 versus retro-auricular skin.

Immunohistochemical staining of p53

The retro-auricular skin samples showed 0.8% to 19.2% p53-positive cells in the basal layer. Most of the staining for p53 was found in the cells of the basal layer of the cholesteatoma epithelium, with some staining in cells in the connective tissue (Fig.1C.). The proportion of p53-positive cells in the cholesteatoma epithelium varied greatly, ranging from 0.3% to 39.1%. The percentage of p53-positive cells was significantly increased compared to that in retro-auricular skin ($p=0.007$). The average percentages of p53-positive cells in retro-auricular skin and cholesteatoma epithelium are listed in Table2. We found no difference in p53 expression between inflamed cholesteatoma samples and non-inflamed cholesteatoma samples.

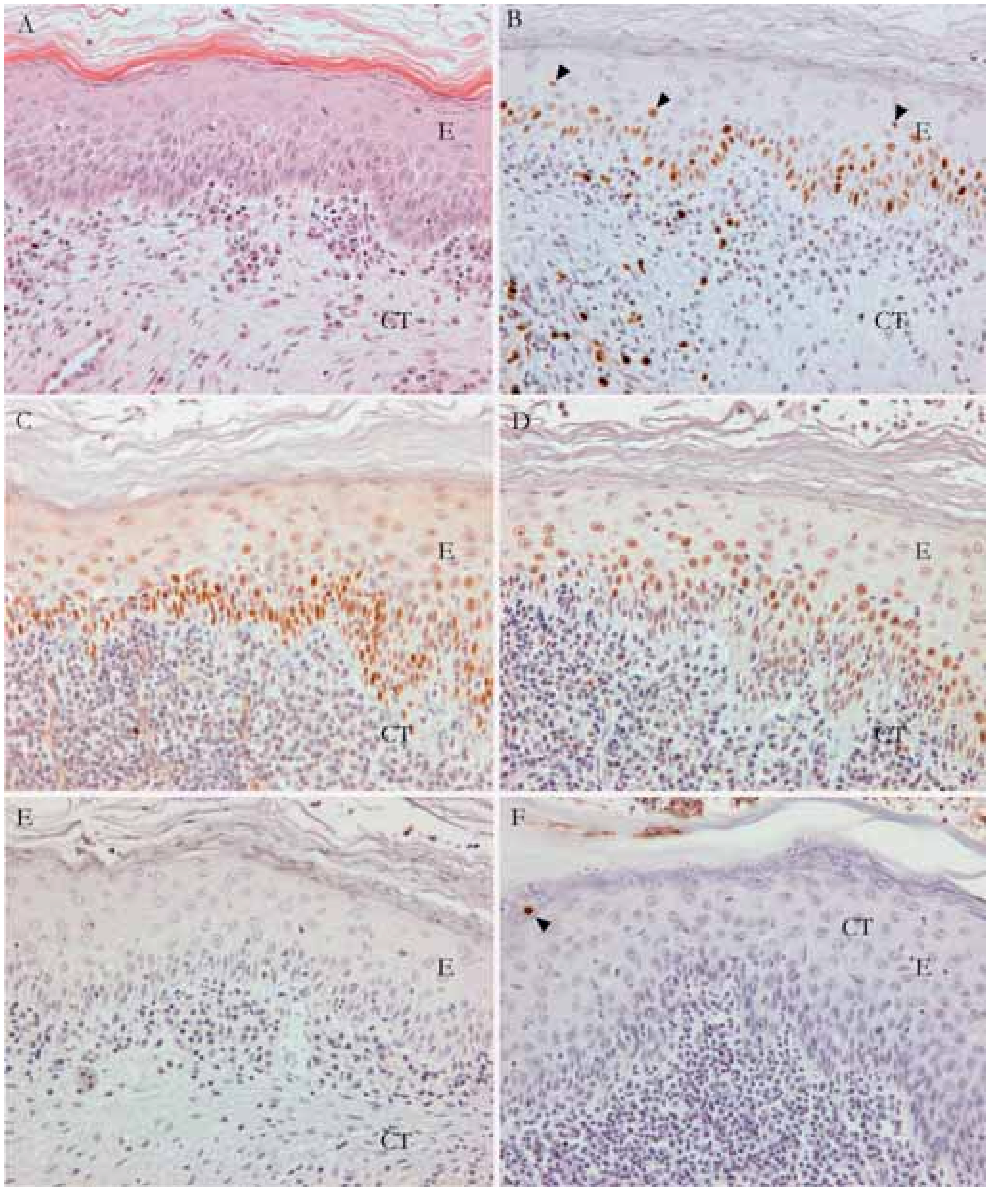

Fig. 1 Immunohistochemical localization of p53, p21 and Ki-67 in serial sections of human cholesteatoma epithelium: (A)HE staining; (B) Ki-67-positive cells are expressed in the basal and suprabasal layers of the epithelium (*arrowheads*); (C) Many p53-positive cells are expressed in the basal layer;(D) p21-positive cells are expressed in the lower suprabasal layers; (E) active caspase3; (F) TUNEL staining showing a positive cell (*arrowhead*). Original magnification x 200. E= epithelium; CT= connective tissue

Immunohistochemical staining of p21

In retro-auricular skin a maximum of 2.1% of the cells were positive for p21. The p21- positive cells of the retro-auricular skin and the cholesteatoma epithelium were locally expressed in the lower suprabasal layers. In some cholesteatoma, p21 expression was also observed in the connective tissue (Fig.1D). p21-positive cells were present in the epithelium of all 15 cholesteatoma samples. The proportion of p21-positive cells varied considerably, ranging from 0.4% to 32.5%. When compared to retro-auricular skin, the percentage of p21-positive cells in cholesteatoma tissue was significantly increased ($p=0.001$). Table 2 lists the percentage of p21-positive cells in retro-auricular skin and cholesteatoma epithelium. We found no difference in p21 expression between samples with or without inflammation.

Co-expression of Ki-67, p53 and p21

Using the Pearson two-tailed correlation test, a significant positive association was observed between p53 and p21 protein expression ($p= 0.016$). We did not find a correlation between any of the other proteins.

Detection of apoptosis

Immunohistochemical staining of active caspase 3

The retro- auricular skin sometimes contained a single positive cell in the granular layer. In the cholesteatoma epithelium samples there were hardly any active caspase 3 positive cells (Fig.1E). In those cells that were positive for active caspase 3, the nuclei showed a fragmented morphology. The data were not used for further calculations as all tissue samples contained a negligible number of positive cells.

TUNEL (modified method)

The retro- auricular skin cells of the granular layer were only occasionally TUNEL-positive. The stromal portion showed a small number of TUNEL-positive cells in 10 cholesteatoma samples. In the stroma of some cholesteatoma samples a few clusters of TUNEL-positive cells were detected, indicating an increase in apoptosis. In some epithelia of cholesteatoma samples a single TUNEL-positive cell was observed (Fig.1F). All TUNEL-positive cells had fragmented nuclei. The samples showed a negligible number of TUNEL-positive cells and therefore these data were not used for further calculations.

Discussion

This is the first report to document, in the same study, cell proliferation, cell cycle arrest and apoptosis in cholesteatoma epithelium. In most cholesteatoma samples we observed active inflammation, involving neutrophils, monocytes, macrophages and newly formed blood vessels. In the epithelial layer of the cholesteatoma we found increased expression of the proliferation marker Ki-67. Concomitantly, the cholesteatoma epithelium showed overexpression of p53. The increased Ki-67 expression was not related to the overexpression of p53. In addition, we found a remarkable increased proportion of p21 in the epithelial layer. We demonstrated a significant positive correlation between p53 and p21. Apoptosis, which is determined

by active caspase 3 expression, was not detected in the cholesteatoma epithelium. This was confirmed by the observation that a negligible proportion of apoptotic cells was determined using the TUNEL technique. The abundant presence of the proliferation marker Ki-67 and the overexpression of p53 in cholesteatomal tissue have been reported before[1]. High expression of p53 has also been mentioned by others, but with a subsequent high level of apoptosis[9], as determined using the TUNEL assay. Our analyses, however, clearly demonstrate that there was no increase in apoptosis in the cholesteatoma samples. Our study demonstrates cell proliferation, characterized by increased Ki-67 protein expression, but also an increase in p53 protein level, which suggests a halt to proliferation by means of G1 blockade[7]. These unexpected results might be caused by a dysfunctioning p53 protein. p21, however, is upregulated in cholesteatoma, indicating a transcriptionally active p53 protein[16]. In addition, we have demonstrated that apoptosis in cholesteatoma can be adequately blocked. This is only possible if there is a combination of normal functioning p53 and p21 proteins[17]. Such a concept is also supported by previous reports showing that cholesteatomas are genetically stable and have a normal DNA content[1]. This indicates that, in cholesteatoma, apoptosis may not be required as a cellular protection mechanism.

Conflicting results have been established in the cholesteatoma epithelium. The presence of normal functioning p53 and p21 proteins suggests an effective proliferation block. This, however, is not the case. The apparently ineffective proliferation block might be caused by different functions of p21[18]. The p21 molecule initiates the cell cycle arrest by binding to the regulators of the cell cycle, the cyclin/cyclin-dependent kinase (CDK) proteins. It has been demonstrated that the CDK inhibitory function of p21 is regulated stoichiometrically, i.e. only when p21 is in molar excess[19]. In the cholesteatoma epithelium, it is possible that inhibition of CDK activity occurs in cells with a molar excess of p21. When the molar abundance of p21 decreases, because of the short half-life of the p21 molecule[20], the cell cycle is no longer inhibited. This assumption is supported by the observation of suprabasal expression of Ki-67 in the cholesteatoma epithelium[1,2] (Fig 1B.).

The p21 protein itself, however, can act as a positive modulator of cell cycle progression. It has recently been found that, in the presence of calcium, p21 can bind directly to Calmodulin[18]. This interaction appears to be required for nuclear localization of cyclinD/CDK4[21], which switches on proliferation. Most cholesteatoma tissues exhibit a considerable bone resorption. Therefore, high local concentrations of calcium might be present. Under these specific conditions, the p21 protein can initiate cell proliferation in the cholesteatoma. Inflammation or the presence of endotoxins can also trigger proliferation. We therefore assume that, in the epithelial layer of the cholesteatoma, the Ki-67 protein is increased by means of different signals. This is also plausible for the upregulation of the p53 protein. Hyperproliferation can increase p53, but neutrophils can also activate the p53 protein by releasing reactive oxygen. It appears that, in cholesteatoma epithelium, the coordination of the cell cycle has become dysfunctional. However, our results prove that the p53 protein can effectively upregulate p21 expression as protection against apoptosis. In this study we have demonstrated that increased proliferation

in the cholesteatoma is not compensated by apoptosis, but may be associated with cell cycle arrest. Future research will focuss on the role of p21 in stem cell commitment and differentiation in the cholesteatoma.

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