Cellular signaling in human cholesteatoma

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Chapter 4

Sustained extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase signalling is related to increased p21 expression in cholesteatoma epithelium.

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Abstract

Conclusion. These results show for the first time that the RAS/ RAF/ ERK1/2 MAPK signalling pathway is active and involved in p21-mediated cell cycle arrest in human cholesteatoma epithelium.

Objective. In a previous report we have demonstrated that the epithelium in human cholesteatoma, is characterized by high p53-dependent p21 expression. The RAS/ RAF/ extracellular signal-regulated kinase (ERK)1/2 mitogen-activated protein kinase (MAPK) signalling pathway can induce p21 expression and subsequent cell cycle arrest via p53-dependent or -independent mechanisms.
We designed the present study to investigate whether the RAS/ RAF/ ERK1/2 MAPK signalling pathway is involved in p53-dependent and p21-mediated cell cycle arrest in human cholesteatoma.

Material and methods. A total of 18 cholesteatoma samples and 18 paired control retro-auricular skin samples were immunohistochemically stained for p53, p21, phosphorylated ERK1/2 (pERK1/2) and total ERK1/2. Positive cells were counted by means of digital image analysis. Double-label fluorescence immunohistochemistry was performed to demonstrate co-expression of p21 and pERK1/2.

Results. Protein expression of p53, p21 and pERK1/2 differed significantly between cholesteatoma epithelium and retro-auricular skin ($p < 0.01$). In cholesteatoma, co-expression of p21 and pERK1/2 was prominent, whereas in retro-auricular skin there was hardly any co-expression. Positive correlations were found between p53 and p21 ($p = 0.003$) and between p21 and pERK1/2 ($p = 0.013$).

Keywords: *Cell cycle arrest, cell signalling, immunohistochemistry, p53*

Introduction

Cholesteatoma is a non-malignant, destructive ear disease, caused by the presence within the middle ear cleft of keratinizing stratified squamous epithelium. Several theories of the pathogenesis of human cholesteatoma have been described, the most important being the migration, invasion and proliferation theory[1]. The link between these theories is disruption of, or damage to, the lamina propria of the tympanic membrane, which usually occurs in combination with a chronic middle ear infection. The biological properties and inductive forces of the inflammation may lead to the invasion of stratified squamous epithelium into the middle ear cleft, causing the formation of cholesteatoma. Clinical sequela may include destruction of the middle ear ossicles and other structures. Cholesteatoma epithelium deviates from normal epidermis in terms of hyperplasia, aberrant differentiation and progressive accumulation of keratins[2]. In human epidermis, normal turnover of keratinocytes depends on controlled coordination between proliferation and differentiation. This requires a regular supply of de novo differentiated cells. It has been demonstrated that irreversible cell cycle arrest is an early and integral part of epidermal differentiation[3]. A key protein involved in cell cycle arrest is the cyclin-dependent kinase inhibitor (CDKI) $p21^{cip1/waf1}$. Binding

of the p21cip1/waf1 protein to cyclins and cyclin-dependent kinases (CDKs) prevents transition of the cell cycle from the G1 phase to the S phase and from the G2 phase to the M phase[4]. P21cip1/waf1 also inhibits DNA replication by association between the carboxy-terminal domain of p21cip1/waf1 and proliferating cell nuclear antigen (PCNA). At least two DNA- metabolizing enzymes, i.e. Fen1 and DNA (cytocine-5)methyltransferase, have been shown to bind to the same overlapping region of PCNA in competition with p21[5]. Association with cyclins, CDK, and PCNA makes the p21 cip1/waf1 protein an essential component of cell cycle control. Expression of p21 cip1/waf1 is usually controlled at the transcriptional level by p53-dependent and -independent mechanisms[6]. The mitogen-activated protein kinase (MAPK) pathway RAS/ RAF/extracellular signal- regulated kinase (ERK)1/2 has been described as being both a p53-dependent and -independent mechanism of p21 induction[6-9]. Previously, we have demonstrated that the p21 cip1/waf1 protein expression was increased and positively correlated with p53 expression in human cholesteatoma epithelium, compared to normal skin[10]. In cholesteatoma, Huang et al have also demonstrated an increased expression of the RAS protein[11]. Because of the essential role of p21 in cell cycle control, we investigated whether the RAS/ RAF/ ERK1/2 MAPK pathway is involved in p53-dependent, p21-mediated cell cycle arrest in human cholesteatoma epithelium. For this purpose we determined the expression of: (i) p53, (ii) p21, (iii) phospho-activated ERK1/2 (pERK1/2) and (iv) total ERK1/2. In addition, co-expression of pERK1/2 and p21 proteins was investigated by means of double-label fluorescence immunohistochemistry using confocal laser scanning microscopy. The results obtained with cholesteatoma tissue were compared to paired control samples from retro-auricular skin.

Materials and methods

Clinical and histopathological data

Cholesteatoma specimens and biopsies of retro-auricular skin were obtained from 18 patients. The samples were placed in phosphate-buffered saline immediately after the operation. The Committee of Medical Ethics of Leiden University Medical Centre approved the protocol. The specimens were prepared for histological examination by fixation in 4% buffered formaldehyde for 20 hours followed by dehydration in ethanol and embedding in paraffin wax.

Immunohistochemistry

Sections (4-μm thick) were taken from each tissue block. The first and last sections were stained with hematoxylin eosin. The sections were immunostained with antibodies respectively against p53 (clone DO7), p21waf1/cip1 (clone SX118), phospho-ERK1/2 (Thr202/Tyr204) and ERK1/2. Anti-p53 and anti-p21 were monoclonal antibodies purchased from Dako B.V. (Glostrup, Denmark) and anti-pERK1/2 and anti-total ERK1/2 were polyclonal antibodies obtained from Cell Signaling Technology (Beverly, MA). The dilutions used for anti-p53 and anti-p21were 1: 100, 1: 500 and for anti-pERK1/2 and anti-total ERK1/2,1: 200 respectively. Double staining was performed by means of immunofluorescence. Anti-p21 was conjugated to goat anti-rabbit horseradish peroxidase (HRP; Kirkegaard & Perry Laboratories,

Gaithersburg, MD) and labelled with Tyramid-fluorescein isothiocyanate (FITC; 1:100; kindly provided by Dr. C. van Kooten, Department of Nephrology, Leiden University Medical Center). Anti-pERK1/2 was labelled with goat anti-rabbit Ig conjugated to Alexa 546 (1:100; Molecular Probes, Leiden, The Netherlands). Sections from the same tissue served as negative controls, i.e. the primary antibody was omitted.

Expression of single proteins

Expression of p53, p21, pERK1/2 and total ERK1/2 was determined using an indirect immunoperoxidase method. Sections from sigmoid (p21) and colon carcinoma (p53, pERK1/2 and total ERK1/2) were used as positive controls. After deparaffination, the sections were treated with methanol containing 0.3% hydrogen peroxide for 20 minutes in order to inactivate endogenous peroxidase. The sections were rehydrated and subjected to microwave antigen retrieval in boiling citrate buffer (0.01M, pH 6.0, for 10 min)[12]. All sections were washed in Tris-buffered saline (TBS) and incubated with the primary antibody in 0.2% bovine serum albumin (BSA) in TBS. The sections were incubated overnight at room temperature (RT) and then washed four times in TBS. The sections used for the p21 antibody treatment were subjected to a secondary incubation with rabbit anti-mouse in Dako diluent buffer (1:2000) for 20 min at RT. After washing in TBS, the p21, pERK1/2 and total ERK1/2 sections were incubated with ChemMate Envision (anti-rabbit and anti-mouse; Dako) for 30 min at RT and developed with 3,3'-diaminobenzidin chromogen (DAB+; 1:50; Dako), for 5 min at RT. The sections were counterstained with hematoxylin for 1 min. The other specimens were incubated with the appropriate biotinylated secondary antibody for 30 min at RT, washed with TBS and subsequently incubated with peroxidase-conjugated streptavidin (Dako) at RT for 30 min. They were then treated with DAB chromogen containing 0.02% H_2O_2 for 15 min and counterstained with hematoxylin for 1 min.

Co-expression of p21 and pERK1/2

The sections were deparaffinized and treated with TBS containing 0.09% H_2O_2 and 0.1% sodium azide for 20 min, in order to block endogenous peroxidase. They were then rehydrated and subjected to microwave antigen retrieval in boiling citrate buffer (0.01M, pH 6.0 for 10 min)[12]. After washing in TBS the sections were incubated with both primary antibodies against p21 and pERK1/2 in 0.2% BSA in TBS overnight at RT. They were then washed four times in TBS and incubated with goat anti-mouse Ig-HRP and goat anti-rabbit-Alexa 546 for 30 min at RT. The sections were subsequently incubated with Tyramid-FITC in Tris-HCl buffer (0.2 M Tris-HCl, pH 8.8, 0.1 M imidazole, 0.0001% H_2O_2) for 30 min at RT. After washing in TBS, the sections were mounted in Vectashield (Brunschwig Chemie, Amsterdam, The Netherlands). The cross-reactive background of all reagents could be excluded by sequential omission of individual reagents, resulting in either single labelling or absence of labelling.

Morphometric analysis of immunohistochemical data

For each of the immunohistochemical markers studied, DAB positive cells were counted using an image analysis system (Leica Microsystems Imaging Solutions Ltd. Cambridge, UK.). The microscope was a Leica DMLB with N Plan 20 x 0.4 objective and a Leica DC 200 digital camera. The computer-assisted system used to determine the positive immunohistochemical staining has been described elsewhere[13]. For each section, images from at least five different areas were stored as digitised images. For cell counting the same areas of the sections were used , but with various stains. The epithelial compartment was delineated on the screen and the positive and negative cells were counted automatically. In each section > 1000 cells were counted, and the percentage of positive cells was determined.

Confocal laser scanning microscopy was performed using a confocal laser scanning microscope (Zeiss LSM510) in a multi-track setting. FITC was excited at 488nm and detected using a 505-530 band-pass filter. Alexa-546 was excited at 543nm and detected using a 560-615nm band-pass filter. Using these settings the two fluorochromes could be detected separately without any background in the other channels. Each fluorochrome was given an artificial colour: FITC, green; Alexa-546, red. A PH2 Plan-NEOFluar 25 x 0.80 Imm Korr objective was used.

Data analysis

Data are expressed as mean ± SD. Because of a non-Gaussian distribution of parameters, we used the non-parametric Wilcoxon signed rank test for comparison of the differences between protein expression in cholesteatoma and retro-auricular skin. The level of significance was set at $p < 0.05$. The Spearman's rank correlation test was used to calculate correlations. Correlations were considered significant at the 0.05 level. The SPSS10 software package (SPSS, Chicago, IL) was used for the calculations.

Results

Histopathological findings

On macroscopic inspection at surgery 11/18 cholesteatoma were considered clinically infected. In13/18 cholesteatoma samples, microscopic inspection revealed inflammatory cells and newly formed blood vessels in the connective tissue. In three tissue samples there was insufficient connective tissue for analysis. In the retro-auricular skin sections there was no evidence of inflammation.

Expression of p53, p21, pErk1/2 and total ERK1/2

Positive expression of p53 was mainly found in the cells of the basal layer but also in the cells of the suprabasal layers of the cholesteatoma epithelium (Figure 1A). The percentage of p53-positive cells was significantly increased compared to that of retro-auricular skin ($p = 0.003$). The average percentages of p53-positive cells in retro-auricular skin and cholesteatoma epithelium are listed in Table I. p21-positive cells were present in the epithelium of all 20 cholesteatoma samples,. p21-positive cells were locally expressed in the lower suprabasal layers of the

retro-auricular skin and cholesteatoma epithelium (Figure 1B). In cholesteatoma, p21 expression was also observed in the upper suprabasal layers. Compared to retro-auricular skin, the percentage of p21-positive cells in cholesteatoma tissue was significantly increased ($p < 0.001$). Table I lists the percentages of p21-positive cells in retro-auricular skin and cholesteatoma epithelium. Expression of pERK1/2 in cholesteatoma epithelium was mainly nuclear and localized in all layers of the cholesteatoma epithelium. The location of total ERK1/2 in the epithelial tissue was similar, but total ERK1/2 expression was also more cytoplasmatic (Figures 1C and D). These observations were consistent with the expression of pERK1/2 and total ERK1/2 in retro-auricular skin. Total ERK1/2 staining was performed as a control of the location of pERK1/2 and for that reason the cells were not counted. On average, cholesteatoma samples showed a significantly increased percentage of pERK1/2-positive cells compared to retro-auricular skin ($p<0.001$). The average percentages of pErk1/2-positive cells in retro-auricular skin and cholesteatoma epithelium are summarised in Table I.

Associations of p53, p21 and pErk1/2 with inflammation

We investigated whether there was a correlation between the samples which were considered clinically infected and protein expressions, but could not find no such relationship. We also found no relationship between the presence of inflammatory cells and protein expressions.

Associations of p53, p21 and pErk1/2

Using Spearman's rank correlation test, a significant positive association was observed between p53 protein expression and p21 ($p=0.003$) and between p21 protein expression and pErk1/2 ($p=0.013$). We found no correlation between p53 and pErk1/2.

Co-expression of p21 and pErk1/2

In cholesteatoma epithelium, confocal laser scanning microscopy revealed that almost all cells in the basal cell layer were pERK1/2 positive. In this layer hardly any ERK1/2 positive cell showed p21 co-expression. In the suprabasal layers co-expression of pERK1/2 and p21 was prominent. (Figures 1E-H). In retro-auricular skin we found almost no co-expression.

Tissue	p53	p21	pERK1/2
Retro-auricular skin	1.7 ± 1.9	1.9 ± 1.4	0.8 ± 0.7
range	0.2 to 4.6	0.1 to 3.4	0.1 to 2.8
Cholesteatoma epithelium	6.3 ± 4.6	11.1 ± 9.4	10.0 ± 7.5
range	0.8 to 13.7	1.3 to 40.6 .	0.2 to 26.9

Table 1. Percentages of P53, P21and pERK1/2-positive cells in control skin and cholesteatoma epithelium.

Fig. 1

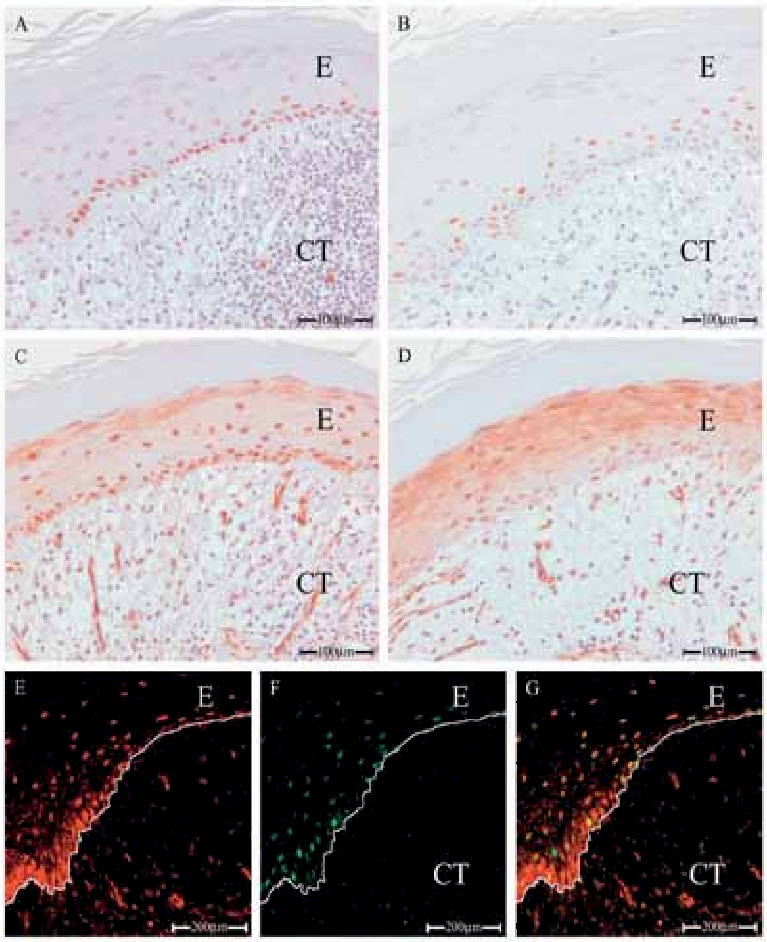

Figure 1. Immunohistochemical localization of P53, p21 and Ki-67 in serial sections of human cholesteatoma epithelium: (A) HE staining; (B) Ki-67-positive cells are expressed in the basal and suprabasal layers of the epithelium (*arrowheads*); (C) Many p53- positive cells are expressed in the basal layer; (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

Discussion

Our results show that in human cholesteatoma epithelium the RAS/ RAF/ ERK1/2 MAPK pathway is involved in p53-dependent increased expression of p21. This is demonstrated by the correlated expression of p53 and p21 and of p21 and active ERK1/2. It has been demonstrated that the RAS/ RAF/ ERK1/2 MAPK signalling pathway is activated in response to many mitogens, such as growth factors, cytokines, bacterial endotoxins, insulin and osmotic stress[14]. It has also been reported that activated RAS elicits premature cell senescence (G1 arrest), which is accompanied by increased expression of p53, p21 and p16[15]. Although transcription factors are important MAPK targets, MAPK can also regulate protein expression through post-transcriptional mechanisms[16,17]. Differential processes may be of importance in this context. The process of post-transcriptional-regulated stabilization of the p53 protein via RAF activation has been reported to result in increased p21 expression[18]. Another post-transcriptional effect of activated MAPK may be upregulation of the p21 protein by preventing of its degradation by the 26S proteasome complex[17].

Curiously, in the MAPK signalling pathway, ERK1/2 activation has been associated with both stimulation and inhibition of cell proliferation. In this context, it has been shown in several studies that the magnitude of ERK1/2 activation determines whether the cellular response includes the induction of p21[6,9,18]. Thus, the induction of p21 expression requires a stronger ERK1/2 signal than ERK1/2-mediated induction of proliferation. It has been demonstrated that with high and sustained ERK1/2 activity, p21 binding to the proteasome complex is inhibited[18], i.e. the proteasomal degradation of p21 is reduced, which may result in high expression of p21[9,17].

Roper et al. have shown that, for proper RAS/ RAF/ ERK1/2 MAPK-induced cell cycle arrest, both p53 as well as p21, need to be present[19]. Increased stability of p53 after RAF activation may then lead to transcription of p21. Roper et al. observed that this RAF-mediated induction of p21 is lost in $P53^{-/-}$ cells. This group also reported that p53 is not induced at the RNA level after RAF activation. This may indicate that RAF regulates p53 at the level of protein stabilization[19], which has also been confirmed by others[20]. Reciprocal to this, Lee et al. showed that p53 can also mediate MAPK activation[21]. This may imply a positive feedback loop in which permanent growth arrest could be augmented by sufficient upregulation of either p53 or MAPK pathways. Moreover, Lee et al. found that p53-mediated ERK1/2 activation is higher and more sustained than that without p53 involvement[21]. These reports fit well with the expression profile observed in cholesteatoma epithelium. Increased RAS expression has previously been demonstrated in human cholesteatoma epithelium[11] and high expression of activated ERK1/2 implies an active RAS/ RAF/ ERK1/2 MAPK signalling pathway. Furthermore, increased p53 and p21 expression was detectable in all suprabasal layers in cholesteatoma epithelial tissue, indicating an extended lifetime of both proteins. The correlation between p53 and p21 expression may imply a direct involvement of p53 in p21 expression. Finally, the co-expression of pERK1/2 with p21 and their correlation, although less than that between p53 and p21, indicates that in human

cholesteatoma pERK1/2 is also involved in the mechanism of p21 upregulation.
We speculate that, in human cholesteatoma, activation of MAPK is mainly caused by external factors, such as inflammation-induced growth factors, cytokines and bacterial endotoxins. These factors may also be responsible for primary upregulation of p53 with subsequent transcription of p21. Low or intermediate concentrations of pERK1/2 may result in progression through the cell cycle. When triggering is prominent, e.g. as a result of different mitogenic stimuli, strong RAS/ RAF/ERK1/ 2 activation may occur[9]. Stabilization of p53 by RAF may then induce positive feedback to produce a sustained increased level of activated ERK1/2 (Figure 2). The latter factor is a major condition for the prevention of p21 breakdown, which is a prerequisite for proper cell cycle inhibition[9]. In cholesteatoma epithelium, these differential processes were visualised by single pERK1/2 expression in the proliferating compartment. In the suprabasal layers, when pERK1/2 expression was sustained the cells were also found to be positive for p21(Figures 1E-G).
We postulate that, in cholesteatoma cell cycle arrest, posttranscriptional mechanisms such as p53 stabilization and prevention of p21 breakdown, play prominent roles. Prolonged MAPK-induced cell cycle inhibition causes accumulation of epithelial cells, which are in G-phase arrest[18]. In human cholesteatoma, prevalent

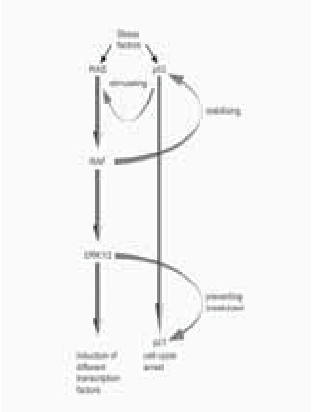

Figure 2. Induction of p21 transcription in human cholesteatoma epithelium. External triggers, like growth factors initiate the activation of the MAPKinase pathway and the transcription of different proteins involved in proliferation. External triggers may also initiate the production of p53. When there are different stimuli, both the MAPKinase pathway and the production of p53 are initiated. RAF activation may then result in stabilisation of p53, which may lead to a further activation of RAS and a sustained ERK1/2 phosphorylation. This prevents p21 breakdown, which is central to an increase in p21 concentration and cell cycle arrest

cell cycle arrest may contribute to epithelial hyperplasia.
MAPK signalling, however, is not only restricted to a special cell type, which led us to presume that different stromal cells are also activated. The study of MAPK signalling in all cholesteatoma cells may, in our opinion, help us in the understanding of processes such as invasion, bone resorption, re- inflammation, etc.
Our next study will concern the specific role of the different mitogenic stimuli in the activation of the RAS/ RAF/ ERK1/2 MAPK pathway in human cholesteatoma epithelium.

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