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Expression of Rb in Human Middle Ear Cholesteatoma

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= 국문초록 =

중이진주종에서 Rb 단백질의 발현

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목 적 : 중이귀진주종의 병리조직학적 특징은 진주종 상피의 과다증식과 비정상적인 과각질화라고 요약되는데 이러한 현상은 진주종 상피를 형성하는 각질세포의 증식, 분화 및 세포 사멸로 이루어지는 세포주기의 항상성 변화로 기인한다. 본 연구는 세포자멸사(apoptsis) 및 세포 주기의 조절에 중요한 영향을 미치는 것으로 알려져 있는 Rb의 가운데귀진주종 상피 내에서의 발현 양상을 알아보고자 시행되었다.

방 법 : 만성 진주종성 중이염으로 수술을 시행 받았던 환자 10예의 진주종 상피와 정상 뒤귓바퀴 및 목의 피부조직 5예를 대상으로 Rb에 대한 Western blot analysis를 시행하였고, 중이 진주종 조직 6예와 정상 뒤귓바퀴 조직 4예에서 Rb의 발현 양상을 면역조직화학적 검사를 통하여 관찰하였다.

결 과 : Western blot analysis 상 가운데귀진주종 상피에서는 10예 중 9예에서 Rb가 발현되었고 대조군으로 사용된 정상 피부조직에서는 5예 중 1예에서만 양성 반응을 보였다. 면역조직화학적 검사에서 가운데귀진주종 상피에서는 Rb 양성인 상피 세포가 바닥층과 바닥위층에 넓게 분포하고 있는 것이 관찰되었고 Rb의 발현 정도는 정상 상피에 비하여 증가되어 있었다. 반면 정상 상피에서는 Rb 양성인 상피 세포는 바닥층에서만 부분적으로 관찰되었고 그 발현의 정도는 진주종 상피에 비하여 감소되어 있었다. 이는 통계적으로 유의하였다. 이상의 결과로 Rb가 가운데귀진주종 상피에서 비정상적으로 과발현 되고 있음을 확인할 수 있었다.

결 론 : 진주종의 중요한 특징인 상피 세포의 과증식과 비정상적인 과각질화는 각질세포의 세포주기 항상성 및 세포사멸 현상의 이상에 의한 것으로 생각되었으며 세포세포자멸사 및 세포 주기의 조절에 중요한 역할을 하는 것으로 알려진 Rb가 중이 진주종의 병인에 중요한 역할을 할 것으로 판단되었다.

중심 단어 : 진주종 · Rb · 과다증식.

Introduction

Human middle ear cholesteatoma has a characteristics of hyperproliferation and hyperkeratinization of the epithelium. This phenomenon is due to disturbance of the cell cycle in epithelial homeostasis[1]. Tissue homeostasis require regulatory co-ordination between cellular proliferation and apoptosis. Apoptosis is a process of programmed cell death that plays an important role in physiological processes such as immune- and nervous-system development, and contributes to defense mec-

hanism important for the prevention of infectious illness and cancer[2]. The co-ordinated structural changes that make up the process of apoptosis are driven by a set of molecular interactions, called the terminal effector events. It is known that most, if not all, living cells contain the molecules that participate in these evens, but in a form that requires activation. The process of death is the result of an interaction between initiating stimuli and factors that determine the susceptibility of the cell to activation of the terminal events[3].

Apoptosis is a genetically controlled process and genes important for the regulation of apoptosis have recently bene identified. Myc, bax, or retinoblastoma genes are known to be concerned in the transcription of DNA in cell cycle[4]. Among these, the retinoblastoma gene was identified over a decade ago as the first tumor suppressor. Although the gene was initially cloned as a result of its frequent mutation in the rare pediatric eye tumor, retinoblastoma[5)6)], it is now thought to play a fundamental role in cellular regulation. The retinoblastoma gene encodes a 928-amino acid phosphoprotein, Rb, which arrests cells in the G1 phase[7]. Rb is phosphorylated and dephosphorylated during the cell cycle. The hyperphosphorylated (inactive) form predominates in proliferating cells, whereas the hypophosphorylated (active) form is generally more abundant in quiescent or differentiating cells[8]. The hyperphosphorylated Rb is known to make complex with E2F in early G1 stage and suppress the processing of apoptosis[9].

So far few reports have investigated the expression of Rb in human middle ear cholesteatoma epithelium, and this study was designed to identify the expression of Rb in cholesteatoma by western blot analysis and immunohistochemistry.

Materials and Methods

1. Tissue specimens

For western blot analysis, tissue specimens of cholesteatoma epithelium were obtained from ten adult patients at the time of surgery for cholesteatoma performed at Chungbuk National University Hospital, Cheongju, during the period from April 1999 through April 2000. As a control, four normal skin samples were obtained from the postauricular area in the above-mentioned cholesteatoma patients during surgery, and one normal skin sample in the neck was harvested from a patient at the time of surgery for submandibular gland. Another six human cholesteatoma specimens and four normal postauricular skin samples were used for the immunohistochemistry.

2. Immunohistochemistry

The specimens were fixed in a 10% buffered formalin solution for 12hours, subjected to dehydration and paraffin embedding, and sliced into 4 μm sections. After deparaffinization and washing, endogenous peroxidase of the sections were inhibited with a methanolic solution of 0.3% H_2O_2 and washed with phosphate buffered solution (10mM, pH 7.4) for 5minutes. After rehydration with ethanol, the slide was microwaved for 15minutes with antigen retrieval solution (DAKO chemo., Glostrup, Denmark). After rinsing in 10mM PBS for 5minutes, the slide was treated with blocking antibody at room temperature to reduce non-specific antibody binding. Then the section was incubated for 60minutes at 25℃ with monoclonal mouse anti-human-Rb (Novocastra, Newcastle, UK). This primary antibody was used in dilution of 1 : 20. After being washed throughly with PBS, the slide was reacted with UltraTek Anti-polyvalent (DAKO Chem., Glostrup, Denmark) for 10minutes. After washing with PBS, the slide was visualized with 3-amino-9-ethylcabazole chromogenic substrate (ScyTek, Logan, USA). Finally, the section was lightly counterstained with hematoxylin.

Percentage of labelled cells : Under microscopic observation at magnification ×400, 500 cells per visual field in five visual fields were inspected. The percentage of positive cells in the 500 cells was calculated, and the results were expressed in terms of mean±SD. The differences in mean values between cholesteatoma specimens and normal skin samples were tested by Wilcoxon's test, and a difference at a P value of 0.05 or smaller was defined as statistically significant.

3. Western blot analysis

Frozen tissues were thawed in homogenization buffer (50-mmol TRIS-HCL, pH 7.6, 100 μg/ml phenylme-

thysulfonyl chloride). Then tissue was homogenized by sonication using ultrasonic homogenizer (Sonicator, B. Braun 2000, USA). The tissue homogenates were centrifuged at 12,000rpm for 30minutes at room temperature, and the resultant supernatants were collected for immunoassay.

Each supernatant involving 20 μg/ml protein was mixed with 2X SDS gel-loading buffer (200mM Tris-HCL, 400mM dithiothreitol, 8% SDS, 0.4% bromophenol blue, 40% glycerol), boiled for 5minutes at 100℃, and analyzed by SDS-PAGE using 4% stacking gel and 8% resolving gel at 3mA for 3hours. Proteins were electrophoretically transffered to Immobilon-P transfer membrane (Millipore, Bedford, MA, USA) at 50 V for 2hours, blocked by PBS involving 0.1% Tween 20 and 3% Skim milk for 30minutes. After washing, nitrocellulose filters were incubated with an monoclonal mouse anti-human-Rb antibody (Novocastra, Newcastle, UK) at 4℃ for overnight. This primary antibody was used at a final dilution of 1 : 100. The secondary peroxydase labeled goat anti-mouse IgG antibody (DAKO A/S, Denmark) was used at a dilution of 1 : 10,000. Immunodetection was performed using ECS kit (Amersham Life Science, Buckinghamshir, England).

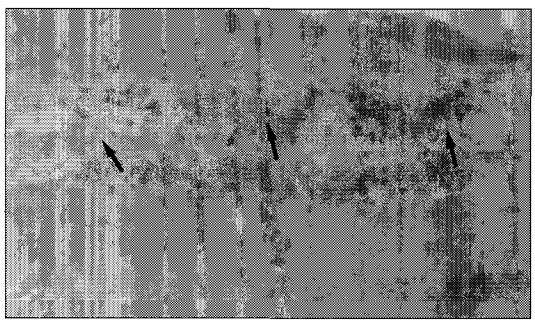

Fig. 1. Rb immunoreactivity in a cholesteatoma specimen. Diffuse Rb positive cells were observed in the basal and suprabasal layers (arrows), and the expression of Rb was relatively strong compared to the normal skin samples (×400).

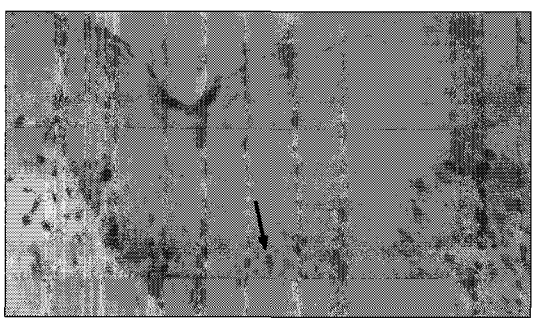

Fig. 2. Rb immunoreactivity in a normal skin sample. Focal Rb positive keratinocytes (arrow) were observed only in the basal layer and the expression of Rb was relatively weak compared to the cholesteatoma epi-thelium (×400).

Results

1. Immunohistochemistry

Cholesteatoma epithelium showed diffuse Rb positive cells in the basal and suprabasal layers (Fig. 1). In contrast, postauricular skin samples showed focal Rb positive keratinocytes only in the basal layer (Fig. 2). Expression of Rb was relatively strong in the cholesteatoma specimens compared to normal skin samples, and calculated percentages of the positively stained cells were 4.96%±1.12 in the basal layer and 3.40%±0.92 in the suprabasal layer. In case of the normal postauricular skin samples, weak staining for Rb was detected in the basal layer (0.70%±0.21) and suprabasal layer (0.10%±0.03). Their differences were statistically significant.

2. Western blot analysis

Nine of ten human cholesteatoma specimens showed definite positive bands around 110kDa (Fig. 3). In contrast, only one of five normal skin samples showed definite expression of Rb (Fig. 4).

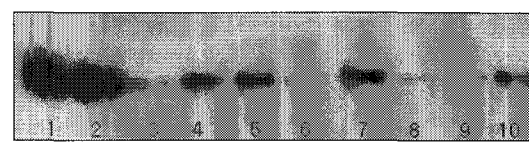

Fig 3. Western blotting for Rb in cholesteatoma specimens. Nine of ten human cholesteatoma specimens showed definite positive bands around 110kDa.

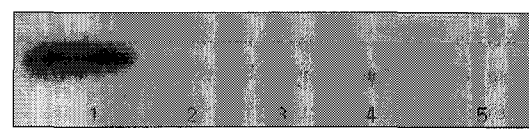

Fig. 4. Western blotting for Rb in normal skin samples. Only one of five normal skin samples showed definite expression of Rb.

Discussion

Normal epidermis consists of several layers of keratinocytes, but only the keratinocytes in the basal layer proliferate. These cells then leave this layer and begin to migrate toward the surface of epidermis. When the keratinocytes reach the granular layer, they enter a destructive phase in which they begin to lose their organells and, in the process of differentiation, leave keratin filaments as the sole cytoplasmic survivors. This programmed cell death is known as apoptosis, which is a mechanism used by different tissues to adjust cellular content by suppressing cells that are dysfunctional or no longer necessary[2)10)].

Apoptosis of keratinocytes involves keratinization and cornification, essential parts of the differentiation process. And in case of cholesteatoma, keratin debris abnormally accumulates due to an increased rate of cell death, driven by the enhanced differentiation of keratinocytes[1)]. Many cytokines and growth factors are known to be localized on keratinocytes in the epithelium of cholesteatoma, and it is suggested that they stimulate the migration, proliferation, and terminal differentiation of keratinocytes in a concerted fashion. These growth factors include IL-1, TNF-α, granulocyte-macrophage colony stimulating factor, EGF, and platelet-derived growth factor[11-14)].

The product of the retinoblastoma gene is a 110kDa phosphoprotein termed Rb. Rb exists in three general form : unphosphorylated Rb, present in G0 cells and when Rb is newly synthesized; hypophosphorylated Rb, present in contact-inhibited cells and in early G1 phase ; and hyperphosphorylated Rb, that is inactive and present in late G1, S, G2, and M phases of cycling cells[8)15)]. Thus in cycling cells, Rb alternates between a hypophosphorylated form present in early G1 to a hyperphosphorylated form after passage through the restriction point in late G1 and continued through S, G2, M phases.

Recent studies have pieced together an important G1 phase cell cycle regulatory pathway involving the INK4 kinase inhibitors that negatively regulate complexes of cyclin D1, D2, and D3 bound to Cdk4 or Cdk6 (referred to as cyclin D : Cdk4/Cdk6 complexes) that phosphorylate the retinoblastoma tumor suppressor gene product (Rb). Cyclin D : Cdk4/Cdk6 complexes associate with Rb pocket domain and then proceed to hypophosphorylate Rb in early G1, and likely throughout the entire cell cycle. hypophosphorylated Rb is active and binds to transcriptional factor, such as E2Fs. The interaction of hypophosphorylated Rb with E2F may block the function of E2F as a transcriptional factor. The initial hyperphosphorylating inactivator of Rb is likely cyclin E : Cdk2 complexes expressed and activated with the passage through the late G1 restriction point. Hyperphosphorylation of Rb results in the dissociation of E2Fs and subsequent activation of E2F-specific promotors, such genes required for DNA synthesis. E2F sites are found in the promoters of many genes that are important for cell cycle progression. Rb appears to repress transcription of these genes through its interaction with E2F, and hyperphosphorylation of Rb followed by activation of E2F may play a fundamental role in cell cycle progression and inhibition of apoptosis[16-22)].

Based on the immunoblot assay, the authors of this study found that Rb was highly expressed in cholesteatoma epithelium compared to normal epithelium. Because the immunoblot assay did not demonstrate the sites of Rb expression, localization of this protein expression was accomplished by immunohistochemical studies using anti-Rb antibody. Immunohistochemical studies demonstrated that Rb staining was relatively strong in the nuclei of keratinocytes in the basal and suprabasal layers of cholesteatoma epithelium. In contrast, Rb was barely expressed in normal epithelium and it was faintly stained in the basal and suprabasal layers of the normal epidermis. From these results, it was suggested that Rb might play an important role in abnormal proliferation of keratinocytes, enhanced differentiation, and suppression of programmed cell death in cholesteatoma epithelium, which results in the accumulation of keratin debris during the growth of cholesteatoma.

The authors have formulated that cytokines known to be concentrated in cholesteatoma epithelium may be involved in the phosphorylation of Rb, and this inactive form of Rb allows the keratinocytes to proceed through the cell cycle. However, such relationship of these cytokines to Rb in cholesteatoma epithelium is needed to be confirmed. Also, this study did not demonstrate whether

expressed Rb in cholesteatoma was hypophosphorylated or hyperphosphorylated form, and further investigation of such issue is needed.

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