ORIGINAL ARTICLE

Utilization of Caspase-14 Promoter for Selective Transgene Expression in Squamous Layers of Cholesteatoma in the Middle Ear

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Objective: Cholesteatoma consists of squamous layers of keratin cells with accumulation of differentiated keratin debris. The lesion is pathologically not malignant. But when inflammation occurs, it rapidly grows and infiltrates to the surrounding tissue. The primary treatment is operation. Generally, total removal of cholesteatoma brings acceptable outcome to the patients. However, some cases are very difficult to treat because of the invasive manner of the progression. Such cases cause serious complications. Therefore, a new treatment to control cholesteatoma is required. Caspase-14 is a member of the caspase family protein which associated with terminal epidermal differentiation. Caspase-14 is specifically expressed in the differentiated keratinocytes. When the cholesteatoma was immunostained, caspase-14 was expressed in suprabasal layers. Since the expression is selective, we try to explore the possibility to use its promoter for selective transgene expression in squamous layers of cholesteatoma in the middle ear.

Materials and Methods: We examine the upstream of caspase-14 nucleic sequences and predicted the promoter region. In order to confirm the selective promoter activity, we constructed recombinant adenoviral vector carries predicted caspase-14 promoter and marker green fluorescent protein (GFP). We also quantified the expression using luciferase marker gene with dual luciferase vectors system.

Results: When cells were infected, GFP was expressed only in the keratinocyte and SCC9 cells. In the fibroblast, expression was hardly detected. Luciferase was highly expressed in SCC9 cells compared to the fibroblast.

Conclusions: Based on the results obtained, we suggest utilizing the promoter for future gene therapy to refractory cholesteatoma.

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Introduction

Surgical removal is the basic approach to treatment of middle-ear cholesteatoma. However, even for an experienced surgeon, it can be very difficult to completely excise the cholesteatoma epithelium while preserving the function at sites that are anatomically complex, such as around the facial nerve, petrous apex etc. For patients who undergo repeated surgery for excision of residual cholesteatoma, but which in the end is unsuccessful, there is a clear need for development of a new, non-surgical treatment method.

Recent years have seen advances in the development of gene therapies for cancers and other malignant tumors and congenital metabolic disorders, and some such treatments have become established clinical options as a result of verification of their safety. In the past, only fatal diseases were considered to be candidates for gene therapy, but recently there has been broadening of the scope of application of gene therapy to include nonfatal diseases such asParkinson disease, arterial occlusion and so on. In addition, promoters from viruses such as cytomegalovirus (CMV), were used in the past to achieve expression of genes transduced into all cells. Recently, however, there has been progress in the analysis of promoters that work in specific tissues or tumors in order to treat cancers. There has thus been development of

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treatments that use specific promoters that do not cause damage to normal tissues. As a result, today it is possible to carry out more refined treatments.[1,2]

In light of this background, it can be thought that, if it were possible to apply procedures such as transduction of genes specific for the epithelial cells of cholesteatoma, it would raise the possibility of development of new treatments for cholesteatoma.

It is said that, pathologically, middle-ear cholesteatoma tissue shows hyperproliferation and hyperdifferentiation, but structurally it is basically similar to normal skin. In addition, unlike in tumors, cholesteatoma epithelial cells usually proliferate slowly, but it is thought that epithelial cell proliferation caused by inflammation is involved in the formation and growth of cholesteatomas. We postulated that, if we could identify a promoter that worked specifically in cholesteatoma epithelial cells, we might be able to use it in a gene therapy that would eliminate cholesteatoma epithelium.

Caspase-14 is a member of the caspase family of apoptosis-related genes. Unlike the other caspases, caspase 14 has not yet been clearly demonstrated to be invalved in apoptosis. However, it is known to play a unique role in the cornification of keratinocytes in normal epithelium [3,4,5]. In addition, caspase 14 protein is specifically expressed in keratinocytes, a fact that indicates that a promoter that controls the expression of the caspase 14 gene must beworking in those calls. However, the location and sequence of the caspase 14 promoter have not yet been elucidated. Accordingly, we estimated the caspase 14 promoter region that could be used in therapy and investigated whether or not the promoter worked specifically in keratinocytes. Our results indicated that the caspase 14 promoter worked more specifically in keratinocytes than in fibroblasts and provided evidence in support of the potential use of this promoter in gene therapy for cholesteatoma.

Materials and Methods

Cells

NHEK (normal human epidermal keratinocytes) (KURABO, Japan) were employed as cultured epidermal cells. NHDF (neonatal normal human dermal fibroblasts) (KURABO) were employed as cultured fibroblasts. SCC9 cells (ATCC, Va) were established from a tongue cancer and used as cultured squamous cell cancer cells. In addition, HEK293 cells were used for virus preparation. The keratinocytes were grown in keratinocyte culture medium, while the fibroblasts and SCC9 were cultured in DMEM (Gibco, Ma). Ten percent FBS (Sigma, Mo) was added at the time of culture.

Immunolocalization of Caspase-14 Protein

Formalin-fixed samples of cholesteatoma and normal skin were embedded in paraffin, the paraffin block was then sliced to a thickness of 5 ❍m and the slices were immunostained. After deparaffinization, the slices were reacted with 1,000-fold mouse anti-caspase 14 monoclonal antibody (Imgenex, Inc., San Diego, Ca) at room temperature for 1 hour, followed by reaction with 500-fold biotinylated anti-mouse goat secondary antibody at room temperature for 1 hour. Color development was then performed using the ABC method (ABC reagent: Vector Laboratories, Ca).

Immunoblotting

The protein fraction was extracted from the cultured cells using Chaps buffer (Gibco), and then 10 ❍g of protein was subjected to 15% SDS polyacrylamide gel electrophoresis. After transfer of each blot to a nitrocellulose membrane (Hybond ECL: Amersham Bioscience Corp., Piscateway, NJ), reaction was performed with 500-fold mouse anti-caspase 14 monoclonal antibody and then 10,000-fold anti-mouse goat secondary antibody. Next, an ECL Detection kit (Pierce, Rock ford, IL) was employed, and the membrane was exposed to an X-ray film. Expression of caspase 14 protein was compared with the expression of ♌actin as a control.

Promoter Prediction

It is known that the caspase 14 gene is located in region 6425836 of human chromosome 19. In order to determine the caspase 14 promoter region, we carried out prediction of the caspase 14 promoter with regard to the upstream genes. The promoter region base sequence and its predicted score were calculated using the PROSCAN Version 1.7 program (Bioinformatics& Molecular Analysis Section, MD, USA) developed by Dr. Dan Prestridge of the University of Minnesota for predicting the polymerase II promoter region of

eukaryotes and the Promoter Scan.jsp of Dr. Frederick Ausubel of Harvard University and Massachusetts General Hospital's Molecular Biology Department. These methods yielded the following results for the location of the sequence predicting the promoter: a score of 0.994 for the upstream 0.7-Kb base pair portion of the caspase 14 gene, and a score of 0.569 for the upstream 1.0-Kb basepair portion. Then we cut out the respective base sequences (0.7 Kb and 1.0 Kb) from those portions up to the transcription initiation site of the caspase 14 gene. These fragments were then analyzed to determine whether they actually had promoter activity and whether they worked specifically in keratinocytes.

Next, these fragments were templates by PCR for oligo Forward: casp14pr(0.7Kb)+SpeI+KpnI+(actagtggtaccgtgtaggaaaaggcaaagagatgggga) and Reverse: casp14pr(0.7Kb)+XhoI-(tttctcgagcagcaccttgtctgatccttggagt). The PCR products and luciferase reporter vectors pGL.4.26 (Promega) and pGL4.14, after treatment with restriction enzymes KpnI and XhoI, were ligated using Ligation High (Toyobo, Japan). This generated luciferase expression vectors casp14pr(0.7)-pGL4.26 and casp14pr(0.7)-pGL4.14, which included the luciferase gene downstream of the caspase 14 promoter. In addition, casp14pr(1.0)+SpeI+KpnI+(actagtggtacctgcagtggctcacatctataatcccag) was used to generate casp14pr(1.0)-pGL4.26 and casp(1.0)-pGL4.14 in the same manner. (Figure 1)

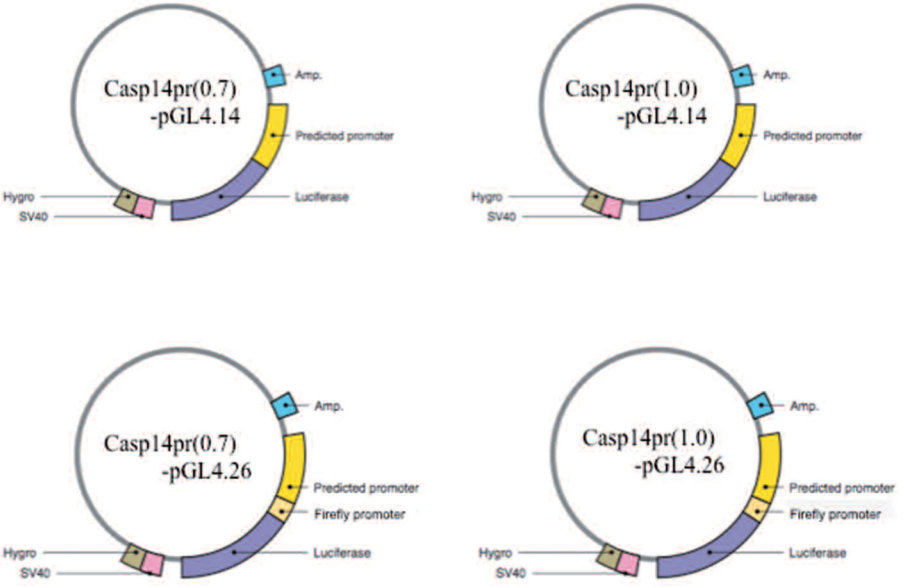

Figure 1. Constructions of luciferase expression vectors

Vector Transfection

The luciferase expression vectors prepared as described above (casp14pr(0.7)-pGL4.26, casp14pr(0.7)-pGL4.14, casp14pr(1.0)-pGL4.26 and casp14pr(1.0)-pGL4.14) were transfected into cultured SCC9 cells and fibroblasts. Then the expression levels of the protein products of the marker genes were measured with a luminometer. Here, the SCC9 cells were employed as an epithelial cell model instead of keratinocytes, which show poor transfection efficiency. In addition, the fibroblasts were employed as subepithelial cells.

Twenty-four hours prior to transfection, the fibroblasts and SCC9 cells were dispensed into 24-well plates at 3x104 cells/well. The luciferase expression vectors were transfected into the cells using Lipofectamine (Invitrogen), and 48 hours later the luminescence was measured using the Dual-Luciferase Reporter Assay(Promega).

EGFP Adenovirus Construction

The transfection efficiency is poor in the case of keratinocytes, and it was thus impossible to determine an accurate value for activation of the caspase 14 promoter in those cells. Next, adenovirus vectors were constructed because they show better gene transduction efficiency compared with lipofection, and the specificity of the promoter was investigated in keratinocytes and fibroblasts.

Casp14pr(0.7) and casp14pr(1.0) were inserted into the pEGFP-1 cloning vector(Clontech, USA), which carries the EGFP reporter gene cassette. Then cleavage was performed with the SpeI and NotI restriction enzymes, and the cleavage products were ligated with the adenovirus shuttle vector pCMVβ-P, which includes an adenovirus homologous recombination domain. The resultant vectors were then cotransfected into HEK293 cells together with pJM17, which contains the adenovirus type 5 genome.[6,7,8,9,10] This completed the construction of adenoviruses containing both the caspase 14 promoter region and the EGFP reporter gene: Ad-casp14pr(0.7) EGFP and Ad-casp14pr(1.0)EGFP. (Figure 2)

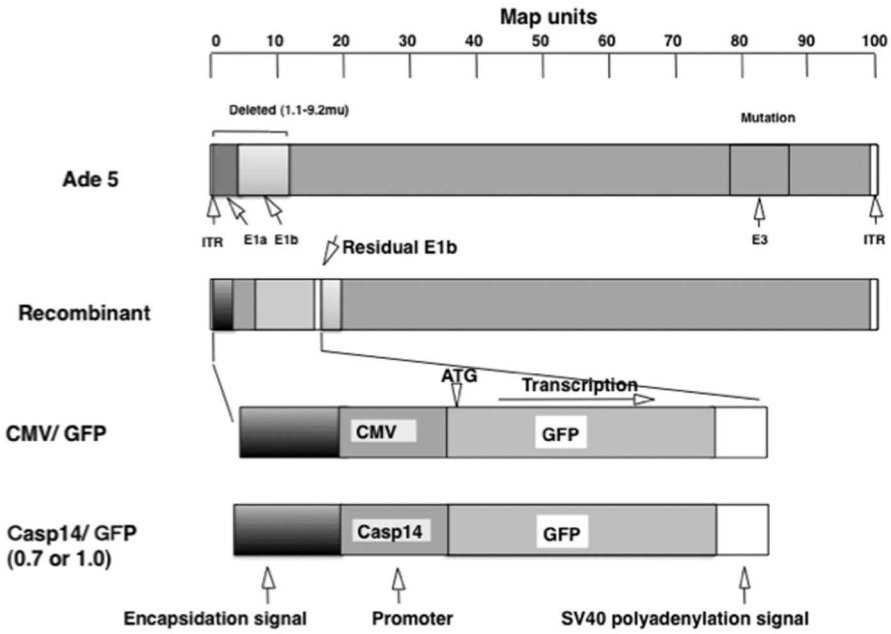

Figure 2. Constructions of recombinant adenovirus shuttle vectors with CMV promoter and caspase-14(0.7Kb, 1.0Kb) promoter

Adenoviral Infection

Twenty-four hours prior to infection, the keratinocytes, SCC9 and fibroblasts were dispensed into 6-well plates at 106 cells/well. DMEM, 2 ml, was added to each well, followed by incubation. The cells were then infected with virus at a multiplicity of infection (MOI) of 10. An adenovirus containing the CMV promoter (AdCMVpr) was used as a control promoter.

The culture medium was changed at 24 hours post infection, and the GFP fluorescence of each well was visualized by laser-scanning fluorescence microscopy at 48 hours postinfection.

In addition, quantification of the fluorescence was performed using Photoshop Elements® histograms, by randomly selecting 3 sites in regions showing similar pixel counts. The fluorescence intensity was calculated for each cell type, and the data were compiled as mean values.

Results

Localization of Caspase-14 Protein in Normal Skin and Cholesteatoma. (Figure 3)

The localization of caspase-14 protein by immunostaining showed that, in normal skin from the back of the ear that was used as the control, it was expressed in the cornified layer, prickle cell layer and granular layer of the epidermis. These findings confirmed that caspase 14 protein is expressed in differentiated epithelial cells of normal skin. For middle-ear cholesteatoma epithelium, as well, caspase 14 protein was observed to be expressed in the cornified, prickle cell and granular layers of the epidermis. However, the cornified layer of the middle-ear cholesteatoma skin was seen to be markedly thickened, and caspase 14 protein expression was *especially increased in that layer.*

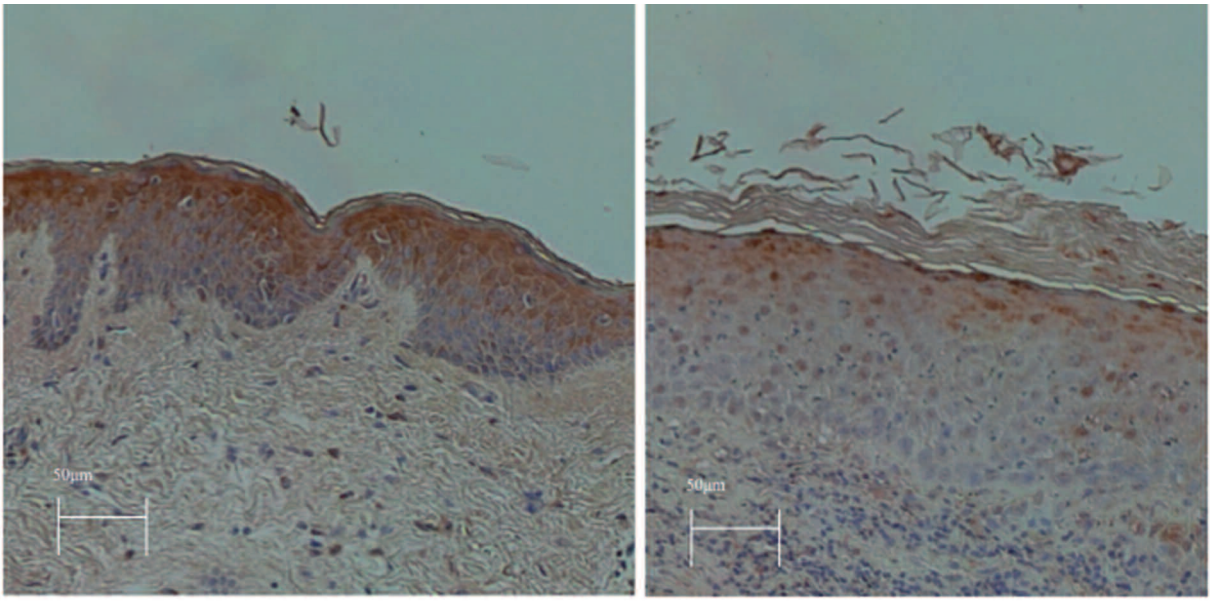

Figure 3. The result of immunostaining of caspase-14
a.Normal skin: casapse-14 was expressed in cornified layer, prickle cell layer and granular layer of the epidermis
b.Middle-ear cholesteatoma: caspase-14 protein was observed to be expressed in the cornified layer, prickle cell layer and granular layer of the epitherium.

Immunoblotting of Caspase-14 Protein

Since expression of caspase 14 protein had been demonstrated in both normal skin and middle-ear cholesteatoma, we next investigated its expression in the three types of cultured cells (NHEK, NHDF and SCC9). Western blotting found that caspase 14 protein was expressed in the cultured keratinocytes (NHEK) and cultured SCC9 cells, but, as had been anticipated, it was not expressed in the cultured fibroblasts (NHDF). (Figure 4)

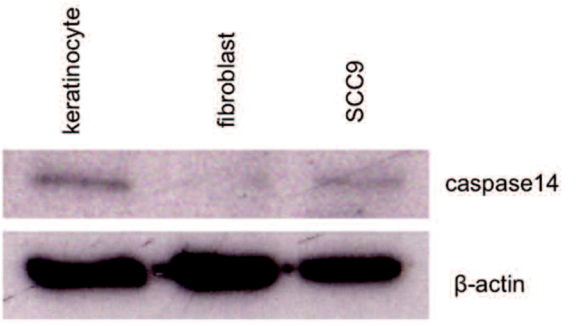

Figure 4. The result of immunoblotting of caspase-14 protein. Caspase-14 protein was expressed in the cultured keratinocytes(NHEK) and cultured SCC9 cells but was not expressed in the cultured fibroblasts(NHDF).

Transfection Using the Luciferase Expression Vectors

The luciferase expression vector casp 14pr(0.7)-pGL4.14, which included the caspase 14 promoter, was transfected into the cultured cells by the Lipofectamine method, and assay of the luciferase activity showed it to be stronger in the SCC9 cells than in the fibroblasts. In addition, when the SCC9 cells were transfected using luciferase expression vector casp14pr(0.7)-pGL4.26, which contained not only the caspase 14 promoter but also a firefly promoter, the luciferase activity was higher than with the luciferase expression vector casp14pr(0.7)-pGL4.14 containing only the caspase 14 promoter. In contrast, there was no difference in the luciferase activity expression level of the fibroblast regardless of whether casp14pr(0.7)-pGL4. 14 or casp14pr(0.7)-pGL4.26 was trasnfected. (Figure 5)

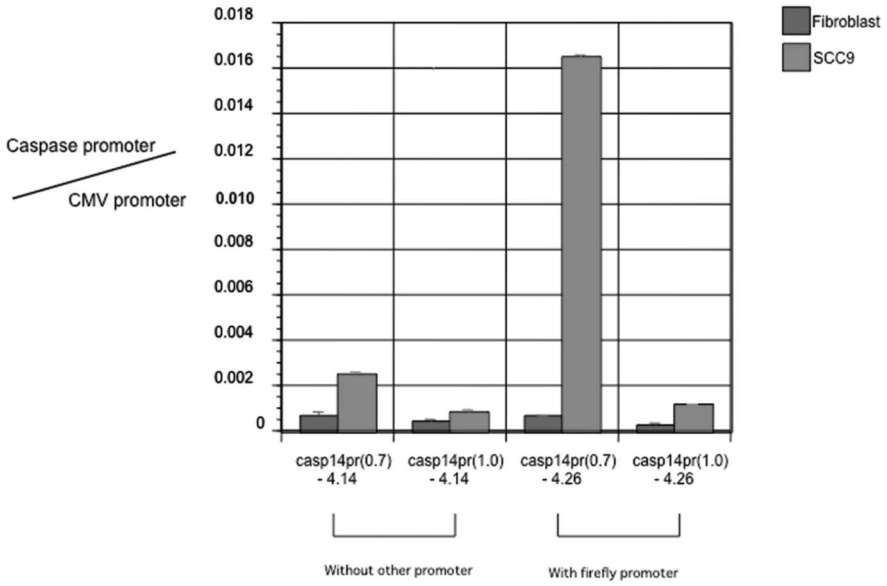

Figure 5. The result of luminometer.
It shows that the caspase-14 promoter, especially 0.7Kbp, works in squamous cell carcinoma and does not work in fibroblast.

Infection Using Adenoviruses

First, keratinocytes, fibroblasts and the SCC9 cells all expressed GFP following infection with adenovirus Ad-CMVprEGFP, which contained the CMV promoter. These results showed that infection with an adenovirus improved the gene transduction efficiency of these cell types. Next, the cultured cells were infected with the previously constructed Ad-casp14pr(0.7)EGFP and Ad-casp14pr(1.0)EGFP adenoviruses that contain both the caspase 14 promoter region and the EGFP reporter gene. As a result, both the keratinocytes and SCC9 cells expressed GFP, but GFP expression by the fibroblasts was very slight. (Figure 6) Quantification of the fluorescence using Photoshop® histograms confirmed that GFP expression was clearly stronger in the keratinocytes than in the fibroblasts. (Figure 7)

These results were similar to those seen with SCC9 cells using luciferase expression vectors.

Discussion

In the early stages of gene therapy, the gene transduction was carried out by transducing genes into all cells, as in the case of CMV promoter. More recently, progress has been made in identifying promoters that function specifically in cancer tissues or tumors. There has thus been development of treatments that use specific promoters and make it

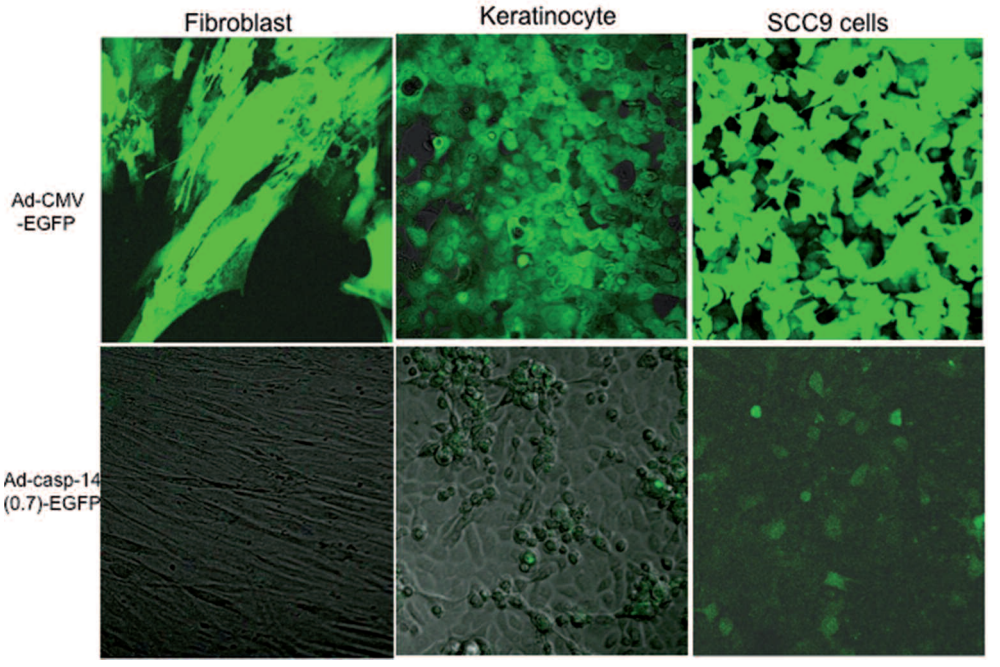

Figure 6. The result of adenoviral infection to keratinocyte, fibroblast and SCC-9 cells.
AdCMV-EGFP infection shows the upper line.
The lower line shows the result of Ad-casp-14pr(0.7)-EGFP.
Keratinocyte and SCC9 are revealed by EGFP but fibroblast is not.

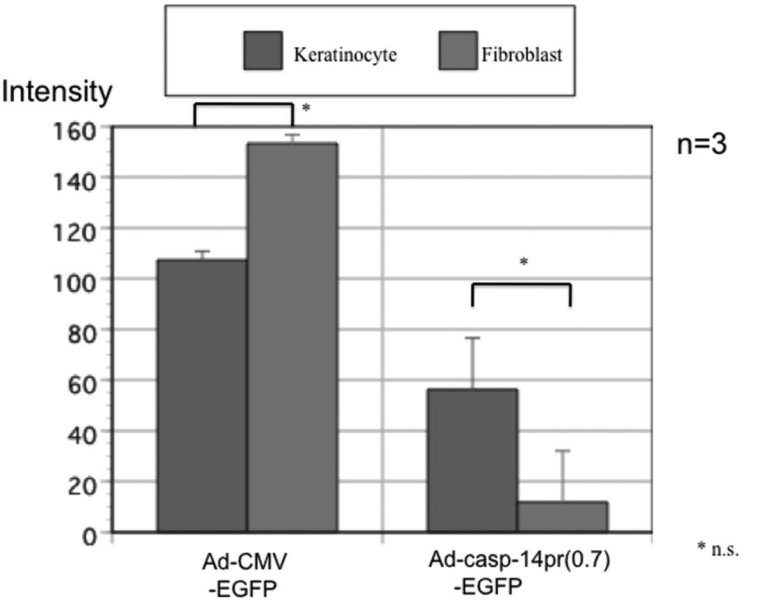

Figure 7. The result of quantification of the fluorescence in GFP expression GFP expression was clearly stronger in the keratinocytes than in the fibroblasts.

possible to minimize damage to normal tissues. As a result, it is now possible to carry out more refined treatments. Examples of such treatments include those using the DF3/MUC1 promoter for breast cancer[11], and the fibroblast growth factor 18 (FGF18) promoter and urokinase plasminogen activator receptor (uPAR or PLAUR gene) promoter for colon cancer[12,13]. In addition, research is being carried out in regard to application of the progression elevated gene-3 (PEG-3) promoter and the prostate-specific membrane antigen (PSMA) promoter to treatment of prostate cancer [14,15]. Possible approaches to the treatment of middle-ear cholesteatoma include suppression of the proliferation of cholesteatoma epithelial cells and promotion of their apoptosis. The structure of cholesteatoma epithelium is basically almost the same as that of skin, and for that reason it can be thought that the techniques of gene therapy for skin epithelial cells are potentially applicable to middle-ear cholesteatoma.

Several promoters are known to act specifically on dermal epithelial cells, including the promoters for keratin-5 and keratin-6. However, the keratin 5 promoter is long, having a full length of 14 Kb, which can be thought to make it difficult to transduce into cells. In addition, although the keratin 6 promoter functions at the time of cell proliferation, it doesn't at the time of nonproliferation, and we thus surmised that it would not be suited for application to gene therapy for cholesteatoma.

In consideration of the above background, we focused on the base sequence upstream of caspase 14 promoter. Based on our present results, it can be concluded that caspase 14 promoter activity is located in the 0.7 Kb or 1.0 Kb region, and that it functions specifically in relation to keratinocytes and SCC9 cells. Gene transduction can be thought to be easy with a DNA base sequence of this length. In addition, if the region downstream of the caspase 14 promoter were transduced as gene therapy, it might be possible to halt the proliferation of keratinocytes. Potential candidates for gene therapy directed at proliferating cells include such suicide genes as thymidine kinase (TK), cytosine deaminase (CD)(2) and deoxycytidine kinase (dCK)[15], etc., and such apoptosis regulatory molecules as survivin antisense[16,17,18], etc. Among those candidates, TK and dCK only function in relation to cells in the S phase of mitosis, meaning that they would be at a disadvantage in gene therapy of cholesteatoma that is in the nonproliferating stage.

Accordingly, CD might be a good choice because it would act even on non-dividing cells and since it shows a strong bystander effect[19].

However, there is a problem with casp-14pr(0.7Kb) and casp-14pr(1.0Kb) because they have low activity. For clinical application, this problem of low activity might be able to be overcome if research on tissue-specific promoters shows specificity in a tissue, and a system can be developed that induced strong local expression of transduced genes even if the activity is weak, for example, by use of Cre-Lox P etc.

In addition, the middle ear presents special problems due to its anatomical structure. That is, middle-ear cholesteatomas develop in an anatomically complex site, in close proximity to the cochlea, vestibule, semicircular canals, facial nerve, dura mater, etc. It is very important that any administered therapy avoid causing damage to these tissues and their cells.

In summary, we have demonstrated the specificity of the caspase 14 promoter in relation to keratinocytes compared with fibroblasts. However, studies must still be conducted to clarify its activity in relation to endothelial cells and nerve cells. Nevertheless, we think that our present findings of the specificity of the caspase 14 promoter's action on keratinocytes is a potentially very important step in regard to future development of gene therapy for middle-ear cholesteatomas.

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