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Differentiation Characteristics of Cholesteatoma Epithelium Determined by Expression of Transglutaminase Isoenzymes

Transglutaminase (TGase) isoenzymes are involved in the process of the differentiation and cornification of keratinocytes in the epidermis. This study investigates the presence and localization of three TGase isoenzymes to elucidate the nature and differentiation status of the squamous epithelium in human aural cholesteatoma. Twenty cholesteatoma specimens were used. The presence and localization of three TGase isoenzymes were studied by reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry. mRNA expression of three TGase isoenzymes were detected in the tested cholesteatomas with variable levels. The immunohistochemical staining patterns of three TGase isoenzymes showed variations within specimens, relating to keratinizing activity. TGase K is the most abundant among three isoenzymes. Keratinizing epithelium of cholesteatoma have similar expression profiles of TGase isoenzymes with those of epidermis of the skin. Other areas, particularly those showing non-keratinizing epithelium, showed weak immunostaining of TGase E and C, suggesting its different maturation status from keratinizing epithelium. The results of this study indicate that epithelium of cholesteatoma undergoes same direction of maturation and differentiation characteristics as the epidermis of skin, evidenced by similar expressions of TGases both in mRNA level and immunochistochemistry.

Key Words: *Cholesteatoma; Protein-glutamine gamma-glutamyltransferase; Isoenzymes*

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INTRODUCTION

Cholesteatoma is a chronic middle ear disease characterized by proliferation of squamous epithelium associated with chronic inflammation and bone destruction. The squamous epithelium of cholesteatoma becomes terminally differentiated and cornified, and then finally desquamated. Abnormal accumulation of keratin debris within the tympanic space resulted in persistent chronic inflammation and bone destruction (1, 2). The keratinizing activity of cholesteatoma has been thought to play an important role in the pathogenesis of continuing inflammation as well as surrounding bone destruction. However, very little information is available on the differentiation characteristics of the squamous epithelium in cholesteatoma and its origin is still controversial through migration theory is predominent (3, 4).

During recent years, distinct expression profile of three isoenzymes of transglutaminase (TGase) were identified in the epidermis in the terminal differentiation of keratinocyte: TGase K, a membrane associated isoenzyme of 103 kDa, is expressed in full layers of the epidermis (5, 6). TGase C, a soluble form of about 80 kDa, is expressed in basal and spinous layers (7, 8). TGase E, a proenzyme of about 78 kDa, is expressed mainly in the granular layer (9, 10). TGases are calcium dependent enzymes which are thought to be involved in the formation of a cornified cell envelope of the epidermal keratinocyte by cross-linking the various substrate proteins such as loricrin, involucrin, small proline-rich (SPR) filaggrin and keratin intermediate filament (11). Although the exact role(s) of the three TGases during the terminal differentiation of the keratinocyte is still obscure, the data on tissue distribution, location of expression in the epidermis, substrate specificity and mode of activation of each TGase suggests that the expression patterns of TGase K and TGase E are important markers in the terminal differentiation of epidermal keratinocyte (12). The cholesteatoma matrix is made up of multiple layers of epithelial cell with keratinized surface layers (13, 14). Because this morphological character of the cholesteatoma matrix is similar to the epidermis, it is interesting to study the

expression pattern of TGase isoenzymes in cholesteatoma in comparison with those of the epidermis.

In this paper, we studied the mRNA expression and localization of TGase isoenzymes in the cholesteatoma tissues by reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry in order to understand the status of epithelial differentiation and the biological characteristics of the cholesteatoma.

MATERIALS AND METHODS

Cholesteatoma specimens

Cholesteatoma specimens were obtained from twenty patients during middle ear surgeries and divided immediately into two portions. The one portion of each specimen was snap frozen immediately and stored in liquid nitrogen for RNA preparation. The other portion of specimens was processed for paraffin embedding after fixation in buffered 10% formalin solution, then sliced into 4-5 μm sections by microtome.

Detection of TGases by RT-PCR

Total RNAs were purified from cholesteatoma by the Chomczynski and Sacchi method (acid guanidinium thiocyanate-phenol, chloroform extraction method) (15). The pellets were resuspended in Diethyl Pyrocarbonate (DEPC) treated water at a concentration of 1 μg/μL as determined by optical density 260 nm. Random primed cDNAs were synthesized from 500 ng of total RNAs by using the Superscript reverse transcriptase kit (Gibco-BRL). The cDNAs were then amplified by two consecutive heminested PCR with specific oligo primers (Table 1). The first PCR amplification was carried out using outer (1F) and reverse (R) primers then subsequent nested PCR was performed using nested (2F) and reverse primers with 1000-fold diluted first PCR product. Primers were chosen to amplify across an intron boundary to ensure amplification of mRNA. Product sizes of nested PCR were 304 bp for TGase K, 378 bp for TGase C, and 324 bp for TGase E, which were confirmed by comparison with plasmids containing human cDNAs for TGase isoenzymes.

Table 1. Sequences of oligonucleotide primers used for heminested PCR

Primer	Sequence	PCR product	Reference
TGase K			
K1F	5′-GAGTCCATCAAGAATGGGCT		
K2F	5′-CCACAAGATGGGGCCGGTAT	304 bp	3, 14
KR	5′-CACATCCTCCGCTGAGCCCC		
TGase C			
C1F	5′-GTCTTTGCCCACATCACC		
C2F	5′-GTGCGGGCCCTCCTCGTG	378 bp	5, 15
CR	5′-TTAGGCGGGGCCAATGAT		
TGase E			
E1F	5′-GCAGTAGGCAAAGAAGTC		
E2F	5′-ATCACAGCGGTGTGCAAG	324 bp	8, 15
ER	5′-CTTGTTGCAGGAGAAGTC		

The 100 μL of first PCR reaction was optimized with 200 μg of each primer, 7 μL of 10X buffer II (Perkin Elmer), 4 μL of 25 mM $MgCl_2$, 1 μL of 10 mM dNTP, 0.5 μL of *Taq* DNA polymerase, and 20 μL of RT reaction product. The 20 μL of second nested PCR reaction was performed in standard conditions. Samples were heated to 95℃ for 5 min, followed by 35 cycles of denaturation at 94℃ for 30 seconds, annnealing at 48℃ for 30 seconds, and extension at 72℃ for 1.5 min. The final extension was at 72℃ for 10 min. PCR products were analyzed by 1.5% SekemGTG (FMC Bioproducts) agarose gel electrophoresis.

Detection of TGases by immunohistochemistry

Three primary antibodies for each isoenzymes of TGase were used in this study. Polyclonal antibody to TGase K was prepared in a goat with purified recombinant human TGase K expressed in bacteria (16). And antibodies to TGase C and E were prepared in rabbit with purified guinea pig liver TGase C and guinea pig skin TGase E, respectively (9). All antibodies were partially purified by affinity chromatography as described previously (9).

The paraffin-embedded cholesteatoma tissues were serially cut at 4-5 μm thickness and mounted on the poly L-lysine coated slides. Immunohistochemical procedure was done according to the standard ABC (Avidin-Biotin peroxidase complex) method using LSAB kit (Dako). In brief, the sections were deparaffinized and endogenous peroxidase was blocked by 0.03% H_2O_2. Non-specific reaction was blocked by bovine serum albumin and non-immune serum. Application of respective each mono-specific polyclonal antibody of anti-TGase K (1:400), C (1:400) and E (1:400) was done. Detection of respective immunoglobulins by biotin conjugated anti-goat or anti-rabbit immunoglobulins followed by ABC with substrate and 3′,3′ diaminobenzidine (DAB) as a chromogen. Negative control slides for each TGase were also run with same procedure without respective antibody, and reveals no positive staining. Relative staining intensity was scored subjectively on the light microscope as follows: − = no or very weak staining, 1+ = low intensity with pale brown color, 2+ = moderate intensity, 3+ = strong intensity with dark brown color.

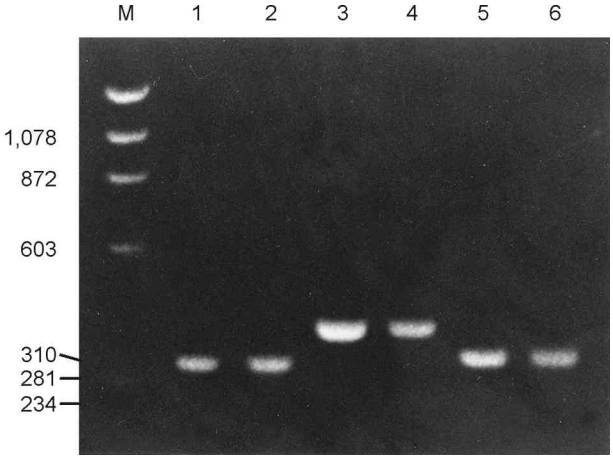

Fig. 1. Reverse transcription PCR analysis showing expression of transglutaminase K, C, and E mRNA. Total extracted RNAs were reverse transcribed, and heminested PCR using specific primers were done. PCR product was loaded on lane M, DNA size marker; lanes 1, 3, 5, cDNA positive control for TGase K, C and E; lanes 2, 4, 6, cholesteatoma derived RT-heminested PCR product of TGase K, C, and E, respectively.

RESULTS

Expression of TGase isoenzymes detected by RT-PCR

In order to study the differentiation characteristics of cholesteatoma epithelium, the expression of three TGase isoenzymes that mediate cross-linking of envelope proteins were examined in the terminal differentiation of epidermal keratinocyte. To achieve this goal, RT-PCR method was adopted because it is more sensitive than Northern blot analysis. From 20 frozen cholesteatoma tissues (10-20 mg), 0.5 μg-4 μg of total RNAs were purified. However, PCR amplification of cDNA produced by RT of total RNA failed to yield detectable amounts of product despite of increasing the cycle number to 35. Therefore, we designed the heminested PCR to increase the sensitivity and specificity of PCR amplification (Table 1). Fig. 1 shows RT-heminested PCR detection of mRNA of TGase isoenzymes, TGase K, TGase C, and TGase E in cholesteatoma epithelium. Lanes 1, 3 and 5 represent control amplifications using a plasmid containing full-length cDNA clones of TGase isoenzyme. Lane 2, 4, 6 represent the cholesteatoma derived RT-heminested PCR product of TGase K, C, and E, respectively. mRNA of three TGase isoenzymes were detected in virtually all of the 20 cholesteatoma tissues.

Localization of TGase isoenzymes by immunohistochemistry

The types of epithelium were divided into two groups; mature keratinizing (cornifying) and immature non-keratinizing (non-cornifying) types, and analyzed the differential expression of TGase isoenzymes in respective epithelium types. Fig. 2 shows the differentiation related expression patterns of TGase isoenzymes in the squamous epithelium of the cholesteatoma. In the keratinizing epithelium, TGase K was strongly stained all the layers of the squamous epithleium of cholesteatoma (Fig. 2A). The TGase C was also strongly stained more in the upper spinous layers than basal layer resulted in grading intensity (Fig. 2B). TGase E was strongly stained in the super-

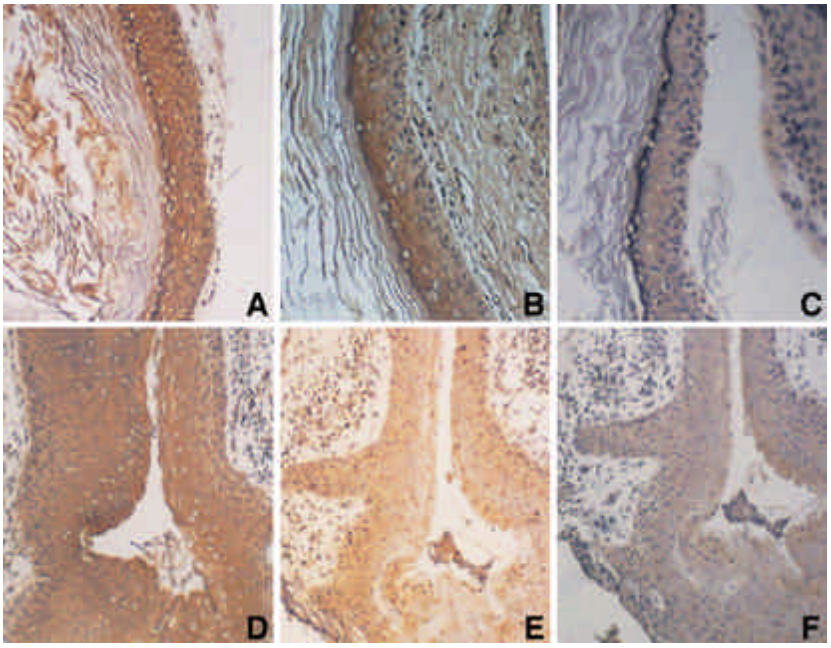

Fig. 2. Immunohistochemical staining of transglutaminase isoenzymes in keratinizing epithelium (A, B, C) and non-keratinizing epithelium (D, E, F) of the cholesteatoma. Figures reveal diffuse and intense staining of TGase K (A, D), similar intensity with TGase K and grading pattern of TGase C (B, D) and TGase E stained predominantly in the granular layer of keratinizing epithelium (C, F) (Immunohistochemical staining with respective polyclonal antibodies of TGase isoenzymes by universal ABC method, ×200).

Table 2. Relative immunostaining intensity of transglutaminase isoenzymes in keratinizing (K) and non-keratinizing (Non-K) epithelium of the cholesteatoma

Cell layers	TGase K		TGase C		TGase E	
	K	Non-K	K	Non-K	K	Non-K
Granular layers	+++	+++	+++	++	+++	−
Spinous layers						
upper	+++	+++	+++	++	++	−
lower	+++	+++	++	+	−	−
Basal layers	++	++	+	+	−	−

Relative staining intensity was scored subjectively: −, no staining; 1+, weak (low) intensity with pale brown color; 2+, moderate intensity; 3+, strong (high) intensity with dark brown color

ficial granular layers (Fig. 2C). These staining patterns were found consistently in all keratinizing epithelial regions of twenty cholesteatomas (Table 2). TGase K is most abundant followed by TGase C and TGase E in decreasing order. So, the different staining patterns of TGase isoenzymes in the keratinized epithelial layers were noted. In the non-keratinizing epithelium, the TGase K antibody stained all the layers of the squamous epithelium of cholesteatoma in a similar pattern with the cornifying (keratinizing) epithelium (Fig. 2D). The TGase C antibody stained differently with reduced intensity (Fig. 2E). The TGase E antibody was not stained (Fig. 2F). Therefore, the localization pattern of TGase E was to be related with the presence of superficial granular layers in the differentiated keratinizing squamous epithelium. Different staining pattern of TGase isoenzymes expression was also noted in between keratinizing and non-keratinizing epithelium of the cholesteatoma tissue. Immunohistochemical localization and staining pattern of TGase isoenzymes were summarized in Table 2.

DISCUSSION

We evaluated the differentiation charateristics of squamous epithelia in cholesteatoma using transglutaminase isoenzymes as keratinization markers in order to understand its nature of origin and differentiation. Thus, the aim of the present study was to determine the type(s) and locations of TGase isoenzymes expressed in aural cholesteatoma tissue. In the epidermis, three TGase isoenzymes are known to be expressed with distinct localization patterns (9, 17) during the terminal differentiation in stratified squamous epithelium especially in type E (III). As revealed in a genetic study, lamella ichthyosis (LI), an autosomal recessive skin disorder, characterized by generalized large scales, is caused by the point mutation of TGase K resulted in improper keratinization process (18). Mutation analysis of TGase K in LI showed that cross-linking activity of TGase K is required for a normal differentiation of the keratinocyte and could not be substituted with TGase C or TGase E activity. Therefore, it is thought that all three TGases are required for a proper keratinization with each TGase having a specific role during terminal differentiation of the keratinocyte.

In this report, the espression of the three TGase isoenzymes; TGase K, TGase C, and TGase E, was demonstrated by RT-PCR and the localization of TGase isoenzymes showed a similar distribution pattern with that of the epidermis by immunohistochemical staining in the aural cholesteatoma tissue. These results indicate that the squamous epithelium of cholesteatoma has an same differentiation characteristic as the epidermal keratinocyte, and suggest its origin of keratinizing squamous epithelium.

With respect to the relative expression level of TGase isoenzymes, we found that TGase K is the most abundant isoenzyme followed by TGase C and TGase E. However, it has been shown that most of the total cross-linking activity determined by dansylcadaverine coupling method were localized mostly in the granular layers in the epithelium of cholesteatoma (19, 20). The above active staining pattern of the TGase could be different from the immunohistochemical stain, because the latter does not differentiate its status of enzyme activity. The active stain reveals that the most active site of TGase is the upper layer of the epithelium. But isoenzymes of the TGases cannot be differentiated with the active stain. This discrepancy between abundance and cross-linking activity indicates that there is a specific activation mechanism for each TGase isoenzyme (21, 22). Therefore, the immunostaining intensity of TGases cannot be used as a parameter to predict the cross-linking activity.

One of the interesting results concerning the distribution of TGase isoenzymes is the difference of TGase E staining pattern between the keratinizing and non-keratinizing regions of cholesteatoma tissues. Although TGase E expression patterns were variable within and between specimens, the staining intensity of TGase E antibody is highly correlated to keratinizing (cornifica-

tion) activity in the keratinizing epithelium of the cholesteatoma. A previous report showed that TGase E is not expressed in the immortalized human keratinocyte cell line or can not be induced even if it is cultured in high calcium medium to activate the differentiation (8). TGase E is known as a proenzyme that requires activation by proteolytic cleavage. Because the activity of TGase E is increased 10-15 fold by activation and its location of expression is confined mostly to the granular layer, it is, therefore, believed that a significant portion of the cross-linking activity in the granular layers is contributed by TGase E (9). This suggests that the amount of cornification of keratinocytes could be closely correlated with the activity of TGase E.

In this immunohistochemical study, the polyclonal antibody against the purified recombinant human TGase K decorated all layers of the cholesteatoma epithelium diffusely, but with an increased intensity toward the granular layers. In conflict with these findings, a previous study on the localization of TGase K in cholesteatoma tissues using B.C.1 monoclonal antibody showed that immunostaining was located only in the region of the middle spinous and upper spinous layers (6). These conflicting results regarding the localization of TGase K expression were due to the different TGase K antibodies used in this study. Since recent reports have shown that the B.C.1 monoclonal antibody recognizes the small proline rich protein 2 (SPR2) rather than TGase K, our data has confirmed that the location of TGase K expression in cholesteatoma tissue was identical to that of the epidermis.

Although the biological function of TGase C has been extensively studied, the physiological role of this ubiquitous cytosolic TGase on epidermal differentiation is less well understood. A transfection study has shown that TGase C has been implicated in cell morphology, cellular adhesion and apoptotic cell death (8). The present immunohistochemical observation demonstrates that the expression of TGase C was extended into all cell layers in both keratinizing and non-keratinizing region of cholesteatoma. This pattern is similar with the TGase K distribution in this immunohistochemical study, which may be attributed by the characteristics of immunohistochemistry because immunohistochemical stain does not reveal the activity but the presence of proteins. This altered expression profile between the keratinizing and non-keratinizing epithelium was rather more correlated with maturation and keratinization than proliferation in the epithelium of cholesteatoma.

In conclusion, all three TGase isoenzymes were expressed in the epithelium of cholesteatoma and have similar expression profiles during the process of differentiation with that of the epidermal keratinocyte. These results suggest that the epithelium of human aural cholesteatoma has an identical differentiation process with that of epidermal keratinocyte.

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