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Expression of transforming growth factor-α (TGF-α) in cholesteatoma

MASANORI SHIWA, M.D., HIROMI KOJIMA, M.D., HIROSHI MORIYAMA, M.D.

Abstract

This study aims at elucidating the role of cytokines in the mechanism of proliferation of cholesteatoma epithelium by investigating the mode of expression of epidermal growth factors, such as TGF-α. The subjects of this study were patients who had undergone operation for middle ear cholesteatoma. Skins of the bone region of the external ear canal (normal skin) of the same patients were used as the negative control. The mode of expression of TGF-α was studied by immunohistochemistry and *in situ* hybridization. In the immunohistochemical study, there were no conspicuous differences observed between cholesteatoma tissues and normal skin. After *in situ* hybridization, expression of TGF-α mRNA was mainly observed in the epidermal basal cell layer in the normal skin, while in the cholesteatoma epidermis with severe inflammatory cell infiltration, expression of TFG-α mRNA was observed up to layers superior to the basal cell layer. The expression of TGF-α mRNA is greatly affected by subepithelial connective tissue, strongly suggesting involvement of paracrine regulation in proliferation of cholesteatoma epithelium.

Key words: Cholesteatoma; Cytokines; TGF-alpha

Introduction

With the objective of elucidating the mechanism of proliferation of cholesteatoma, we have been studying the relationship of cholesteatoma epidermis and subepithelial connective tissue with cytokines and growth factors, mainly employing immunohistochemical techniques and *in situ* hybridization. Our study indicated involvement of IL-1α produced by subepithelial macrophages in the proliferation of cholesteatoma, but no clear correlation could be proven between clinical pathology and expression of IL-1α (Shiwa *et al.*, 1995). However, clear differences were observed in the mode of expression of mRNA of the EGF-receptors (EGF-R mRNA) between cholesteatoma epithelium and the skin of the normal external ear canal. While expression of EGF-R mRNA was observed mainly in the epidermal basal cell layer in the normal external ear canal, strong over-expression of EGF-R mRNA was observed in almost all layers in the cholesteatoma epithelium. This suggests the possibility of involvement of EGF-R in the mechanism of proliferation of cholesteatoma epithelium (Kojima *et al.*, 1994).

In the present study, we investigated the transforming growth factor-α (TGF-α), which is the ligand of EGF-R and is a growth factor of keratinocytes. In terms of amino acid sequence, TGF-α has a 30 to 40 per cent homology with EGF, and can bind to EGF-R and promote proliferation of various kinds of cells (Coffey *et al.*, 1987; King *et al.*, 1990). In order to understand the mode of involvement of TGF-α in proliferation of cholesteatoma epithelium, we conducted a study using immunohistochemistry and *in situ* hybridization.

Materials and methods

Subjects

The subjects of this study were 12 patients selected from patients who had undergone operation for cholesteatoma of the middle ear (flaccid part type) for the first time at the Department of Otorhinolaryngology of a hospital of Toyko Jikei University School of Medicine during the period from October 1992 to July 1996. They consisted of nine males and three females, and their ages ranged from 18 to 54 years with the mean age of 39 years. As the control, four skin samples simultaneously collected from the bone-region of the normal external ear canal of each patient were used.

Immunohistochemical method

The cholesteatoma tissue samples and skin tissue samples from the external ear canal were immedi-

From the Department of Otorhinolaryngology, The Jikei University School of Medicine, 3-25-8 Nishishinbashi Minato-ku, Tokyo 105-0003, Japan.
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ately fixed in a 10 per cent buffered formalin solution at room temperature overnight. The SAB technique was employed. A 50-fold dilution of mouse anti-human TGF-α monoclonal antibody (Oncogene Science, USA) was employed as the primary antibody, and histofine biotin-conjugated rabbit ant-mouse IgG+IgA+IgM (Nichirei, Japan) was employed as the secondary antibody. After colour development with DAB, the specimens were counter stained with haematoxylin.

In situ hybridization

Human transforming growth factor-α (R&D System Europe Ltd., UK) was used as the probe. This probe is a synthetic oligonucleotide DNA cocktail probe consisting of Exon 1 (26mer), Exon 2 (26mer), Exon 3A (30mer), Exon 3B (29mer) and Exon 5 (27mer), with their 3′ and 5′ terminals being labelled with biotin.

Immediately after collection, the specimens were fixed in a 10 per cent buffered formalin solution overnight, embedded in paraffin, sliced into 3–4 μm sections, and placed on a slide coated with 3-amino-propyl-triethoxylsilane (Yatron Co. Japan). After deparaffinization, the sections were treated with proteinase K (17 μg/ml, 37°C, 20 minutes), and post-fixed with 0.4 per cent paraformaldehyde/PBS. Then the sections were dehydrated, dried, subjected to prehybridization (37°C, one hour), hybridization (250 ng/ml, 37°C, 16 hours), washed with SSC solution, allowed to react with streptoavidin-alkaline phosphatase, and subjected to colour development with NBT-BCIP. *In situ* Work Station Kit (British Bio-Technology Products Ltd., UK) and *In situ* Hybridization and Detection System (GIBCO BRL, Life Technologies, Inc., USA) were used as the reagents. A specimen pretreated with RNase to decompose mRNA and a specimen in which the probe was not used in the hybridization process were employed as the negative controls.

Results

In the immunohistochemical study, all epidermal layers of both normal skin tissue and cholesteatoma tissue underwent staining in all subjects. In both tissues, strong staining was observed near the granular layer. There were no conspicuous differences observed between cholesteatoma tissues (Figure 1) and normal external ear canal skin tissues (Figure 2).

In the study on the mode of expression of TGF-α mRNA using *in situ* hybridization, a high signal was observed mainly in the basal cell layer and suprabasal cell layer in five of the 12 subjects. Two of these five subjects showed much infiltration of the sub-epithelial connective tissue by inflammatory cells,

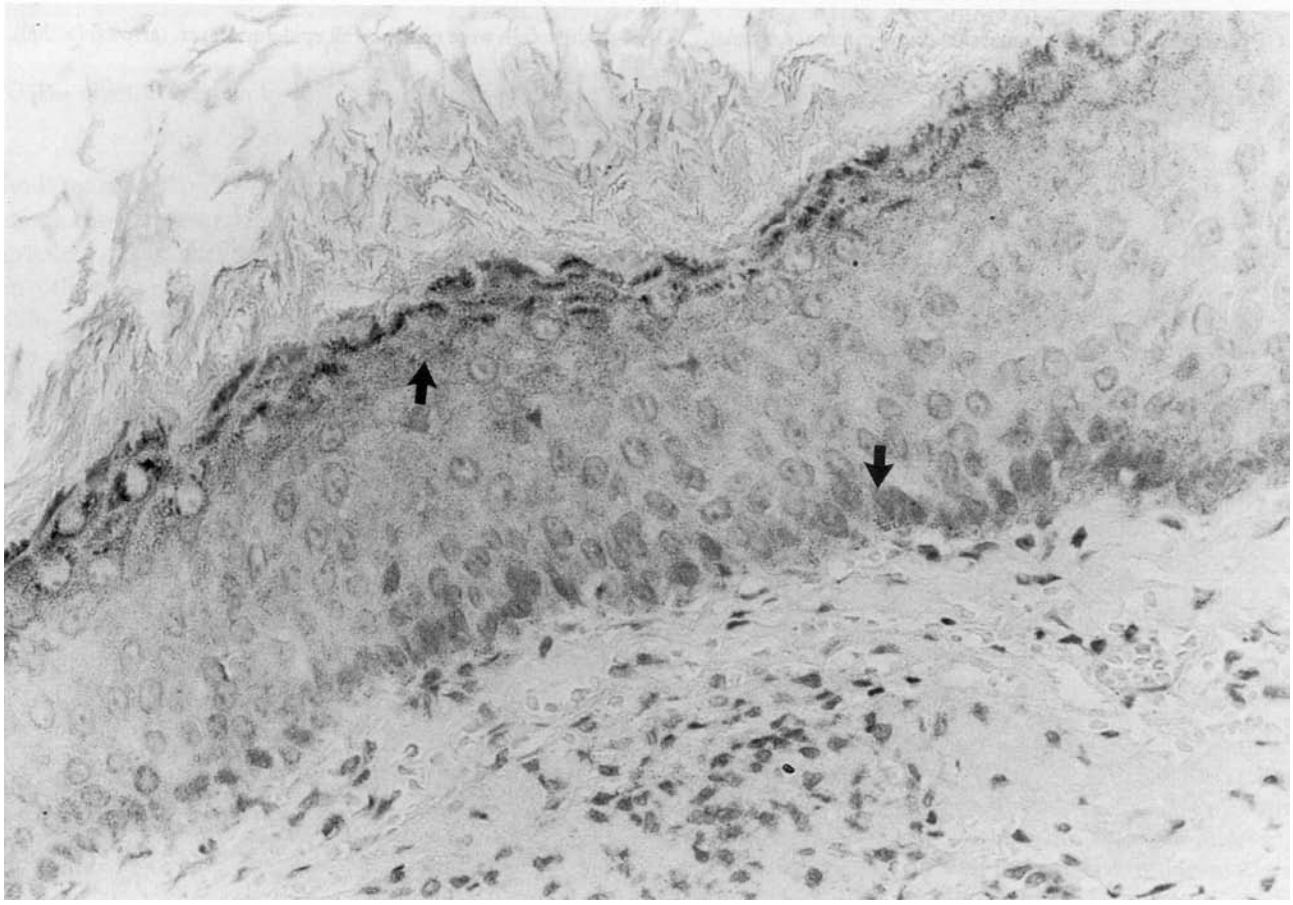

FIG. 1

TGF-α immunostaining of cholesteatoma. : TGF-α-positive cells (arrows) were observed all epidermal layer, especially, strong staining were observed in granular cell layer (× 200).

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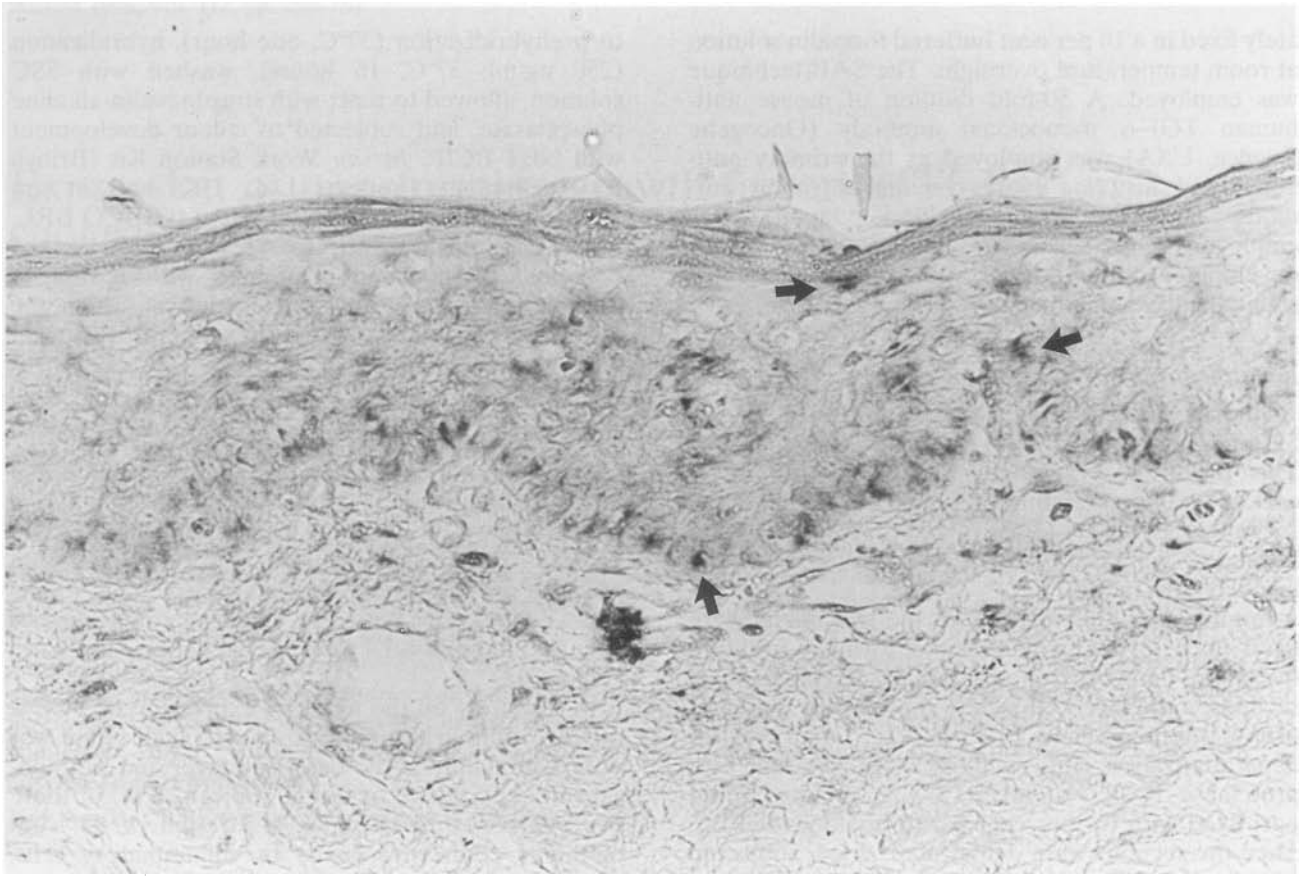

FIG. 2
TGF-α immunostaining of normal skin of the external ear canal. : TGF-α-positive cells were observed all epidermal layer. (arrows) (× 200).

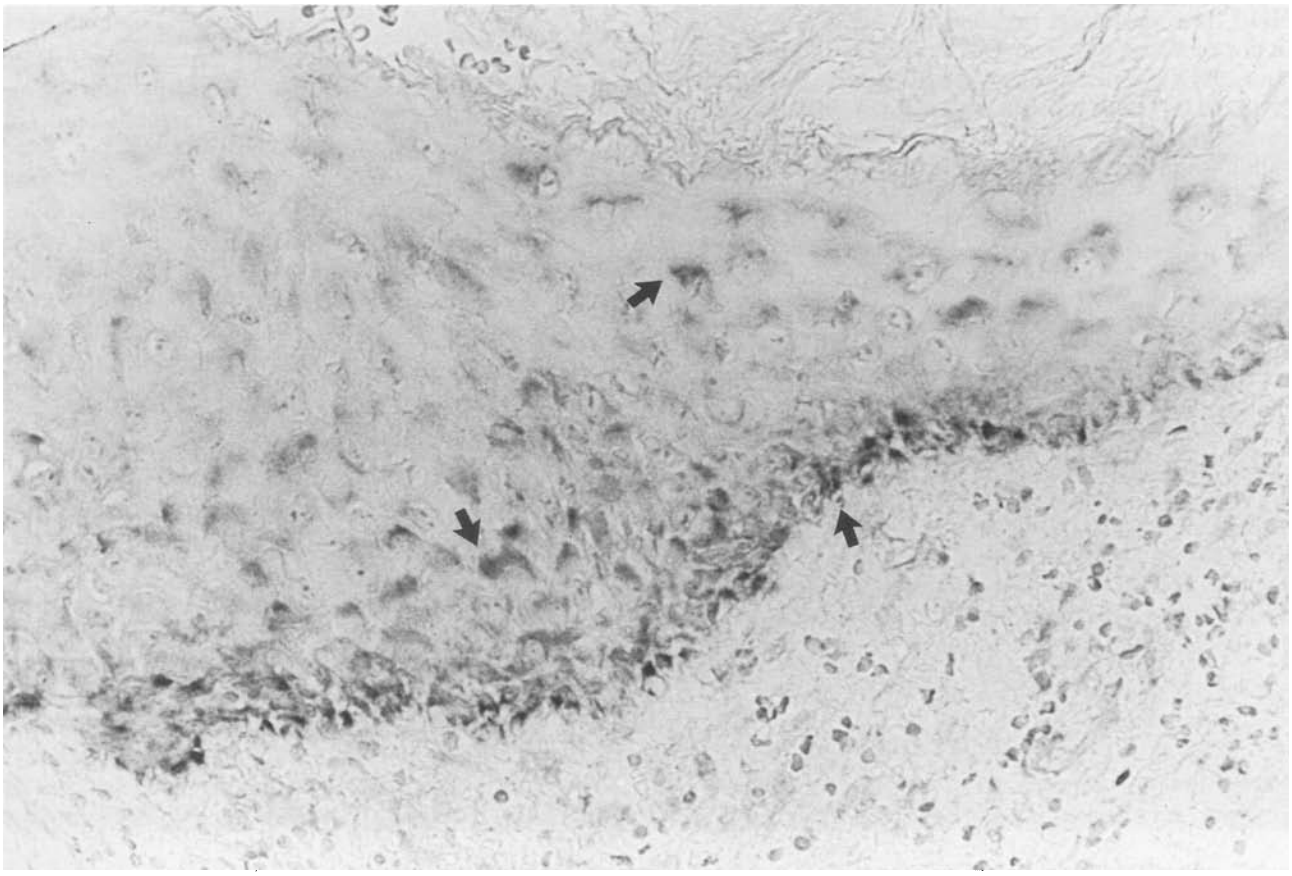

FIG. 3
TGF-α mRNA detection by ISH in cholesteatoma. : Expression of TGF-α mRNA were observed all epidermal layer, mainly signal expression were observed in basal cell layer and supurabasal cell layer (arrows) (× 200).

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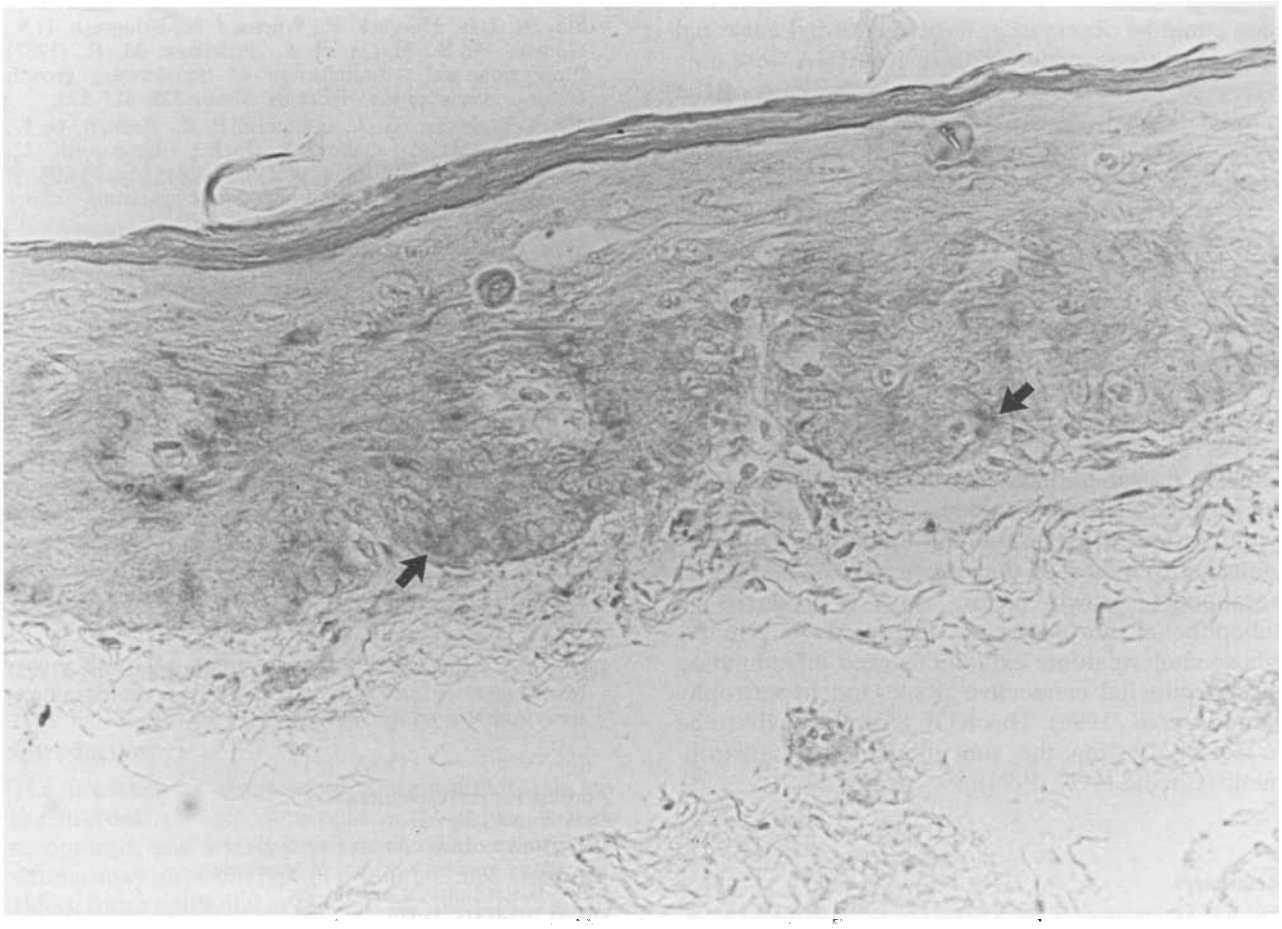

FIG. 4

TGF-α mRNA detection by ISH in normal skin of the external ear canal. : Expression of TGF-α mRNA were observed in the scattered manner mainly in the basal cell layer. (arrows) (× 200).

and marked epidermal hypertrophy. Their specimens showed strong signal expression mainly in the granular layer, and the expression was also observed in other superior layers (Figure 3). In the normal skin tissue, only scattered weak signals were observed mainly in the basal cell layer (Figure 4).

In summary, there were no differences in the distribution of TGF-α protein between cholesteatoma tissue and normal skin tissue. However, at the mRNA level, while expression of mRNA was limited to the basal cell layer in the case of normal skin tissue, signal expression was also observed in the granular layer, which is superior to the basal cell layer, in the case of cholesteatoma tissues (Table I).

TABLE I
EXPRESSION OF TGF-αmRNA

Epithelium	Basal layer	Para basal layer	Upper layer
skin of external ear canal (4 cases)	+ (4/4)	+ (1/4) − (3/4)	− (4/4)
cholesteatoma (12 cases)	+ (10/12) − (2/12)	+ (5/12) − (7/12)	+ (2/12) − (10/12)

+: positive expression. −: negative expression

Discussion

A study on the proliferation of keratinocytes using TGF-α has indicated that there is autocrine regulation between TGF-α and EGF-R (Coffey *et al.*, 1987). Also, enhancement of expression of TGF-α and EGF has been reported in diseases which are based on vigorous division of the epidermis, such as psoriasis and malignant epidermal tumours (Elder *et al.*, 1989). Accordingly, middle ear cholesteatoma, which involves excessive epidermal proliferation, is thought to have a similar proliferation mechanism (Shulz *et al.*, 1993).

In the present study, the signals of TGF-α mRNA were strongly expressed up to the granular layer, a layer superior to the basal cell layer, in subjects exhibiting severe subepithelial inflammation and hypertrophy. This suggests that expression of TGF-α is closely related to the condition of the subepithelium. In our earlier study on localization of IL-1α in cholesteatoma epithelium, as well, IL-1α tended to be strongly expressed in cases with abundant subepithelial inflammatory granulation. This also suggests a relationship with inflammation (Shiwa *et al.*, 1995). Moreover, in a study of EGF mRNA and EGF-R mRNA, expression of EGF-R mRNA was observed along the basal cell layer in the case of cholesteatoma, while almost no such expres-

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sion could be observed in normal external ear canal skin. In contrast, signals of EGF-R mRNA were only observed in the basal cell layer in the case of the normal external ear canal skin, while marked signals were observed in all layers of cholesteatoma epithelium (Kojima *et al.*, 1994).

When all the above findings are taken into consideration, it is inferred that over-expression of so-called ligands of epidermal growth factors, such as TGF-α and EGF, is affected by the inflammatory condition of the subepithelium, and that abnormal expression of EGF-R, the receptor of the said ligands, may be an important factor in the regulation of proliferation of cholesteatoma epithelium.

The expression of TGF-α mRNA is greatly affected by subepithelial connective tissue, strongly suggesting involvement of paracrine regulation in proliferation of cholesteatoma epidermis. Our previous study indicated that expression of mRNA of keratinocyte growth factor, which is produced by subepithelial fibroblasts, is increased in patients whose cholesteatoma exhibits marked inflammation of subepithelial connective tissue and hypertrophy (Kojima *et al.*, 1996). This KGF is also thought to be a factor affecting the subepithelial microenvironment (Chedid *et al.*, 1994).

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Address for correspondence:
Dr Masanori Shiwa,
Department of Otorhinolaryngology,
The Jikei University School of Medicine,
3-25-8 Nishishinbashi Minato-ku,
Tokyo 105-0003, Japan.

Fax: +81-3-3435-8436