Expression of Transforming Growth Factor (TGF)–β 1 and its Type I and Type II Receptors in Carcinoma and Leukoplakia of the Tongue: Immunohistochemical and immunoelectron microscopic studies

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TGF-β 1, TGF-β RI and TGF-β RII were examined in 80 cases of squamous cell carcinoma (SCC), 26 cases of leukoplakia and 10 cases of early carcinoma in leukoplakia of the tongue by immunohistochemical methods. Nine cases of SCC were analyzed immunoelectron microscopically. Expression of TGF-β 1 was enhanced in SCC compared with early carcinoma in leukoplakia and without leukoplakia (P<0.05). Expression of TGF-β RI and TGF-β RII was significantly reduced in SCC compared with leukoplakia (P<0.01). Early carcinoma in leukoplakia did not show enhanced expression of TGF-β 1 but expression of TGF-β RI and TGF-β RII was reduced, compared with leukoplakia (P<0.05). Immunoelectron microscopically, TGF-β 1 immunostaining was scattered in the cytoplasm of tumor cells and TGF-β RI and TGF-β RII stainings were present on the tumor cell membrane and in the capillary endothelial cells of the stroma. In conclusion, tumorigenesis in the tongue may involve escape from cellular regulation of TGF-β 1 by reduced expression of TGF-β RI and TGF-β RII.

Key words: TGF-β 1, TGF-β RI, TGF-β RII, tongue, carcinoma, leukoplakia

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Introduction

TGF-βs are dimeric polypeptides of 25 KDa belonging to a large family of related proteins, which regulate a number of cellular processes including growth inhibition and differentiation of epithelial cells and accumulation of the extracellular matrix proteins (1, 2). Three isoforms of TGF-βs have been identified in mammals, and TGF-β 1, the predominant form in humans, is widely expressed. TGF-βRI and TGF-βRII are transmembrane serine/threonine kinase receptors and TGF-β 1 binds directly to TGF-βRII, which recruits TGF-βRI into a heteromeric complex (3-5). Phosphorylation of TGF-βRI by TGF-βRII activates its kinase activity and activates the downstream substrate, such as the Smad family for the signaling transduction (6).

TGF-β is a multifunction cytokine that can either stimulate or inhibit cell proliferation and differentiation via autocrine and paracrine mechanisms. It has been demonstrated that TGF-β 1 levels are elevated in the cancers of stomach (7, 8), colon and rectum (9, 10), breast (11, 12), pancreas (13) and lung (14). However Comerci et al. (15) described how decreased expression of intracellular TGF-β in neoplastic epithelium and increased expression of extracellular TGF-β in stroma were associated with invasive cervical carcinoma.

SCC of the tongue is the most frequent cancer in the oral cavity and leukoplakia is considered a precursor for SCC (16, 17). In order to examine of roles of TGF-β 1 in the tumorigenesis of the tongue, we analyzed expression of TGF-β 1 and its type I and type II receptors in the carcinoma, leukoplakia and early carcinoma in leukoplakia, by immunohistological and immunoelectron microscopic methods.

Materials and Methods

1. Tissue specimens

A total of 124 tissue specimens were collected from the archives of 1992 to 1997 in the Laboratory Medicine and Oral Pathology, Tokyo Medical and Dental University. Among them, 80 cases were squamous cell carci-
noma which occurred on the lateral border of anterior 2/3 of the tongue and did not receive any chemotherapy or radiotherapy before. According to the WHO’s criteria of histologic differentiation, we classified 30 cases into grade 1, 30 cases into grade 2 and 20 cases into grade 3. Twenty-six cases were leukoplakia and 10 were early carcinoma in leukoplakia. Eight cases were normal mucosa of the tongue.

For immunoelectron microscopic observations, 5 cases of SCC were analysed by pre-embedding method and 4 cases of SCC were analysed by immunogold staining method.

2. Immunohistochemistry

1) Immunohistochemical staining

A histofine SAB-PO kit was used. After deparaffinization, dehydration in the increasing dilutions of ethanol, and immersion in phosphate-buffered saline (PBS), the endogenous peroxidase activity was blocked in 0.3 % H2O2 methanol for 30 min. The sections were then treated with blocking reagent (normal serum of rabbit or mouse) for 15 min. The primary antibodies used were anti-TGF-β 1 (mouse monoclonal antibody, 1: 100; Antigenix America Inc.), anti-TGF-βRI (rabbit polyclonal antibody, 1: 200; Santa Cruz Biotechnology Inc.) and anti-TGF-βRII (rabbit polyclonal antibody, 1: 200; Santa Cruz Biotechnology Inc.). Incubation with the primary antibodies was performed in a humid chamber overnight at 4°C. Following incubation biotinated secondary antibodies were applied for 15 min. The peroxidase-labelled streptavidin was added for 10 min. The visualization was done by using peroxidase substrate 3, 3’-diaminobenzidine tetrahydrochloride (DAB) for 5 min and counterstaining was done by methyl-green.

2) Standard

Two pathologists divided intensity of stainability into -, +, ++, ++++. In the cases of SCC and leukoplakia, the positive staining in 5–30% were+, 30–70% were++, 70–100% were +++ and below 5% were -. The specimens were fixed in 2% paraformaldehyde solution, embedded in O.C.T compounds, and quickly frozen in liquid nitrogen (18). Frozen sections (6 μm) were placed on silane-coated glass slides and kept at 0°C until use. The staining procedures were the same as those in immunohistochemical staining except omission of the blocking step. In order to preserve ultrastructure of the tissues, all the reactions were done under 4°C. After the staining procedures, the samples were post-fixed with 1% glutaraldehyde, and then were subjected to DAB reactions. Later, the sections were fixed in 1% osmium tetroxide for 1 h, dehydrated in the graded ethanol, and embedded in epon 812 resin. Ultrathin sections without electron staining were observed with Hitachi H-7100 electron microscopy.

2) Immunogold staining method

The specimens were fixed in 1% paraformaldehyde solution for 2 h, were cut in 40 μm with microslicer (DTK-1000). After immersion in PBS, the sections were treated with blocking reagent (1% normal serum of goat in 0.05% saponin PBS) for 30 min. The sections were incubated with the same primary antibodies diluted in 0.05% saponin PBS in a humid chamber overnight at 4°C, then immunogold (10 nm) diluted in 0.05% saponin PBS (1: 20) were applied and incubated for 24 h. After staining procedures, the samples were fixed and embedded according to usual electron microscopic method. After electron staining, ultrathin sections were observed with Hitachi H-7100 electron microscopy.

Results

1. Immunohistochemistry

Immunohistochemical results of TGF-β 1 and its receptors TGF-βRI and TGF-βRII are summarized in Table 1.

1) TGF-β 1

Enhanced expression of TGF-β1 was present in SCC, in which immunoreactivity was present in the cytoplasm of cancerous spinous and keratotic cells in the cancer nests and the peripheral basal cells were not stained (Fig. 1). The stronger immunoreactivity was seen in the less differentiated carcinoma. Positive staining was also

### Table 1: Immunohistochemical results of TGF−β1, TGF−βRI and TGF−βRII expression

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th>TGF−β 1 (%)</th>
<th>TGF−βRI (%)</th>
<th>TGF−βRII (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCC of tongue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>30</td>
<td>21 (70%)</td>
<td>4 (13%)</td>
<td>4 (13%)</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>26 (87%)</td>
<td>8 (27%)</td>
<td>3 (10%)</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>17 (85%)</td>
<td>3 (15%)</td>
<td>4 (20%)</td>
</tr>
<tr>
<td>Total</td>
<td>80</td>
<td>64 (80%)</td>
<td>15 (19%)</td>
<td>11 (14%)</td>
</tr>
<tr>
<td>Leukoplakia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slight</td>
<td>10</td>
<td>4 (40%)</td>
<td>6 (60%)</td>
<td>7 (70%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>8</td>
<td>6 (75%)</td>
<td>7 (88%)</td>
<td>5 (63%)</td>
</tr>
<tr>
<td>Severe</td>
<td>8</td>
<td>5 (63%)</td>
<td>4 (50%)</td>
<td>6 (75%)</td>
</tr>
<tr>
<td>Total</td>
<td>26</td>
<td>15 (58%)</td>
<td>17 (65%)</td>
<td>18 (69%)</td>
</tr>
<tr>
<td>Early carcinoma in leukoplakia</td>
<td>10</td>
<td>5 (50%)</td>
<td>3 (30%)</td>
<td>3 (30%)</td>
</tr>
<tr>
<td>Normal epithelium</td>
<td>8</td>
<td>6 (75%)</td>
<td>6 (75%)</td>
<td>6 (75%)</td>
</tr>
</tbody>
</table>

Histological grade by WHO’s criteria  Degree of dysplasia
Fig. 1: TGF-β1 immunoreactivity in grade 1 SCC (×440)
TGF-β1 is diffusely stained in the cytoplasm of cancerous spinous cells.

Fig. 2: Early carcinoma in leukoplakia (H. E. ×50)

Fig. 3: TGF-βRI immunoreactivity in grade 2 SCC (×440)
TGF-βRI is stained strongly on the cellular membrane and diffusely stained in the cytoplasm of cancer cells.

Fig. 4: TGF-βRII immunoreactivity in leukoplakia with moderate dysplasia (×300)
TGF-βRII is stained on the cell membrane of spinous cells.

Fig. 5: Statistical correlation of TGF-β1, TGF-βRI and TGF-βRII expression in SCC, leukoplakia and early carcinoma in leukoplakia of the tongue.

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found in the stromal cells, especially cells of capillaries and fibroblasts around cancer nests.

In leukoplakia, immunoreactivity was observed in the cytoplasm of the spinous and keratotic cells, while basal cells were not stained. In the early carcinoma in leukoplakia, immunoreactivity was weak (Fig. 2). In normal mucosa, spinous cells were stained intracellularly.

2) TGF-βRI and TGF-βRII

Stainability was basically the same between TGF-βRI and TGF-βRII. In SCC, the cancerous spinous and keratotic cells in the cancer nests were stained in the cytoplasm and, the cell membrane was more strongly stained in some cells (Fig. 3).

In leukoplakia, the positive staining of TGF-βRI and TGF-βRII was observed in the cell membrane of the spinous cells, while staining was not seen in the basal cells (Fig. 4). In the early carcinoma in leukoplakia, expression of TGF-βRI and TGF-βRII was remarkably reduced.

3) Statistical analysis of immunohistochemical stainability

Rate of expression of TGF-β1, TGF-βRI and TGF-βRII in SCC, leukoplakia and early carcinoma in leukoplakia was shown in Fig. 5. Significant increase of TGF-β1 expression was seen in SCC, compared with leukoplakia and early carcinoma in leukoplakia (p<0.05). There was no significant difference of TGF-β1 expression in comparison between leukoplakia and early carcinoma in leukoplakia. Significant reduced expression of TGF-βRI and TGF-βRII was found in SCC compared with leukoplakia (p<0.01). Reduced expression of TGF-βRI and TGF-βRII was also found in early carcinoma in leukoplakia compared with leukoplakia (p<0.05).

2. Immunoelectron microscopy

1) TGF-β 1

In both pre-embedding and immunogold staining methods, immunoreactivity of TGF-β1 was found in the cytoplasm of the tumor cells, which was not specifically located in the cell organelles (Fig. 6). Sometime immunoreactive gold particles were found on the filaments. The cytoplasmic membrane was not stained.

2) TGF-βRI and TGF-βRII

In both pre-embedding and immunogold staining
methods, the immunoreactivity of TGF-βRI and TGF-βRII was found in the cell membrane and membrane of the organella, especially rough surfaced endoplasmic reticulum of the tumor cells (Figs. 7, 8). Intense immunoreactive gold particles of TGF-βRI and TGF-βRII were located in the cell membrane of capillary endothelial cells and membrane of organella of stromal cells (Fig. 9).

Discussion

Results of the present study of the tongue carcinoma demonstrated that TGF-β1 was overexpressed and TGF-βRII was remarkably reduced compared with leukoplakia and normal epithelium of the tongue. There was seen an inverse correlation between the levels of expression of TGF-β1 and histological grading of the tongue carcinoma. Microscopic early carcinoma in leukoplakia did not show elevated expression of TGF-β1, although expression of TGF-βRII and TGF-βRII was remarkably reduced. In the immunoelectron microscopic observations, TGF-β1 was present predominantly in the cytoplasm of the tumor cells but also in the stroma cells. TGF-βRI and TGF-βRII were located on the cell membrane of carcinoma cells and stroma cells. In the stroma, intense immunoreactivity of TGF-βRII and TGF-βRII was observed in the capillary endothelial cells mainly in the areas surrounding cancer nests. Taken together, these observations provided a valuable insight regarding the potential role of TGF-β1, TGF-βRI and TGF-βRII in the tumorigenesis of the tongue carcinoma.

TGF-β is synthesized and secreted in a latent form that is combined with latent TGF-β binding proteins (LTBPs) (19-21). The cleavage of LTBp by proteinase releases soluble TGF-β and it can be stored in the extracellular matrix (22, 23). Taipale et al. (24) mentioned that latent TGF-β1 and its binding protein were components of extracellular matrix microfibrils. Ultrastructurally in the gastrointestinal carcinoma, LTBp was located in the extracellular matrix around fibroblasts and smooth muscle cells (25). It is considered that tumors with the ability to activate latent form of TGF-β might be those with poor prognosis. However, antibodies used in the present study did not distinguish between latent and active TGF-β1s. Moreover it should be remembered that the mere presence of TGF-β1, TGF-βRII and/or TGF-βRIII does not necessarily point to autocrine function, since there may be no effect when ligand and receptor are produced separately.

In tongue carcinoma, growth inhibitory effect on the tumor epithelial cells by elevated levels of TGF-β1 could be escaped by decreased expression of TGF-βRII and TGF-βRII. With reduction of the expression of two receptors, the signal transduction pathway may be significantly impaired and the autocrine and/or paracrine growth control could be destroyed. Eisma et al. (26) reported decreased expression of TGF-β receptor on head and neck squamous cell carcinoma. Reduced expression of TGF-β receptors has been reported in the cancers of stomach (27), colon (28-30), esophagus (31), breast (32), kidney (33), urinary bladder (34), prostate (35-37), pancreas (38), thyroid (39), salivary gland (40) and brain (41). However, Lu et al. (42) reported that increased levels of TGF-βRI and RII correlated with disease progression of pancreatic cancer. Another possible mechanism of escape could involve genetic changes in the TGF-β receptor gene itself or altered expression of its mRNA, as seen in the cancers of stomach (43), colon and rectum (44) and prostate (45). Garrigue-Antar et al. (46) and Muñoz-Antonia et al. (47) reported missense mutations of TGF-βRII in squamous cell carcinoma, in vivo and in vitro.

Adequate nutrient supply through the neovascular networks in the stroma may be a prerequisite for proliferation of the tongue carcinoma. TGF-β1 is an important moderator of angiogenesis with the potential for promotion of neovascularization in the stroma and production of extracellular matrix in the cancer tissue (48). Therefore it is interesting that in the present study immuno-electron microscopic observations showed intense immunoreactivity of TGF-βRI and TGF-βRII in the capillary endoepithelial cells around cancer nests. Poclopoulos et al. (49) described that there is a positive correlation between histological differentiation, TGF-β expression and the elaboration of extracellular proteins in rat squamous cell carcinoma.

In the present study, expression of TGF-β1 in the early carcinoma in leukoplakia was not enhanced and TGF-βRI and TGF-βRII expression was remarkably reduced. This suggested that TGF-β1 did not play a critical role and reduction in the receptors was more important in the early stage of tumorigenesis of tongue carcinoma.

In summary, it could be concluded that TGF-β1 is one of the growth factors which have been implicated in the processes of progression and differentiation rather than initial development in the tumorigenesis of tongue carcinoma. Remarkably reduced expression of TGF-βRII and TGF-βRIII might prevent the downregulation of tumor cell proliferation by TGF-β1 and the resulting increased levels of TGF-β1 in the tumor microenvironent could enhance tumor progression by stimulating angiogenesis and other mechanisms. Loss of responsiveness to TGF-β1 might be important in the progression of leukoplakia to invasive carcinoma.

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