Laryngeal keratinocytes show variable inhibition of replication by TGF-β

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Summary

The response of secondary human laryngeal epithelial cells to transforming growth factor-β (TGF-β) was investigated, and this response was compared with that of epithelial cells derived from virally induced laryngeal papillomas. In most cases, both normal laryngeal epithelial cells and those derived from laryngeal papillomas exhibited growth inhibition in response to 10 ng ml⁻¹ TGF-β. Response was not a function of cell density or proliferation rate when cells were in a low-calcium medium, but was reduced in high calcium. Using keratinocytes derived from several different tissue explants, we found that cells grown from different explants show marked variation in response to TGF-β.

Key words: TGF-β, laryngeal, keratinocytes, papilloma.

Introduction

Transforming growth factor-β (TGF-β) is a regulator of cell growth and differentiation that affects a number of different cell types and elicits a wide variety of cellular responses (for review, see Sporn et al. 1987). TGF-β stimulates the growth of fibroblastic cells (Leof et al. 1986). In contrast, the proliferation of foreskin keratinocytes and many other normal epithelial cell types is inhibited by TGF-β (Moses et al. 1985; Shipley et al. 1986; Jetten et al. 1986; Lin et al. 1987; Masui et al. 1986).

Little is known about the response of human laryngeal epithelial cells to growth regulators such as TGF-β. The goals of the present study were to investigate the response of these cells to TGF-β and to compare their response to that of epithelial cells derived from laryngeal papillomas. Papillomas of the larynx are benign neoplasms caused by one or more of the human papillomaviruses. The papillomas frequently recur and can be life-threatening because they obstruct the airway. Histologically, the papillomas exhibit hyperplasia of the spinous layer, leading to an overall thickening of the epithelium. We were interested in knowing whether the escape from normal growth controls exhibited by papillomas is due to an altered response to TGF-β.

Our findings show that, in most cases, both normal laryngeal epithelial cells and those derived from laryngeal papillomas exhibit growth inhibition in response to TGF-β. However, cells derived from different tissue explants vary in their levels of response to this growth regulator.

Materials and methods

Cells

Primary human laryngeal epithelial cells were cultured as previously described (Steinberg et al. 1982) from biopsy fragments of normal and papillomatous tissues.

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Assay for bromodeoxyuridine incorporation

Experimental cultures were plated on glass coverslips in MCDB 153 (Clonetics; Boyce and Ham, 1983) supplemented with epidermal growth factor, retinoic acid, bovine pituitary extract, insulin, transferrin and hydrocortisone. All experiments were done in this complete medium and in 0.1–0.2 mM calcium unless otherwise stated.

When cultures neared confluence, they were exposed to 10 ng ml⁻¹ TGF-β (Collaborative Research) for 4 days and to 3 μg ml⁻¹ bromodeoxyuridine (BrdU) for 24 h during the last day of exposure to TGF-β. Control cultures received BrdU alone. Paired coverslips were always plated, fed, labeled and stained at the same time. The cells were fixed with 70% ethanol at 4°C and stained for BrdU using an indirect immunofluorescence technique. The primary anti-BrdU antibody was purchased from Becton Dickinson and used as directed. The secondary antibody was purchased from Dako. Brightly fluorescent nuclei were scored as positive. At least 10 fields were counted per coverslip. The significance of the difference between treated and control means was determined by Student’s t-test. In most cases, response was measured on a single set of coverslips for each explant. When sufficient cells were available for duplicate coverslips, they were in very good agreement.

Involucrin and keratin stains

Cultures to be stained for involucrin or keratins were plated on glass coverslips. For the involucrin stain, cells were fixed with 4% formaldehyde in phosphate-buffered saline and stained for involucrin using the Vectastain ABC immunoperoxidase procedure (Vector Laboratories). The primary antibody used was purchased from Becton Dickinson and used as directed. The secondary antibody was purchased from Dako. Brightly fluorescent nuclei were scored as positive. At least 10 fields were counted per coverslip. The significance of the difference between treated and control means was determined by Student’s t-test. In most cases, response was measured on a single set of coverslips for each explant. When sufficient cells were available for duplicate coverslips, they were in very good agreement.

Results

An immunological method for the detection of incorpo-
Fig. 1. Indirect immunofluorescence staining, using an anti-BrdU antibody, of laryngeal epithelial cells labeled with BrdU for 24 h (x390). A. Bright field. B. Immunofluorescence of same field.

...ated 5-bromodeoxyuridine (BrdU; Gratzner, 1982) can be used as a measure of cell proliferation. Davison et al. (1979) have reported that thymidine incorporation may not be a valid indicator of keratinocyte proliferation. They found that [3H]thymidine incorporation into DNA was inconsistent with direct counts of cell number in studies on the proliferation of epidermal keratinocytes. Their data suggest that rapidly growing keratinocytes switch from a salvage pathway for DNA biosynthesis to de novo nucleotide synthesis. Because of the limitation in the use of [3H]thymidine as an indicator of keratinocyte proliferation, we used BrdU labeling as a measure of proliferation of normal laryngeal keratinocytes and those derived from laryngeal papillomas. Fig. 1 shows normal laryngeal epithelial cells that were incubated with BrdU for 24 h and then stained for BrdU using the immunofluorescence technique described in Materials and methods. The cells having brightly fluorescent nuclei were in the S-phase of the cell cycle at some point during the 24-h labeling period.

This study was conducted using cells grown from biopsies of human laryngeal epithelium. All biopsies were taken from either the true or false vocal cords, which are covered by a non-keratinizing stratified squamous epithelium in vivo. The cultures derived from these biopsies contained greater than 95% epithelial cells, as determined by morphology and staining with anti-keratin antibodies

Fig. 2. Cultured normal human laryngeal epithelial cells. A. Indirect immunofluorescent staining using anti-keratin antibodies AE1 and AE3 (x500). B. Phase-contrast micrograph of live cells growing in MCDB 153 with 0.1 mM calcium (x80). C. Indirect immunoperoxidase staining using an anti-involucrin antiserum (x190).

(Fig. 2A). The cells seen in Fig. 2B were cultured in the serum-free medium MCDB 153 containing 0.1 mM calcium. They exhibit the 'cobblestone' appearance typical of epithelial cells in culture. When the laryngeal cells are grown in 1 mM calcium, the individual cells show marked flattening, the culture stratifies, and some of the cells synthesize involucrin (Fig. 2C), which is a precursor pro-
tein of the keratinocyte’s cornified envelope. The response of laryngeal keratinocytes to high calcium concentration is similar to that exhibited by keratinocytes derived from human epidermis (Boyce and Hamb, 1983). It should be noted that cells cultured from laryngeal papilloma biopsies have previously been shown to contain human papillomavirus DNA (Steinberg et al., 1983).

To determine the extent of TGF-β on the growth of normal human laryngeal epithelial cells, bromodeoxyuridine incorporation was measured in cultures exposed to 1 ng/ml (400 pm), 5 ng/ml (200 pm), and 10 ng/ml (400 pm) TGF-β. The results for cells derived from a single tissue explant (Fig. 3) show that significant inhibition of proliferation occurs at a TGF-β concentration of 10 ng/ml. For this reason, 10 ng/ml was the concentration used in all subsequent experiments.

We investigated whether cells at different densities would respond similarly to TGF-β (Table 1). Normal laryngeal epithelial cells derived from a single tissue explant were seeded at 5 x 10^3 cm^-2 and allowed to grow for 3, 7 or 10 days before a 4-day exposure to TGF-β. At 3 days after seeding, cultures were sparse. At 7 days, they were approximately half-confluent, and at 10 days they were confluent. Cells were labeled with BrdU for 24 h during the last day of exposure to TGF-β. Labeled nuclei and total nuclei in 15 fields were counted, and labeling indices, rather than total cell counts, were measured.

Table 1: Effect of TGF-β on BrdU incorporation in normal laryngeal epithelial cells at different cell densities

<table>
<thead>
<tr>
<th>Cells cm^-2 after labeling period</th>
<th>Labeled nuclei*</th>
<th>Control</th>
<th>+TGF-β</th>
<th>Control</th>
<th>+TGF-β</th>
<th>Total nuclei*</th>
<th>Control</th>
<th>+TGF-β</th>
<th>Control</th>
<th>+TGF-β</th>
<th>Labeling index (%)</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td></td>
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<tr>
<td></td>
<td>4.3 x 10^3</td>
<td>1.6 x 10^4</td>
<td>427</td>
<td>31</td>
<td>507</td>
<td>216</td>
<td>(37 ± 14)</td>
<td>(1.8 ± 2.9)</td>
<td>1794</td>
<td>1716</td>
<td>(114 ± 28)</td>
</tr>
<tr>
<td></td>
<td>(28 ± 12)</td>
<td>(2.1 ± 1.7)</td>
<td>(5.7 ± 2.7)</td>
<td>(1.8 ± 2.9)</td>
<td>(1.8 ± 2.9)</td>
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<td></td>
<td>1.5 x 10^6</td>
<td>5.8 x 10^4</td>
<td>842</td>
<td>86</td>
<td>1997</td>
<td>753</td>
<td>(133 ± 50)</td>
<td>(50 ± 11)</td>
<td>2555</td>
<td>1671</td>
<td>(114 ± 28)</td>
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<td></td>
<td>(56 ± 13)</td>
<td>(5.7 ± 2.7)</td>
<td>(1.8 ± 2.9)</td>
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<td>C</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>2.0 x 10^6</td>
<td>1.3 x 10^4</td>
<td>262</td>
<td>27</td>
<td>2594</td>
<td>1716</td>
<td>(173 ± 20)</td>
<td>(114 ± 28)</td>
<td>2694</td>
<td>1716</td>
<td>(114 ± 28)</td>
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<td>(17 ± 9)</td>
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</table>

Cells were seeded at 5 x 10^3 cm^-2 and allowed to grow for 3 (row A), 7 (row B), or 10 (row C) days before a 4-day exposure to TGF-β. Cultures were labeled with BrdU for 24 h during the last day of exposure to TGF-β. Controls were exposed to BrdU alone. * Values given are totals for 15 fields. Numbers in parentheses are mean ± S.D. per field.

Reiss and Sartorelli (1987) have shown that in the absence of epidermal growth factor (EGF), the proliferation of foreskin keratinocytes is not inhibited by TGF-β, implying that actively growing cultures are most susceptible to the effects of TGF-β. Although we saw no marked difference in inhibition as a function of rate of proliferation (Table 1), mitogenically stimulated cells might show an altered response to TGF-β compared to unstimulated cells. Bovine pituitary extract and retinoic acid, like EGF, promote cell division of keratinocytes. The effect of TGF-β on four secondary normal laryngeal cultures in the ab-

Laryngeal keratinocytes and TGF-β
epithelial cells. Experimental cultures were exposed to 10 ng ml⁻¹ TGF-β for 4 days and to BrdU for 24 h during the last day of exposure to TGF-β. Control cultures were exposed to BrdU alone. (*) difference between experimental and control values was not statistically significant. Column A, cells were grown in the complete medium described in Materials and methods. Column B, cells were grown in the absence of EGF, bovine pituitary extract and retinoic acid.

**Table 2. Effect of TGF-β on BrdU incorporation in papilloma-derived laryngeal epithelial cells under various culture conditions**

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Labeled nuclei (% of control)</th>
<th>0.1 mM calcium</th>
<th>1 mM calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+EGF</td>
<td>-EGF</td>
<td>+EGF</td>
</tr>
<tr>
<td>Specimen 1</td>
<td>38*</td>
<td>10*</td>
<td>67†</td>
</tr>
<tr>
<td>Specimen 2</td>
<td>49*</td>
<td>40*</td>
<td>69†</td>
</tr>
</tbody>
</table>

* P<0.003, compared to control without TGF-β.
† P<0.05, compared to control without TGF-β.
‡ Did not differ significantly from control.

The presence of EGF, bovine pituitary extract and retinoic acid was determined (Fig. 4B). Under these conditions, three of the four cultures still showed significant inhibition in response to 10 ng ml⁻¹ TGF-β.

Calcium concentration is also known to affect growth and differentiation of keratinocytes (Boyce and Ham, 1983; Rubin and Rice, 1986; Hennings et al. 1980). Table 2 shows the effect of 10 ng ml⁻¹ TGF-β on two different laryngeal papilloma specimens grown in 0.1 mM or 1 mM calcium and with or without EGF. It appears that TGF-β has a more inhibitory effect in the presence of 0.1 mM calcium than in 1 mM calcium. The response of specimens 1 and 2 to TGF-β in 0.1 mM calcium is another example of the variability in the response to TGF-β seen with cells derived from different primary cultures. Unfortunately, sufficient primary cells were not available to determine if there were any differences in EGF receptor expression between the two specimens. As with normal laryngeal keratinocytes (Fig. 4B), Table 2 also shows that EGF does not need to be present for papilloma-derived cells to show growth inhibition in response to TGF-β.

Treatment of laryngeal epithelial cells with TGF-β induced changes in cellular morphology. Treated cells exhibited some increased spreading and flattening when compared with untreated controls (data not shown). However, this morphology does not indicate an effect of TGF-β on differentiation, as control cultures and cultures treated with TGF-β for 4 days showed approximately equal numbers of cells that were positive for involucrin when cultured in 1 mM calcium (data not shown).

Since normal laryngeal epithelial cells derived from different tissue explants showed a variable response to TGF-β (Fig. 4), we investigated whether the degree of response to TGF-β was related to diagnosis or age of the patient. Note that in no case was the biopsy taken at the site of the lesion. For example, if a vocal cord polyp was present on the left side of the larynx, the biopsy was taken from the opposite side. Table 3 lists eight different tissues in order of decreasing inhibition of DNA synthesis in response to TGF-β. The age and diagnosis of the patient are also given. No obvious relationship was found between either of these variables and the response to TGF-β. There was also no correlation between the level of proliferation in the control cultures and inhibition by TGF-β. This was not surprising in light of the data presented in Table 1.

**Discussion**

Using cells grown from several different tissue explants, we have found that, in most cases, TGF-β inhibits the proliferation of both normal laryngeal epithelial cells and epithelial cells derived from laryngeal papillomas. This is in agreement with previous reports that have demonstrated that TGF-β inhibits the proliferation of a variety of epithelial cell types, including human foreskin keratinocytes (Mosen et al. 1985) and human bronchial cells (Masui et al. 1986). Moreover, there was no difference between the normal cells and those derived from the virally induced neoplasms. However, cells derived from different tissue explants responded to varying degrees. All of the cultures studied contained greater than 95% epithelial cells, as determined by morphology and staining with anti-keratin antibodies. Therefore, the variable response to TGF-β seen from culture to culture is not due to differences in the number of fibroblasts present in each culture. It also cannot be due to variation in the ability of different cultures to activate TGF-β (Lyons et al. 1988), because TGF-β as supplied by Collaborative Research is the active

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*Fig. 4. Effect of TGF-β on DNA synthesis in different explant cultures of normal (○) and papilloma-derived (●) laryngeal epithelial cells. Experimental cultures were exposed to 10 ng ml⁻¹ TGF-β for 4 days and to BrdU for 24 h during the last day of exposure to TGF-β. Control cultures were exposed to BrdU alone. (*) difference between experimental and control values was not statistically significant. Column A, cells were grown in the complete medium described in Materials and methods. Column B, cells were grown in the absence of EGF, bovine pituitary extract and retinoic acid.*
form of the molecule. We do not believe that it reflects experimental error, because on those occasions when sufficient cells were available for duplicate coverslips, there was good agreement (data not shown).

Three structurally distinct cell-surface glycoproteins that specifically bind TGF-β have been identified in a variety of cell types, and these three types of TGF-β receptors bind ligand with different affinities (Massague, 1986; Boyce and Shikowitz, 1985; Chichefetz et al., 1985). One likely explanation for the variable response of laryngeal epithelial cells to TGF-β is that cells derived from different explant cultures may possess variable numbers of TGF-β receptors, or their receptors may bind TGF-β with different affinities. This hypothesis was not tested here, because the limited number of primary cells available from a laryngeal explant culture precludes performing both proliferation and receptor assays on the same sample. Methods are available for the expansion of human keratinocyte cultures to larger cell numbers (Green et al., 1979). However, all experiments described here were done on primary cells that had been cultured for as short a time as possible. This was done because extended time and passage in culture could result in the selection of a subpopulation of cells whose response to TGF-β would not be representative of the original explant culture. In most cases, only a single dose of TGF-β (10 ng ml⁻¹) was used in this study. Therefore, it is possible that differences in receptor number and ligand affinity could account for the variable response. Higher doses might have had an effect on the cultures that did not respond to 10 ng ml⁻¹ TGF-β. However, the limited number of cells available did not allow multiple concentrations to be tested in most cases.

The precise mechanism by which TGF-β inhibits epithelial cell growth is largely unknown, and the intracellular signaling mechanism activated by TGF-β-receptor interaction has not yet been elucidated. However, another possible explanation for the variable response of laryngeal keratinocytes to TGF-β is that there are variations from specimen to specimen that occur distal to TGF-β ligand-receptor binding.

Calcium concentration is known to have an effect on the proliferation of human keratinocytes in culture. Boyce and Ham (1983) have shown that the growth rate of human epidermal keratinocyte cultures increases between 0.1 and 1 mM calcium, and a calcium concentration of 1 mM growth rate is not changed. Similarly, we have found that the proliferation of human laryngeal keratinocytes is increased when the calcium concentration is raised from 0.1 mM to 1 mM (data not shown). Table 2 indicates that TGF-β has a more inhibitory effect on laryngeal keratinocytes in the presence of 0.1 mM calcium than in 1 mM calcium. It may be that those cells that continue to divide even in the sub-optimal calcium concentration of 0.1 mM are somewhat more sensitive to the effect of TGF-β than is the general population. It is also possible that calcium plays some role in the regulation of receptor expression or the intracellular signaling mechanism triggered by TGF-β-receptor interaction.

Although further work must be done to determine the cause or causes for the variability in response of laryngeal keratinocytes to TGF-β, the data reported here do indicate that, when working with tissue explants, conclusions cannot necessarily be drawn on the basis of the study of a single explant culture. It is important to repeat experiments with samples from different sources before conclusions are drawn.

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References


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