Telomerase Activity and p53 Expression in Pterygia

Shigeto Shimmura, Misaki Ishioka, Kazuomi Hanada, Jun Shimazaki, and Kazuo Tsubota

PURPOSE. To investigate telomerase activity and p53 expression in pterygial tissue.

METHODS. Pterygia tissue was obtained during excisional surgery from 35 eyes of 35 patients, and superior bulbar conjunctival tissue from the same eye was also sampled as control when possible. Fluorescence telomeric repeat amplification protocol was used to measure telomerase activity in whole pterygium samples from 9 cases and in the epithelium and stroma of pterygium from another 10 cases. p53 protein content was measured by enzyme-linked immunosorbent assay (ELISA) in tissues obtained from 7 eyes, as well as in epithelial cell suspensions collected by brush cytology in 8 eyes. Six samples were also analyzed for UV-specific mutations in the p53 gene by the single-strand conformation polymorphism technique and DNA sequencing. A conjunctival epithelial cell line was irradiated with sublethal levels of UV-B to investigate whether telomerase activity can be induced in vitro.

RESULTS. In all, 63% of pterygium samples demonstrated telomerase activity, whereas all 10 paired conjunctival control samples were negative (P = 0.05, chi-square test). Of the 10 samples in which telomerase activity was measured separately in the epithelium and stroma of pterygium, 5 samples were positive in the epithelium, only 1 of which had activity in the stroma. Average telomerase activity in positive samples was 18.44 ± 8.77 U/μg protein, compared with telomerase activity measured in a carcinoma in situ patient (33.73 U/μg), and in an immortalized conjunctival epithelial cell line (50.72 ± 15.55 U/μg). Telomerase activity was not upregulated in this cell line by UV-B exposure. All 6 pterygia samples tested for p53 mutations did not reveal the UV-specific mutations in exons 5, 6, 7, or 8. No statistical significance was observed in the pterygium or conjunctiva p53 protein levels in epithelial cells collected by brush cytology, while p53 protein level was lower in pterygia when measured in whole tissue samples.

CONCLUSIONS. Telomerase activity was detected in some pterygia, mostly in the epithelium. Pterygia was not associated with an increase in epithelial p53 protein content measured by ELISA. (Invest Ophthalmol Vis Sci. 2000;41:1364–1369)

Pterygia are benign invasive lesions of vascularized abnormal conjunctival tissue on to the peripheral nasal cornea. Although the pathogenesis of pterygia is still not clear, an association with UV radiation has been reported.1 UV radiation is also responsible for several dermatologic conditions ranging from benign solar elastosis, or photoaging,2 to more malignant lesions such as squamous cell carcinoma. Both UVB (290–320 nm) and UVA (320–400 nm) can contribute to UV-induced carcinogenesis,3 and recent studies have shown that UV-exposed skin suffers specific mutations in the tumor suppressor gene, p53, at codons 245 and 247/248.4 UV-induced p53 mutations were also found in actinic keratosis, a precancerous state in the progression of squamous cell carcinoma,5 and squamous cell carcinoma was experimentally induced in p53-deficient mice with UVB.6 Cumulative reports show a strong relation between UV-induced p53 mutations and disease caused by UV exposure. Abnormal expression of p53 has also been demonstrated in pterygia by immunostaining with monoclonal antibodies directed against mutant p53 protein,7 suggesting that p53 plays a possible role in the pathogenesis of pterygia.

On the other hand, telomerase activity was found to be higher in sun-exposed skin, suggesting that telomerase activity can also be modulated by UV radiation.8 Human telomerase RNA expression was also expressed in epidermal basal cells in newborns and in samples from psoriasis, contact dermatitis, basal cell carcinoma, squamous cell carcinoma, and melanomas.9 Telomerase is a ribonucleoprotein that catalyzes the de novo synthesis of telomeres, and addition of telomeric repeats to existing telomeres.10,11 Telomeres function in the segregation of chromosomes during cell division, and increased telomere length is associated with prolonged cell survival.12 The high incidence of telomerase activity observed with neoplastic changes has associated the enzyme as a possible tumor marker. The present study investigated the possible involvement of p53 mutation and telomerase activity in the abnormal proliferative properties of pterygia.

METHODS

Telomeric Repeat Amplification Protocol

In all, 35 eyes from 35 patients with primary pterygia were treated by surgical excision with transplantation of free con-
Conjunctival Cell Line Culture and UV Irradiation

An immortalized human conjunctival epithelial cell line (ChWK, clone CCL 20.2) was obtained from the American Type Culture Collection (ATCC), and cultured in TC-199 medium containing 10% fetal bovine serum as previously described. After 2 passages, cells were seeded in 35-mm plastic culture dishes (Iwaki Glass, Tokyo, Japan) until confluence. To determine whether telomerase activity can be upregulated by UV in vitro, cells were exposed to either 0.5 or 1.0 mJ/cm². UVB using a UV lamp (model UL 200; HOYA Schott, Tokyo, Japan) at 65°C/60 minutes, and overnight at 56°C. Sample DNA was extracted by the phenol method and subjected to PCR with Cy-5-labeled primers for exons 5, 6, 7, and 8 of the p53 gene (Pharmacia Biotech). PCR products were heat-denatured at 80°C, 5 minutes, and placed in a 5% acrylamide/5% glycerol gel for electrophoresis in a DNA sequencer (ALFred DNA Sequencer, Pharmacia). Any point mutations in exons 5 to 8 would be reflected in an abnormal fluorescence peak compared with normal control. Tissue samples obtained from an 84-year-old female patient with carcinoma in situ (CIS) of the keratolimbal area were also included as a positive control.

p53 Protein Content

Because p53 protein content is often used as an indicator of p53 mutation, which often causes stabilization of the protein, we measured p53 protein content in pterygia and conjunctival tissue samples from 7 patients and in brush cytology epithelial cell suspensions from 8 patients. p53 levels were measured by a sandwich ELISA kit (p53 pan ELISA, Boehringer Mannheim, Mannheim, Germany) according to the protocol provided. In brief, for whole tissue samples, each sample was washed with PBS immediately after surgical excision. As much of the adherent blood was removed as possible, and then tissue samples were frozen at −80°C until assays. After thawing, samples were placed on ice and mixed with 1 volume of RIPA buffer (20 mM Tris, 0.5 mM EDTA, 1.0% Nonidet P-40, 0.05% sodium deoxycholate, 0.05% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 2 μg/ml leupeptin) and lysed using a microhomogenizer. Samples were subjected to ELISA using a peroxidase-labeled anti-p53 monoclonal antibody, and p53 concentrations were determined by colorimetry readings taken after reaction with tetramethylbenzidine substrate. The primary antibody used recognizes both wild-type and mutant p53 proteins.

The same procedure was used to measure p53 protein in epithelial cell suspensions collected by brush cytology. Brush cytology enables the procurement of approximately 10⁶ epithelial cells from pterygia and normal conjunctiva. Because brush cytology leaves the basement membrane intact, other cellular components from the stroma were excluded.

RESULTS

Table 1 shows the results of telomerase activity in pterygia, conjunctival tissue, and CIS determined by the TRAP assay, as well as the results of SSCP for p53 exons 5 through 8. Of the 9 samples tested for telomerase activity, 7 pterygia samples were positive, whereas all 5 conjunctival control samples were negative. Samples of telomerase positive and negative peak patterns are shown in Figure 1. Pterygium and conjunctival tissue samples of cases 5 through 10 were analyzed for p53 mutations in exons 5 to 8. Of the 9 samples tested for p53 mutations, 5 pterygia samples were positive, whereas all 5 conjunctival control samples were negative. Samples of telomerase positive and negative peak patterns are shown in Figure 1. Pterygium and conjunctival samples of cases 5 through 10 were analyzed for p53 mutations by SSCP, which is also a marker of UV-induced neoplastic activity. None of the cases revealed mutations in exons 5 through 8 of the p53 gene.
analysis on case 10. Interestingly, 3 of the 6 samples tested (5, 7, and 9) still had positive telomerase activity in the same pterygium tissue examined.

Because the first 10 samples showed telomerase activity in pterygium, but not in conjunctiva, an additional 10 cases were examined to see if telomerase activity is differentially expressed in either the epithelium or stroma of pterygia. As shown in Table 2, 5 of 10 samples demonstrated telomerase activity, which was restricted to the epithelium except for 1 case that had activity within the stroma (case 13). In all 20 cases, 63% of pterygia samples demonstrated telomerase activity, whereas the 10 paired conjunctival control samples were negative (P = 0.05, chi-square test). Average telomerase activity in positive samples was 18.44 ± 8.77 U/µg protein, compared with telomerase activity measured in the CISP patient (33.73 U/µg).

Because SSCP failed to reveal any mutations in exons 5 through 8 of the p53 gene, we quantitated the amount of p53 protein present in pterygia and conjunctiva as another means to seek the possibility of altered p53 expression as a possible factor in pterygia pathogenesis. When whole tissue samples were analyzed, p53 protein content was significantly higher in normal conjunctiva (Fig. 3) compared with pterygia. However, p53 content in epithelial samples obtained by brush cytology was essentially the same in both conjunctival and pterygial epithelium (Fig. 4).

Telomerase activity was also detected in the ChWK conjunctival epithelial cell line. However, a single dose of sublethal levels of UVB did not affect telomerase activity after 24 hours (Fig. 5).

**DISCUSSION**

Although UV radiation has been shown to be related to the pathogenesis of pterygium, the correlation is that of an epi-

---

**TABLE 1. Telomerase Activity and p53 Mutation in Pterygium, Conjunctiva, and CISP**

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Telomerase Activity</th>
<th>p53 Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pterygium</td>
<td>Conjunctiva</td>
</tr>
<tr>
<td>1</td>
<td>24.51</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>26.27</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>23.37</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>21.46</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>36.18</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>7</td>
<td>7.88</td>
<td>NA</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>9</td>
<td>20.71</td>
<td>NA</td>
</tr>
<tr>
<td>10</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CISP</td>
<td>33.73</td>
<td>ND</td>
</tr>
</tbody>
</table>

* U/µg protein.
NA, not available; ND, not detected.

---

**TABLE 2. Telomerase Activity in Pterygium Epithelium, Pterygium Stroma, and Conjunctiva**

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Pterygium Epithelium</th>
<th>Pterygium Stroma</th>
<th>Conjunctiva</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>8.78</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>16.99</td>
<td>18.44</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>13.20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>6.06</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>17</td>
<td>15.85</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>18</td>
<td>0</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>19</td>
<td>0</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>0</td>
<td>NA</td>
</tr>
</tbody>
</table>

* U/µg protein.
NA, not available.
demiologic study, and the precise mechanisms involved have not yet been elucidated. On the other hand, the involvement of UV radiation in precancerous and cancerous skin lesions is well established, and many reports have provided evidence that p53 mutations are involved. Because p53 is involved in the regulation of cell division and cell apoptosis, mutations in the gene results in a nonfunctional p53 protein that allows the cell to become immortalized, a key process in cancer formation. However, pterygia are not associated with malignant transformation, or recognized as a precancerous state such as actinic keratosis in the skin. The benign nature of pterygia may explain the fact that p53 point mutations were not found in this study in exons 5 through 8. We also did not detect p53 mutation in exons 5 through 8 in the tissue obtained from a CIS patient, which agrees with previous reports that show no mutations in the p53 gene in Bowen's disease of the skin.

There still may be the possibility that the amount of tissue available for SSCP analysis may not be sufficient for ocular surface pathologies. A more sensitive test may elucidate this point. However, results of ELISA measuring p53 protein levels did not show an increase in pterygium compared with paired conjunctiva. Because increased p53 expression is often used as an indicator of p53 mutation, the ELISA results seem to substantiate the negative SSCP results.

Although previous reports have shown increased p53 protein in pterygial tissue, the results of the present study show that p53 protein levels were the same in epithelial cells obtained from pterygia and conjunctiva of the same eye. When whole tissue samples were assayed, pterygia had lower levels of p53, probably due to greater stromal mass. Although telomerase activity was limited to the epithelium in all but one sample, p53 contents in epithelial cells collected by brush cytology also showed similar levels in both pterygium and conjunctiva. Although these results contradict some previous reports, the discrepancy may be explained by the fact that prior studies relied on histochemical data to qualitatively localize p53 protein. The possibility that mutations may have existed in exons that are not UV-specific cannot be ruled out by the present study. There is also the possibility that mutations may have only occurred in a very limited set of cells, such as limbal-derived cells at the leading edge of pterygia, in which case SSCP and ELISA may not be sensitive enough to detect.
Perhaps the most interesting result of the present study is the demonstration of telomerase activity in pterygium. Telomerase is a key enzyme that is involved in the regulation of cell division and cell senescence. Telomerase is responsible for the elongation of telomeres located at chromosome ends, functioning in the protection of DNA and leading to prolonged cell survival. Telomerase activity has been considered as a tumor marker, because it is often expressed in transformed cells, and because it is usually not detected in normal adult tissue. However, a recent study has shown that telomerase-negative cells transfected with human telomerase catalytic subunit exhibit a much longer life span, without phenotypic or neoplastic changes. Therefore, it is now believed that telomerase activity is not necessarily a marker of tumorigenesis but rather of cell proliferation. However, ours is still a rare case reporting telomerase activity in noncancerous tissue.

Recent reports have shown that UV radiation can cause upregulation of telomerase mRNA and protein both in vitro and in vivo. The same study also revealed that only half of the sun-exposed skin samples that were positive for telomerase activity showed point mutations in the p53 gene specific for UV radiation. Therefore, UV radiation alone, without simultaneous mutations in the p53 gene, can cause telomerase upregulation. The results of the present study have also demonstrated increased telomerase activity in approximately two thirds of pterygium tissue tested, but in none of the conjunctival tissue taken from the superior bulbar conjunctiva of the same patient. Furthermore, cases in which telomerase activity was measured separately in the epithelium and stroma of the pterygium, only the epithelium exhibited measurable activity in all but one exception. This may support the theory proposed by Dushku and Reid, suggesting that pterygia originate from altered limbal epithelial basal cells. However, lack of telomerase activity within stromal tissue may also be due to the scarcity of cell mass and may not necessarily indicate that pterygium is a primarily epithelial disease. The single case with telomerase activity in the stroma was a relatively large pterygium, with over 5 D of induced astigmatism. However, the possibility that epithelial tissue may have contaminated the stromal sample cannot be ruled out. Other clinical parameters were compared between telomerase-positive and -negative cases such as age, astigmatism, surface regularity index, and surface asymmetry index measured by video keratospo (model TMS-1; Computed Anatomy, New York, NY). However, there were no significant differences in these values. Although it is not clear why approximately one third of pterygia samples were negative for telomerase activity, the difference may be due to the stage of disease progression or the inclusion of specific tissue such as the leading limbal basal cells. Although the present study only dealt with primary surgical procedures, it would be interesting to compare telomerase levels in recurring pterygia as a possible marker of proliferative potential.

Recent reports have suggested that telomerase activity can be upregulated in an epithelial cell line by UVB in vitro, however, we were unable to reproduce these results with a conjunctival epithelial cell line using a similar protocol. Multiple exposures to UV may be required for upregulating telomerase activity or other factors such as coexisting fibroblasts or inflammatory cells may also play an important role. It is interesting to note, however, that pterygial tissue samples expressed levels of telomerase activity comparable to a conjunctival epithelial cell line with an extended life span. Telomerase activity was also comparable to that measured in a tissue sample obtained from a patient with CIS, which was included as a positive control.

Although conclusions that can be drawn from the present study are still limited, we have shown that an increase in telomerase activity may be involved in the pathogenesis of some pterygia. Telomerase activity may be a direct or indirect result of UV exposure, probably without the involvement of widespread mutations in the p53 protein, at least not in the more common exons found in skin lesions. Unlike the neoplastic changes observed in precancerous skin lesions, pterygium is more likely a hyperproliferative lesion due to chronic inflammation after repeated exposures to UV radiation.

Acknowledgment
The authors thank Maki Hojo for her excellent technical assistance.

References


