p53 Expression and Relation to Human Papillomavirus Infection in Pingueculae, Pterygia, and Limbal Tumors

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Response: The tumor suppressor gene p53 is expressed without apoptosis in the limbal basal stem cells of all pterygia and limbal tumors and most pingueculae from which these growths seem to originate. Oncogenic human papillomaviruses (HPVs) have been found in pterygia and limbal tumors, and HPV and p53 overexpression commonly coexist in oropharyngeal and penile carcinomas.

Objective: To search for HPV DNA as a cofactor in the development of pingueculae, pterygia, and limbal tumors.

Methods: We examined specimens—1 of pinguecula, 13 of pterygia (7 primary, 1 recurrent, 1 with dysplasia, and 4 primary not tested for p53), and 10 of limbal tumors (2 with actinic keratosis dysplasia, 3 with conjunctival intraepithelial neoplasia, 3 with carcinoma in situ, and 4 with squamous cell carcinoma)—expressing p53. Specimens were tested for the presence of HPV DNA by the polymerase chain reaction using degenerate consensus primers for the highly conserved portion of the L1 region that encodes a capsid protein of the virus. This assay has a wide spectrum with capability of detecting essentially all known HPV types. Nested polymerase chain reaction was performed on all specimens. Primers of the cystic fibrosis gene were used to confirm the presence of genomic DNA and to rule out inhibitors. Purified HPV DNA type 11 was the positive control, and HPV-negative genomic DNA was the negative control.

Results: Using consensus primers for the highly conserved portion of the L1 region, all specimens of pingueculae, pterygia, and limbal tumors studied were negative for HPV DNA by nested polymerase chain reaction.

Conclusions: Human papillomavirus DNA is not required as a cofactor in the development of pterygia and limbal tumors. These data support the theory that increased p53 expression in the limbal epithelia of pingueculae, pterygia, and limbal tumors indicates the probable existence of p53 mutations in these cells as an early event in their development, which is consistent with UV irradiation causation. Thus, due to a damaged p53-dependent programmed cell death mechanism, mutations in other genes may be progressively acquired. This would allow for the multistep development of pterygia and limbal tumor cells from p53-mutated limbal epithelial basal stem cells overlying pingueculae.

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Background: Based on the intermediate-filament immunohistochemical analysis of surgical primary, and recurrent pterygia specimens, we discovered that the pathogenesis of pterygia was a result of an invasion of the cornea by vimentin-expressing altered limbal epithelial basal cells, the pterygium cells, followed by conjunctival epithelium.

Because epidemiologically, UV-B radiation correlates as the etiologic agent for pterygia and limbal tumors, and most pingueculae, indicating the probable existence of p53 mutations in these cells as an early event in their development, which is consistent with their causation by UV irradiation. Because of this damaged p53-dependent programmed cell death mechanism, mutations in other genes are progressively acquired that allow the multistep development of pterygia and limbal tumor cells from p53-expressing cells overlying a pinguecula.

Another known mechanism for the reduction of the functional effectiveness of the wild-type p53 protein, other than point mutations, is by infection with cer-
MATERIALS AND METHODS

TISSUE SPECIMENS

Specimens of pinguecula (n = 1), pterygia (n = 13; 7 with primary, 1 with recurrent, 1 with dysplasia, and 4 primary not tested for p53), and limbal tumors (n = 10; 2 with actinic keratosis dysplasia, 1 with conjunctival intraepithelial neoplasia, 3 with carcinoma in situ, and 4 with squamous cell carcinoma) were examined. For immunohistochemical analysis, fresh normal human surgically removed material of superior (4 specimens) and lateral (1 specimen) limbal epithelia and 1 cadaver specimen of normal human medial corneal, limbal, and conjunctival tissue were used for negative controls. Positive tissue controls included specimens of colon carcinoma and breast carcinoma (BioGenex Laboratories, San Ramon, Calif).

IMMUNOHISTOCHEMICAL ANALYSIS

The primary antibody p53, Ab-6, clone DO-133 (Onco-gene Science Inc, Uniondale, NY), was used to demonstrate p53 protein expression in limbal basal cells in pin-gueculae, pterygia, and limbal tumors. An avidin-biotin complex–immunoperoxidase technique (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, Calif) was selected for staining with the primary antibody.34,35 Hematoxylin was used for counterstaining.

APOTOPSIS TUNEL ASSAY

We performed the apoptosis TUNEL (terminal deoxynucleotidyltransferase–mediated deoxyuridine 5'-triphosphate and biotin nick end labeling) assay on specimens of pingueculae, pterygia, and limbal tumors that had also been stained for p53 expression. This assay was performed according to the procedure of Lovelace et al14 using the TACS 2 TdT-DAB kit from Trevigen, Gaithersburg, Md.

PCR ASSAY

Quality Control

Control specimens were run with each patient specimen. The positive control was purified HPV type 11 isolated from HPV viral culture, and the negative control was DNA that was known to be negative for HPV. Each of these was amplified in the same manner as the patient specimen.26 It was presumed that viral and genomic DNA were isolated simultaneously. As a control for DNA isolation and amplification, each patient specimen that did not amplify for p53 expression. This assay was performed according to the procedure of Loveal et al14 using the TACS 2 TdT-DAB kit from Trevigen, Gaithersburg, Md.

PCR ASSAY

Quality Control

Control specimens were run with each patient specimen. The positive control was purified HPV type 11 isolated from HPV viral culture, and the negative control was DNA that was known to be negative for HPV. Each of these was amplified in the same manner as the patient specimen.26 It was presumed that viral and genomic DNA were isolated simultaneously. As a control for DNA isolation and amplification, each patient specimen that did not amplify for HPV was amplified with a set of primers that amplify a fragment of a human gene.

DNA Analysis of HPV

The DNA was extracted from frozen tissue using DNA isolation reagents (PureGene; Gentra Systems Inc, Research Triangle Park, NC). Total DNA (500 ng) was amplified with primers that amplify a 425-base pair (bp) region of the cystic fibrosis conductance regulator gene. These primers verified the successful isolation of genomic DNA and ruled out the presence of inhibitors. Specimens that did not demonstrate a 425-bp fragment were amplified using nested primers that yield a 295-bp fragment.

A sample of DNA (500 ng) from each specimen was enzymatically amplified to generate “consensus” oligonucleotide primers that amplify a highly conserved portion of the L1 region of the HPV. This assay is capable of detecting essentially all known HPV types. Fragments were amplified in 100 µL containing My09 primer (5'-CGTCCMARRGGAWACTGATC-3' [R = A or G, W = A or T, Y = C or T, and M = A or C]), 0.5 µmol/L; My11 primer (5'-GCMCAGGGWCATAAYATGGG-3'), 0.5 µmol/L; deoxyadenosine 5'-triphosphate, 0.2 mmol/L of each; Taq DNA polymerase, 2 units; magnesium chloride, 1.5 mmol/L; and 1X Taq buffer. To prevent evaporation, the specimens were overlaid with mineral oil, 100 µL. They were then placed in a programmable heating block with an initial denaturation step of 94°C for 2 minutes, followed by 35 cycles at 94°C for 30 seconds, 95°C for 30 seconds, and 72°C for 35 seconds. The specimens were electrophoresed on 1% agarose, 25 mL, and 2% NuSieve genetic technology grade agarose (FMC Corporation Bioproducts, Rockland, Me) in 0.5X buffer (TRIS base, boric acid, and EDTA at pH 8.3 [TBE]) in a mini-sub cell apparatus (Bio Rad Laboratories, Hercules, Calif). Gels were electrophoresed at 10 V/cm for 1 hour. Immediately after electrophoresis, gels were stained in 0.5X TBE containing ethidium bromide, 0.5 µg/mL, for 10 minutes, destained in 0.5X TBE for 10 minutes, and photographed. This fragment is approximately 450 bp. The size of the fragment varies depending on the HPV type present. The presence of the 450-bp fragment indicates that the specimen contains HPV. If the 450-bp fragment was not present, 5 µL of the PCR product from the My09/My11 reaction was amplified in a nested PCR with a set of primers located internal to the My09/My11 primers. Fragments were amplified in 100 µL containing HPV 1 primer (5'-TTGTGTACTGTGATGATAC-3'), 0.5 µM; HPV 2 primer (5'-AAAAATACAAGGATACATC-3'), 0.5 µmol/L; deoxyadenosine 5'-triphosphate, 0.2 mmol/L of each; Taq DNA polymerase, 2 units; magnesium chloride, 1.5 mmol/L; and 1X Taq buffer. To prevent evaporation, the specimens were overlaid with mineral oil, 100 µL. They were then placed in a programmable heating block for 35 cycles at 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 55 seconds. The specimens were electrophoresed on 1% agarose and 2% NuSieve genetic technology grade agarose, 25 mL, in 0.5X TBE buffer. Gels were electrophoresed at 10 V/cm for 1 hour. Immediately after electrophoresis, gels were stained in 0.5X TBE containing ethidium bromide, 0.5 µg/mL, for 10 minutes, destained in 0.5X TBE for 10 minutes, and photographed. This fragment is approximately 139 bp. The size of the fragment varies depending on the HPV type present.

PHOTOGRAPHY

Ethidium bromide–stained gels were photographed under UV light. Sections that immunostained for p53 were photographed with a photoscope (Zeiss Ultra Phot; Carl Zeiss Inc, Thornwood, NY).
tain oncogenic human papillomaviruses (HPVs). Human papillomavirus is the only DNA tumor virus that has been shown18 to be involved in human cancer. The binding to the wild-type p53 proteins of the E6 oncoproteins encoded by HPV types 16 and 18 results in the rapid degeneration of p53 through the ubiquitin-mediated pathway. Low levels of nuclear p53 protein lead to a damaged p53-dependent programmed cell death mechanism, which is similar to that caused by p53 mutations. This HPV mechanism would also permit mutations in other genes to accumulate and allow the multistep development of pterygia and limbal tumors from HPV-infected limbal stem cells.

Human papillomavirus and p53 overexpression commonly coexist in oropharyngeal carcinomas, penile carcinomas, grade III cervical intraepithelial neoplasia, and invasive squamous cell carcinomas of the cervix, but show a negative correlation rate with esophageal squamous cell carcinomas and certain cervical cancers. Because other investigators have found certain strains of HPV in pterygia and limbal tumors, we used the polymerase chain reaction (PCR) to search for HPV DNA as a cofactor in the development of these abnormal growths.

RESULTS

p53 EXPRESSION

In normal control studies, p53 immunostaining was negative in 5 fresh surgical specimens of superior (n = 4) and lateral (n = 1) limbal epithelia and in the medial interpalpebral limbal-corneal epithelia of 1 cadaver eye. All tested specimens of pingueculae (1/1), pterygia (9/9), and limbal tumors (10/10) expressed nuclear p53 in limbal epithelial cells but not in the fibroblasts (Figure 1).

TUNEL ASSAY

For the specimens of pingueculae and pterygia, we found 0% to less than 1% apoptosis, and for the specimens of limbal tumors, we found 0% to less than 5% apoptosis.

PCR ASSAY

Amplification of the 425-bp or nested 295-bp fragment by the cystic fibrosis gene primers in all patient specimens confirmed that genomic DNA had been isolated from all specimens and that all patient specimens were free of PCR inhibitors (Figure 2 and Figure 3). We were unable to detect the 450-bp or the nested 139-bp L1 region HPV DNA fragments in all specimens of pingueculae (1/1), pterygia (13/13), and limbal tumors (10/10) tested (Figure 4 and Figure 5).

COMMENT

UV LIGHT, NOT HPV

Epidemiologically3,6 and histopathologically, HPV DNA fragments in all specimens of pingueculae, pterygia, and limbal tumors. Because oncogenic HPVs have been found by other researchers in pterygia and limbal tumors, we used PCR to search for HPV DNA in p53-expressing pingueculae, pterygia, and limbal tumors.

We found no HPV DNA in any of these growths and conclude that HPV DNA is not required as a cofactor for the etiology of these lesions, either through the control of the action of p53 or through any other mechanism.

BASEMENT MEMBRANES AND STEM CELLS IN LIMBAL EPITHELIUM

Important in the pathogenesis of pterygia and limbal tumors is the histological evidence that the limbal, corneal, and conjunctival epithelia have adjacent and joined basement membranes that allow possible migration of any of their basal cells onto each other's basement membrane.

Although macroscopically the limbal epithelium looks like conjunctival epithelium and previously was considered to be composed of conjunctival epithelial cells, current evidence indicates that the limbal epithelium instead contains corneal stem cells.

Because tumors are thought to arise from stem cells, knowledge of the existence of stem cells in the limbal epithelium, but not in the adjacent differentiated corneal and conjunctival epithelia, is also important in the understanding of the pathogenesis of pterygia and limbal tumors from stem cells. Normally, the parental stem cells in the limbal epithelium next to the conjunctival cells maintain a constant posterior border and do not migrate across that border onto conjunctival basement membrane. Instead, they produce daughter cells that migrate circumferentially onto limbal epithelial basement membranes and centripetally onto corneal basement membrane, where they differentiate into corneal basal cells. The adjacent differentiated conjunctival basal cells originally migrate from conjunctival stem cells located in the palpebral fornices and differentiate into mature conjunctival basal cells as they move onto bulbar conjunctival basement membranes.

LIMBAL STEM CELLS IN PINGUECULAE, PTERYGIA, AND LIMBAL TUMORS

The limbal epithelium over a pinguecula contains stem cells and can be the site of the development of pterygia and limbal tumors. Pterygia originate from a migration of an entire segment of altered limbal epithelial basal stem cells onto corneal basement membrane, followed by conjunctival epithelial cells. Before migration of the limbal epithelial segment, altered limbal basal stem cells (the pterygium cells) infiltrate centrifugally onto adjacent corneal, limbal, and conjunctival epithelial basement membranes. Consistent with these findings is our proposal that pterygia recur because of the incomplete removal of altered limbal basal stem cells that have invaded basal corneal, conjunctival, and circumferential limbal epithelia, and that recurrent pterygia are not a separate process of an invasion of vascular scar tissue in response to surgical injury.

The unusual developmental pattern of pterygia is consistent with the peculiar growth patterns of limbal tu-
mors, such as corneal intraepithelial neoplasia or epithelial dysmaturation of Jakobiec. Stem cell migrations in the postnatal period are not a new biological phenomenon and may occur in such diseases as Barrett esophagus and ectropion of the uterine cervix. Other limbal tumors that originate from limbal basal stem cells do not migrate as a segment of the limbal epithelium onto corneal basement membrane but have stationary limbal epithelia whose altered cells grow centripetally and usually infiltrate locally.

PATHOGENESIS OF PTERYGIA AND LIMBAL TUMORS FROM LIMBAL EPITHELIAL CELLS

Based on our findings of p53 expression without apoptosis in the limbal epithelia of pingueculae, pterygia, and
In pterygia, p53 mutations in the limbal stem cells result in a damaged p53-dependent programmed cell death mechanism, and the overproduction by the pterygium cells of transforming growth factor β (TGF-β) by the p53-Rb-TGF-β pathway. Many of the tissue changes seen in pterygia can be explained by this mechanism. First, in the migrating limbus of pterygium cells, TGF-β production occurs, resulting in a decreased number of cell layers (average, 3-4 layers). Second, among the TGF-β-expressing pterygium cells of the migrating limbus epithelium and in the TGF-β-soaked subepithelial stroma, increased capillaries and vessels occur, indicating angiogenesis. Third, also within the epithelial cell layers (personal observations) and stroma under the pterygium cells, monocytes can accumulate. Fourth, a group of normal-appearing fibroblasts (Figure 1, A) gathers beneath the TGF-β-expressing pterygium cells, producing normal-appearing collagen. Fifth, some of these fibroblasts (Figure 1, A) next to the basal membrane of the pterygium cells to dissolve Bowman’s layer, probably through the up-regulation of collagenase. Sixth, another group of these collagenase-producing fibroblasts (Figure 1, B) migrates anteriorly to the leading edges of the pterygia between Bowman’s layer and the basement membrane of pterygium cells to form little islands of fibroblasts that locally dissolve Bowman’s layer. Seventh, pterygium cells are mobile locally as they move on top of their basement membranes. The parental basal stem cells leave their permanent locations next to conjunctival basal cells and migrate onto corneal basement membrane, followed by conjunctival cells. Excess TGF-β production by the parental stem cells makes these cells more mobile by a mechanism whereby it induces increased production of collagenase that more readily dissolves hemidesmosomal attachments.
cur beneath both limbal and conjunctival epithelia, leading to atrophic flat scars or raised vascular areas of abnormal elastic material production similar to that in the stroma of sun-damaged skin.13,38 However, in the skin, these stromal changes cannot be seen underneath the opaque epidermis, whereas in the interpalpebral area of the eye, the epithelium is transparent and the stromal changes, called pingueculae, are visible.

The limbal epithelium over the limbal portion of a pinguecula has stem cells and can also have UV-induced mutations in the p53 genes (Figure 1, C), leading to vascularized raised pingueculae, limbal tumors, or pterygia. Thus, both the limbal epithelium of pingueculae and the altered fibroblasts in the stroma of pingueculae become storage centers for damaged genes that can lead to growths in the limbal area.

**LIMBAL TUMORS INITIATED BY HPV INFECTION**

Limbal stem cells infected with oncogenic HPV might produce some limbal tumors. The present results indicate, however, that HPV plays a minor role, if any, in the development of these tumors.

The mechanism of HPV DNA limbal tumorigenesis could be related to the rapid degradation of p53 by the ubiquitin pathway.16,17 Low levels of nuclear p53 protein lead to a damaged p53-dependent programmed cell death mechanism, similar to what occurs when p53 is mutated. This would lead to the accumulation of damaged DNA and the multistep development of limbal tumors.

Such a situation probably occurs in viral papillomas at the limbus and limbal dysplasias that arise outside the UV-exposed interpalpebral region. Human papillomavirus infections in these growths could explain the topical antiviral treatment with interferon alfa-2b can lead to the regression of these tumors16-69 by a restoration of the p53-dependent programmed cell death mechanism, which can cause these HPV-infected tumor cells to commit suicide.

Even though we did not find HPV DNA in any of our specimens, HPV DNA may still be a cofactor in certain pterygia and limbal tumor development. Because of HPV DNA's effect on p53 degradation, HPV infection can be a first or early event in tumor development, especially in limbal tumors such as those growths found in immunocompromised patients with pathogenic HPV 16 and 18 ocular infections or in growths found in parts of the world where ocular HPV is endemic.

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**REFERENCES**


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