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Expression of Transcription Factors in Keratoconus, a Cornea-Thinning Disease[1]

R. Brent Whitelock, Yuhong Li, LiLi Zhou, Joel Sugar, and Beatrice Y. J. T. Yue[2]
Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago College of Medicine, Chicago, Illinois

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Transcription factors are known to regulate gene transcription through the recognition and binding of specific DNA sequences in the promoter or enhancer regions of many genes. Keratoconus is a cornea-thinning disease in which upregulated expression of degradative enzymes and downregulated expression of protease inhibitors have been demonstrated. In view of the alteration in gene expression for multiple proteins, five common transcription factors, AP1, AP2, CREB, Sp1, and NF-κB were examined for their possible roles in keratoconus. Immunostaining experiments and Western blotting showed that Sp1 exhibited enhanced expression in keratoconus corneas. Increased binding of Sp1 consensus sequence oligonucleotides with nuclear extracts from the epithelium of keratoconus corneas was also seen by gel mobility shift assays. This is believed to be a first demonstration connecting Sp1 alteration to a human disease. The elevated Sp1 expression may contribute to the enzyme and inhibitor abnormalities found in keratoconus corneas. **© 1997 Academic Press**

Gene expression is often controlled through transcriptional regulation by transcription factors. These DNA-binding protein factors regulate gene transcription via recognition and binding of specific short DNA sequences in the promoter or enhancer regions of many genes (1-4). Transcription factors allow coordinated control of multiple genes, a necessary ability in complex situations such as during developmental stages, cell cycle, or in response to extracellular signals (5-7).

Keratoconus is a noninflammatory disease (8,9) that progressively thins and distorts the central portion of the cornea and leads to visual impairment. The condition commonly begins during the second decade of life. No specific treatment of this disease exists, except to replace the corneal tissue by transplantation when the patient's vision is beyond correction with contact lenses. The exact cause is not clear, although evidence suggests that a genetic component (10,11) is likely involved. Environmental conditions such as excessive eye rubbing (12) and contact lens wear (13) may also be associated with the disease.

Previous protein studies, both in corneal specimens from affected individuals and in cultured cells, indicated that there is a reduction in the amount of total protein present in keratoconus corneas compared with normal controls (14,15), even while protein synthesis proceeds normally in some cases (14,16). This finding led to the formulation of the hypothesis that the abnormality in keratoconus may lie in the degradative pathway of macromolecular constituents in the cornea (14).

This hypothesis has gained support from a number of studies demonstrating that in keratoconus corneas the levels of lysosomal enzymes, including acid esterase and acid phosphatase, are elevated (17) and those of protease inhibitors, including α1-proteinase inhibitor (18) and α2-macroglobulin (19), are reduced. This varied pattern persists up to the level of gene transcription, as has been verified through *in situ* hybridization experiments and quantitative competitive polymerase chain reactions (20).

Speculation that the perturbed pattern of degradative enzymes and inhibitors in keratoconus might be due to coordinated transcriptional regulation led us to examine whether expression of transcription factors was affected in this disease. We examined five of the better understood transcription factors, AP1, AP2, Sp1, CREB, and NF-κB (6,7,21,22), in keratoconus using immunostaining techniques and electrophoretic gel mobility shift assays (EMSA).

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[2] To whom correspondence should be addressed at University of Illinois at Chicago, Department of Ophthalmology and Visual Sciences, 1855 W. Taylor St., Chicago, IL 60612. Fax: 312-996-7773. E-Mail: U24184@UIC.EDU.

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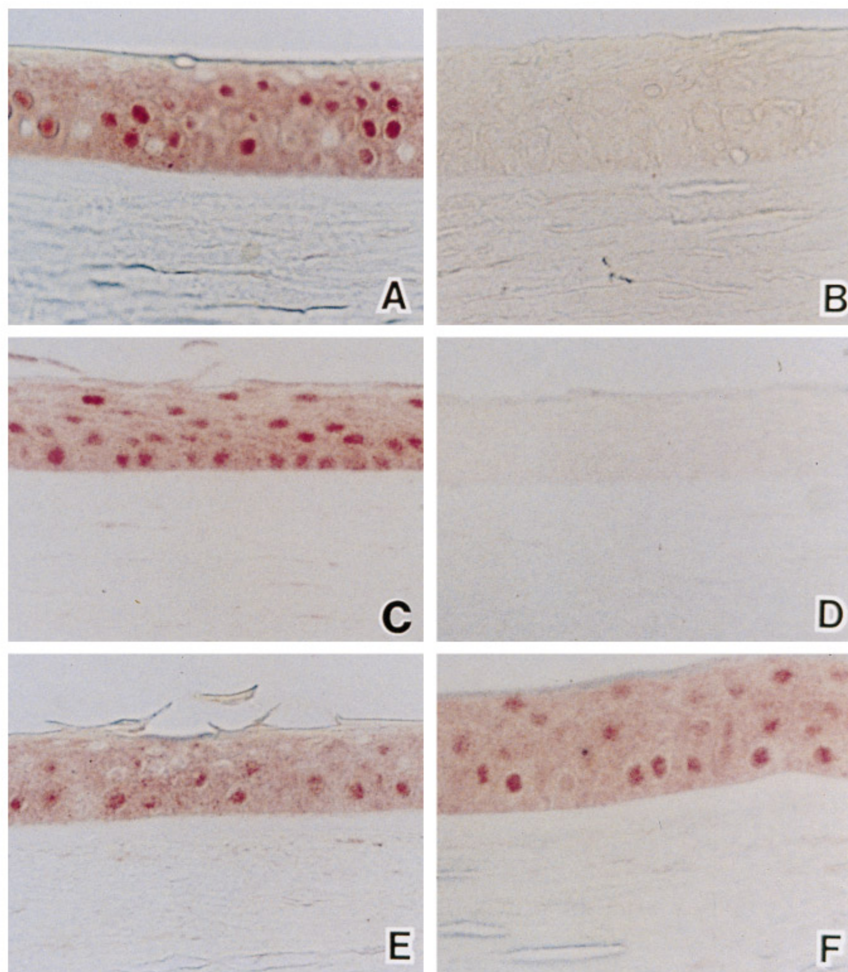

FIG. 1. Immunostaining of corneas for c-fos. Paraffin sections prepared from corneas obtained from a 47-year-old normal individual (A and B), a 56-year-old keratoconus patient (C and D), a 79-year-old patient with pseudophakic bullous keratopathy (E), and a 58-year-old patient with herpetic infection (F). Sections except B and D were stained with polyclonal rabbit anti-c-fos. The specimen in B is a serial section of A and that in D is a serial section of C. They were stained with nonimmune rabbit IgG as negative controls. The avidin-biotin-alkaline phophatase method was used and positive staining appears as pink deposits (original magnification, ×20).

MATERIALS AND METHODS

Twenty normal human eyes were obtained from the Illinois Eye Bank, Chicago. The donors ranged in age from 4 months to 92 years. They did not have any known ocular diseases, and the corneas were all clear. Eleven of these corneas were used for immunostaining. Nuclear extracts were obtained for the remaining nine corneas for EMSA and Western blot analysis.

Half corneal buttons from 24 patients with clinical features typical of keratoconus were collected after transplantation from the Cornea Service at the University of Illinois at Chicago. Patients ranged in age from 23 to 69 years at surgery. Half corneal buttons from 14 patients with other corneal diseases such as pseudophakic bullous keratopathy, Fuchs' corneal dystrophy, herpetic infection, and macular corneal dystrophy were obtained to serve as another set of controls.

Immunostaining. Normal human, keratoconus, and other diseased corneas were fixed in 10% formalin and embedded in paraffin. Immunostaining experiments were performed on 5 μm-thick sections using the ExtrAvidin-alkaline phosphatase complex (Sigma, St. Louis, MO) method as previously described (18,19,23). Primary antibodies included polyclonal rabbit anti-human c-fos, c-jun, CREB, Sp1, or NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA). Tissue sections serving as negative controls received the same dilution of normal rabbit IgG. Biotinylated goat anti-rabbit IgG was used as the secondary antibody and Fast Red TR/Naphthol AS-MX Phosphate (Sigma) was the substrate for alkaline phosphatase. The slides were examined under a light microscope for variations in staining pattern and/or staining intensity and then photographed. For AP2 staining, frozen sections prepared from the corneas were used. Immunostaining experiments were repeated at least three times to confirm the results.

Nuclear extraction, Western blotting, and EMSA. Nuclear extraction was performed as previously described (24). Diseased corneal buttons and half of the 7.5 mm-diameter central portion trephinated from normal human corneas were dissected into the epithelial, stromal, and endothelial layers. The stroma was digested with 1500 U/ml collagenase (Sigma) in minimal essential medium (Sigma) at 37°C

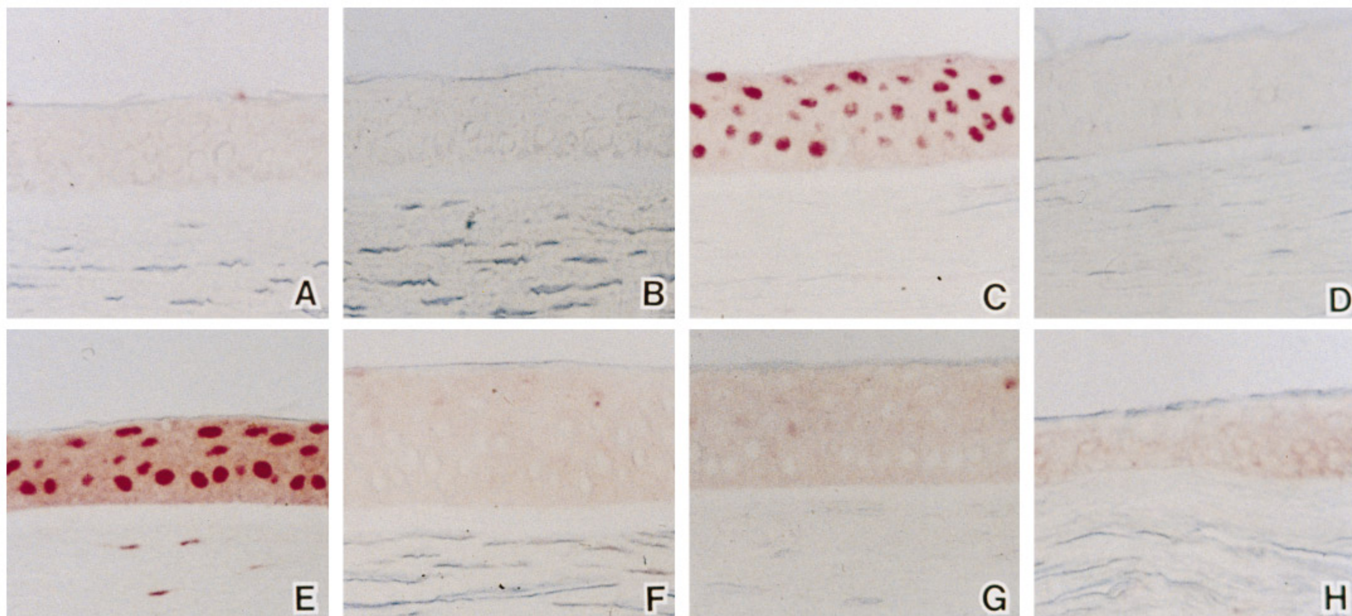

FIG. 2. Immunostaining of corneas for Sp1. Paraffin sections of corneas obtained from a 66-year-old normal individual (A and B), a 67-year-old keratoconus patient (C and D), a 33-year-old patient with keratoconus (E), a 51-year-old patient with Fuchs' corneal dystrophy (F), a 67-year-old pateint with pseudophakic bullous keratopathy (G), and a 55-year-old pateint with herpetic infection (H). Sections except B and D were stained with polyclonal rabbit anti-Sp1. The specimen in B is a serial section of A and that in D is a serial section of C. They were stained with nonimmune rabbit IgG as negative controls. The avidin-biotin-alkaline phophatase method was used and positive staining appears as pink deposits (original magnification, ×20).

with periodic agitation until complete digestion was obtained, usually 4 to 5 hours. The endothelial layer contained insufficient numbers of cells for practical extraction and subsequent analysis, so this layer was not examined. The number of cells obtained from both the epithelial and stromal layers ranged from 1×10^6 to 1×10^7. The total protein in each nuclear extract was determine by BCA assay (Pierce, Rockford, IL) using bovine serum albumin as a standard. Generally between 75 to 100 μg of protein was recovered in each extraction. All nuclear extracts were aliquoted and stored at −70°C.

Western blot analysis was performed as previously described (24), using 40 μg of nuclear extracts from each sample. For EMSA, double stranded consensus binding sequences, each specific for AP1, AP2, CREB, Sp1, or NF-κB (Promega, Madison, WI, or Santa Cruz Biotechnologies) were endlabeled with [γ-^{32}P] ATP (Amersham, Arlington Heights, IL) with T4 polynucleotide kinase (Promega). The EMSA reactions were performed as per manufacturer's protocol (Promega). The HeLa extract was used as a positive control. Four binding reactions (negative, positive, competitive, and non-competitive) were carried out for each extract using from 5 to 7.5 μg total protein per reaction. In addition, some reactions were incubated with 7 μg of anti-human antibodies (Santa Cruz) to specific transcription factor, in supershift reactions to further verify the band shift identity. The binding reactions were electrophoresed on a 6% polyacrylamide gel with an 80:1 ratio of acrylamide to bis-acrylamide (BioRad, Hercules, CA). Gels were dried under vacuum and exposed to XAR-5 film at −70°C. Resulting band shifts were evaluated using an Intelligent Quantifier densitometry system (BioImage, Ann Arbor, MI).

RESULTS

The immunostaining procedure was carried out on a total of 16 keratoconus corneas, eleven normal corneas, and eight disease controls. The staining pattern and intensity for transcription factors AP1 (Fig. 1), AP2, CREB, and NF-κB (photographs not shown) in keratoconus corneas were similar to those found in controls.

Normal human corneas and corneas with diseases

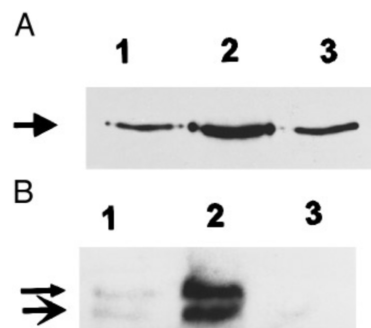

FIG. 3. (A) Western blot analysis with polyclonal rabbit anti-c-fos. Nuclear extracts were prepared from the epithelial layer of a normal human cornea (lane 1), a keratoconus cornea (lane 2), and a cornea with pseudophakic bullous keratopathy (lane 3). Arrow shows the position of the 62-kD c-fos protein. (B) Western blot analysis with polyclonal rabbit anti-human Sp1. Nuclear extracts were prepared from the epithelial layer of a normal human cornea (lane 1), a keratoconus cornea (lane 2), and a cornea with pseudophakic bullous keratopathy (lane 3). Arrows indicate the positions of 106-kD and 95-kD Sp1 proteins.

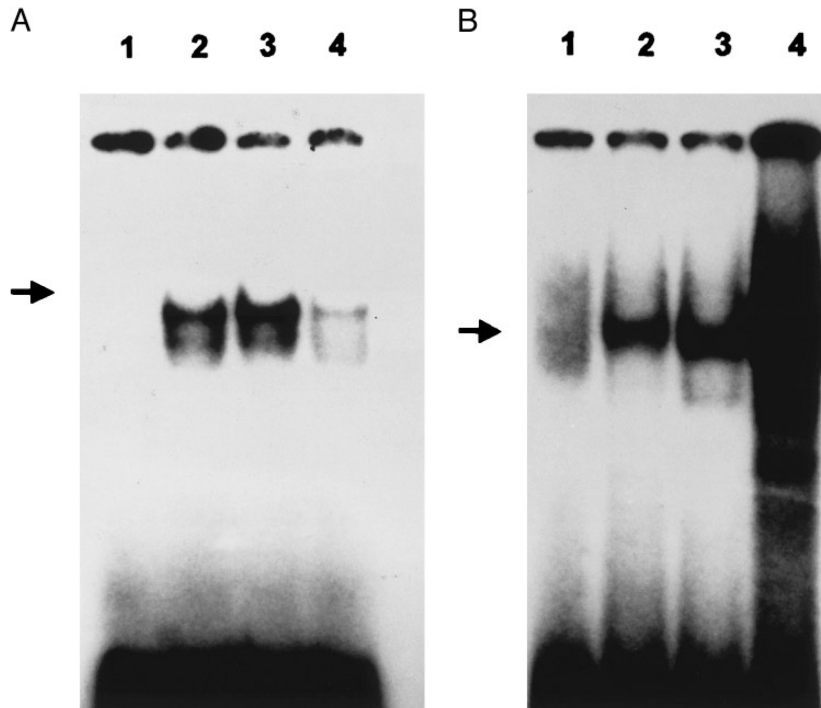

FIG. 4. Electrophoretic mobility shift analysis with corneal epithelium nuclear proteins. (A) ^{32}P-Labeled AP-1 consensus oligonucleotide was incubated with no protein (lane 1, negative control); 7.5 μg of nuclear extracts from the epithelium of a normal human cornea (lane 2), a keratoconus cornea (lane 3), and a cornea with pseudophakic bullous keratopathy (lane 4). (B) ^{32}P-Labeled NF-κB consensus oligonucleotide was incubated with no protein (lane 1, negative control); 10 μg of nuclear extracts from the epithelium of a normal human cornea (lane 2), a keratoconus cornea (lane 3), and 2 μg of HeLa nuclear extract (lane 4, positive control). Arrow denotes specific DNA-transcription factor interactions.

other than keratoconus yielded only basal level immunostaining for Sp1 (Fig. 2). By contrast, strong nuclear SP1 staining was observed, especially in the epithelium of all 11 keratoconus specimens examined.

For AP1, Western blotting assay detected a 62-kD c-fos protein band. A mild increase in staining intensity for the c-fos band was noted in keratoconus specimens compared with normal human ones (Fig. 3A). However, an increase was also observed in disease control corneas. The alteration thus was most likely related to the disease or wound healing condition (23). For Sp1, a 106-kD protein band and a 95-kD band were detected by Western blotting of nuclear extracts (Fig. 3B). Consistent with the immunostaining data, extracts of the keratoconus epithelium yielded much stronger Sp1 bands than that from the normals or disease control corneas.

By EMSA, nuclear extracts from keratoconus and normal human corneal epithelial specimens showed no striking differences in the amounts of DNA-protein complexes with consensus sequences to AP1 (Fig. 4A), AP2, CREB (data not shown) and NF-κB (Fig. 4B). Two DNA-protein complexes were generated with the Sp1 consensus sequence (Fig. 5), as was demonstrated previously (24-26). The binding activity in nuclear extracts prepared from the epithelial layer of keratoconus corneas was visibly much higher than that from normal human or other diseased samples (Fig. 5). The binding was competed specifically with an excess of unlabeled homologous Sp1 oligonucleotide (Fig. 6) and, to a much lesser extent, with a nonhomologus oligonucleotide. The complex formation was also reduced in the presence of anti-Sp1 antibody, and a slower complex was formed instead (data not shown).

Autoradiograms of the polyacrylamide gels were subjected to densitometry and the band intensities found in 8 keratoconus specimens were approximately fourteenfold (14.7 $\pm$ 9.7 fold) of those found in normal controls. Nuclear extracts from the stromal layer of keratoconus corneas and normal human corneas showed no significant differences in the amounts of proteins by Western blotting or levels of DNA-protein complexes by EMSA.

DISCUSSION

The present study demonstrates that the expression of one transcription factor, Sp1, is increased in keratoconus, a disease that causes thinning and scarring of the corneal. Data were obtained from three different

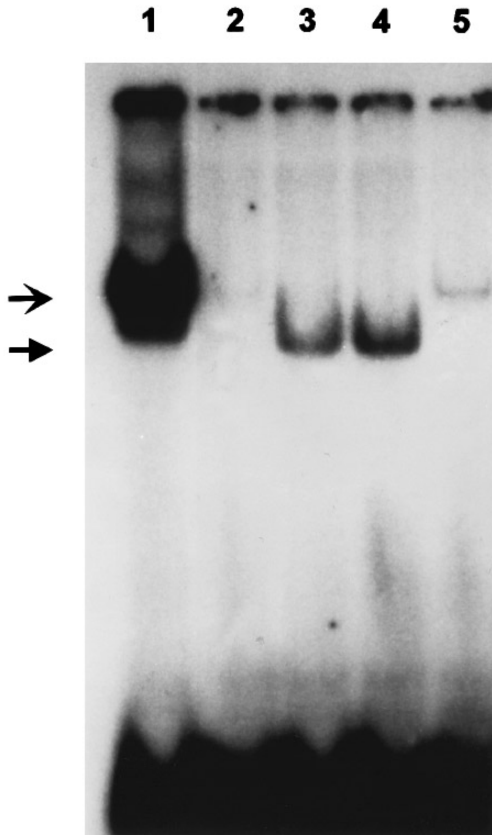

FIG. 5. Electrophoretic mobility shift analyses with corneal epithelium nuclear proteins. ^{32}P-Labeled Sp-1 consensus oligonucleotide was incubated with 1 μg of HeLa nuclear extract (lane 1, positive control); 10 μg of nuclear extracts from the epithelium of a normal human cornea (lane 2), and two keratoconus corneas (lanes 3 and 4), and a cornea with Fuchs' corneal dystrophy (lane 5). The resulting complexes were resolved in a 6% native polyacrylamide gel. Positions of two DNA-protein complexes (arrows) are shown.

methods and results from each corroborated with the others.

Sp1 is a specific factor originally described as required for simian virus 40 (SV40) transcription (27). It interacts with GC boxes in the promoter elements and plays an important role in the expression of many viral and cellular genes including constitutive housekeeping genes and inducible genes (28). Recent investigations have shown that the activity and synthesis of Sp1 are subject to a variety of regulations. For example, Sp1 expression is increased during SV40 infection of the CV1 cells (29,30). Although ubiquitously expressed, the level of Sp1 protein expression varies widely among different cell types in the mouse, and increased Sp1 expression has been associated with late stages of differentiation (31,32). Elevated levels of Sp1 expression have also been noted in gastric carcinoma cells (33). The current study provides the first reported demonstration of an altered Sp1 expression in connection with a human disease.

Previous work from our laboratory have shown that in keratoconus corneas, a number of degradative enzymes exhibit an increased level and/or activity while some protease inhibitors are present in reduced amounts. The abnormal levels of both enzymes and inhibitors may result in accelerated degradation of corneal constituents, leading to pathogenesis of keratoconus. The mRNA levels for genes encoding for these enzymes and inhibitor have also been found to parallel those from protein studies. The pattern of the keratoconus phenotype is thus suggestive of some form of coordinated regulatory control, and the issue of transcriptional control must be examined.

Results presented here indicate that the expression

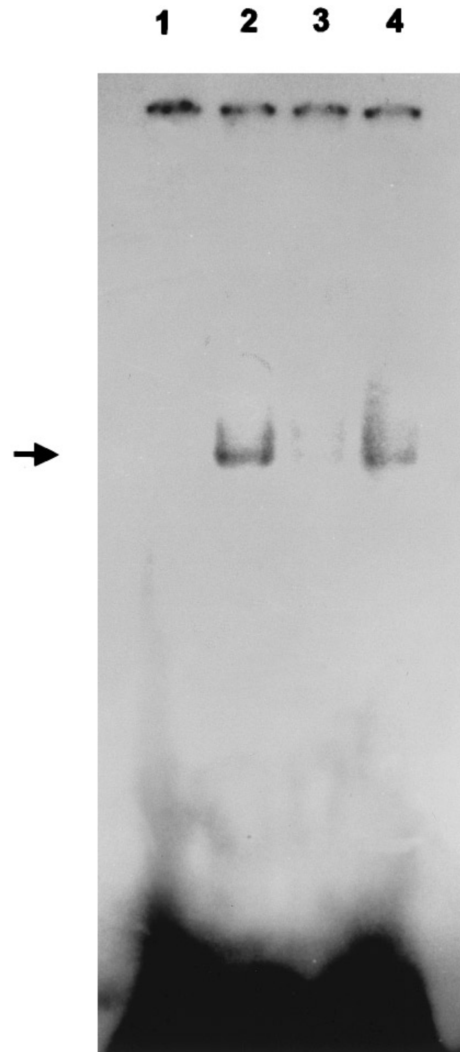

FIG. 6. Competitive EMSA assays with keratoconus corneal epithelium nuclear proteins. Nuclear extracts (7.5 μg each lane) were incubated with radiolabeled Sp1 oligonucleotide in the absence (lane 2), or presence of unlabeled Sp1 probe (lane 3) or unlabeled AP2 consensus oligonucleotide (lane 4). Lane 1 contains no protein as a negative control.

of transcription factors AP1, AP2, CREB, and NF-κB is either only slightly enhanced or not altered in keratoconus. Since these transcription factors, especially AP1 and NF-κB, are known to be induced under stress conditions (34-37), the lack of specific alteration in their expression implies that stress, either environmental or physiologic, is probably not a significant determinant in the pathogenesis of keratoconus (23). This finding argues against the suspicion that environmental or mechanical trauma such as eye rubbing may be a causative factor for keratoconus.

The Sp1 expression that was markedly increased in keratoconus was not observed in other disease controls. Furthermore, the increase is found mostly in the epithelial layer of the keratoconus corneas, a layer previously shown to be involved in keratoconus. The significance of the elevated Sp1 expression in keratoconus is unclear. However, it is of interest that binding sites for this transcription factor have been shown to be present in the regulatory sequences of many of the enzyme and inhibitor genes (38,39) affected in keratoconus. An elevated expression of Sp1 or other members of the Sp1 family (40) in keratoconus corneas could conceivably alter the normal homeostasis, triggering or contributing to disease progression.

In this vein, expression studies using the regulatory regions of the enzyme and inhibitor genes known to be altered in keratoconus and an Sp1 expression vector may allow examination of cell type-specific regulation of these genes by Sp1 and provide insights into the possible roles of increased Sp1 in keratoconus.

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