Macular corneal dystrophy is an inherited corneal disease characterized by corneal opacities resulting from intra- and extracellular deposits within the corneal stroma. Several monoclonal antibodies developed against antigens of corneal fibroblasts were screened for their reactivity with these abnormal deposits in corneas with macular dystrophy using an indirect peroxidase-conjugated immunostaining technique. One of these monoclonal antibodies (designated 8F1-3) reacted very strongly with these abnormal deposits. Although the antigen recognized by this monoclonal antibody was present in the normal corneal stromal and endothelial cells, its concentration in the cells in the corneas with macular dystrophy appeared to be considerably higher, based on the intensity of the immunostaining reaction. Corneal fibroblasts grown in tissue culture were employed for further characterization of the antigen. After fixing with paraformaldehyde and permeabilizing with Triton X-100, immunofluorescent staining of the corneal fibroblasts using these monoclonal antibodies revealed a filamentous pattern of staining which resembled that seen for vimentin filaments. On treatment of corneal fibroblasts with colchicine, the filaments recognized by this antibody were withdrawn from their cytoplasmic array to form a perinuclear cap as also observed for vimentin-containing intermediate filaments. Immunoelectron microscopic studies using colloidal gold-conjugated antimouse IgG indicated that this monoclonal antibody recognized an antigen associated with intermediate-type filament. However, antivimentin antibody did not react with the abnormal deposits in the corneas with macular dystrophy, indicating that the antigen identified in the present study, although associated with intermediate filaments, was not vimentin. Analyses of cytoskeletal antigens by the immunoblotting technique further revealed that this monoclonal antibody recognized two polypeptides with Mr 48,000 and 45,000, while antivimentin antibody reacted with 58,000 Mr polypeptide (vimentin). Invest Ophthalmol Vis Sci 28:1678–1686, 1987

Macular corneal dystrophy is a heritable disorder characterized by abnormalities in the corneal stroma. Decrease in visual acuity due to bilateral corneal clouding progresses slowly from an early age of onset to the advanced stage of the disease in which corneal transplantation is required.1–3 This disorder has been associated with abnormalities in the metabolism of a proteoglycan, proteokeratan sulfate.4–6 In organ cultures of corneas from patients with macular dystrophy, the stromal fibroblasts have either a reduction in or absence of sulfation of keratan chains.6 Recent studies7,8 have demonstrated that the serum of the patients with macular corneal dystrophy lack sulfated keratan sulfate, indicating that the disorder is not limited to the cornea. Pronounced alterations in corneal stromal cells and in endothelial cells are observed in macular dystrophy at both the light and electron microscopic levels.9–16 Extracellular deposits of abnormal material, especially in the subepithelial regions, cause severe visual impairment. Keratocytes and endothelial cells in corneas with macular dystrophy contain numerous intracytoplasmic vacuoles filled with fibrillar material.11–16 The identity of this storage material in macular corneal dystrophy is still not certain. In the present studies, monoclonal antibodies were used to further characterize the intra- and extracellular deposits in the corneas with macular dystrophy.

Materials and Methods

Tissues and Tissue Cultures

Donor eyes obtained from the Medical Eye Bank of Western Pennsylvania (Pittsburgh, PA) were used as the source of normal corneas. Corneas from patients with macular corneal dystrophy were obtained from the Eye and Ear Hospital, Pittsburgh, Pennsylvania, after keratoplasty of three patients aged 35, 44 and 45. Human corneal fibroblasts were established in culture as described by Stoesser et al17 and maintained in Dulbecco’s Modified Eagle’s medium with...
10% fetal bovine serum (DME+S) under 5% CO₂:95% air at 37°C. For immunohistochemical staining, cells were subcultured on chamber slides (Miles Laboratory, Inc., Naperville, IL). To induce the formation of intermediate filament caps, subconfluent layers of cultures in the chamber slides were exposed to 5 × 10⁻⁴M colchicine (Sigma Chemical Co., St. Louis, MO) in culture medium for 18–24 hr at 37°C.

Monoclonal Antibodies

Several monoclonal antibodies, developed using plasma membrane-rich fractions of corneal fibroblasts, were used in the present studies. While some of these antibodies were directed against cell surface-associated antigens, others were either to extracellular matrix antigens or to intracellular components of the cells. Hybridomas secreting these monoclonal antibodies were grown as ascites tumors in Balb/c mice and the ascites fluid was used as a source of highly concentrated monoclonal antibodies.

Indirect Immunohistochemical Staining For Light Microscopy

After the corneas were briefly rinsed in phosphate buffered saline (PBS), they were frozen in Tissue-Tek II O.C.T. compound (Miles Laboratory, Inc.). Cryostat sections (6 μm) of frozen tissue were transferred onto gelatin-coated microscope slides and were used for carrying out indirect peroxidase-conjugated immunohistochemical staining as described earlier.

The same procedure was used for staining cultured cells grown on chamber slides. For immunostaining of cytoskeletons, cells grown on chamber slides were fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100 (Sigma Chemical Co.) and peroxidase-conjugated IgG fraction rabbit anti-mouse IgG (Cappel Laboratories, Cochraneville, PA) was used at 1:40 dilution in place of the peroxidase-conjugated antibody.

Immunoelectron Microscopy

Corneal fibroblasts were grown in 35 mm tissue culture dishes. Cells were fixed and permeabized as described above to obtain cytoskeletal preparations. Using the same procedure as described for peroxidase staining, cytoskeletal structures were immunoreacted with monoclonal antibody followed by colloidal gold-conjugated goat antimouse IgG (AuroProbe EM GAM IgG G10 from Janssen, Beerse, Belgium) as the second antibody, at a dilution of 1:10. Cells were then fixed with glutaraldehyde and processed for electron microscopy. The Epon-embedded cells were peeled from the dishes and sections were cut parallel to the plane of growth using a LKB III ultramicrotome (Bromma, Sweden), stained with osmium tetroxide and viewed with Philips 300 electron microscope. To assess nonspecific binding, a monoclonal antibody against an antigen not present in corneal fibroblasts was used at the same concentration as antibody 8F1-3.

SDS-PAGE and Immunotransblot Analysis

Cytoskeletal preparation of corneal fibroblasts grown in 100 mm dishes were obtained according to Cabral et al., except protease inhibitors (1.0 mM phenylmethyl sulfonyl fluoride, 20 mM EDTA, 2.0 mM n-ethyl maleimide and 1.0 μg/ml peptatin) were included in all the buffers used for the extraction. Briefly, the cells were washed on the dishes with PBS, followed by 5% Triton X-100 in PBS with the protease inhibitors, followed by 0.6 M KCl in PBS with the protease inhibitors. The residues were scraped in PBS with the protease inhibitors and centrifuged at 1000 g for 10 min. The pellet was suspended in the sample buffer and the protein denaturation and electrophoresis on 10% acrylamide SDS slab gels were carried out according to Laemmli. Each lane in the SDS-PAGE contained approximately 100 μg of protein (from one 100 mm dish). Following electrophoresis, proteins in the gels were transferred to nitrocellulose paper and the immunoreactivity of the monoclonal antibody with the protein bands on the transblots was tested according to Towbin et al., with a few modifications. The blots were blocked with 20% fetal bovine serum, monoclonal antibodies were used at approximately 20 μg/ml concentration and peroxidase-conjugated IgG fraction rabbit antimouse IgG (Cappel Laboratories) was used at 1:250 dilution as the second antibody. HRP color development reagent (4-chloro-1-naphthol) from Bio-Rad (Richmond, CA) was employed to detect binding of the second antibody. Nonspecific binding was assessed by reacting the blots with a monoclonal antibody (20 μg/ml) against an antigen absent in corneal fibroblasts.

Results

Selection of Specific Monoclonal Antibodies

Several monoclonal antibodies against corneal fibroblasts were developed from hybridomas derived...
by fusion of a mouse myeloma cell line (SP2/OAg14) with spleen cells from mice immunized with partially purified membrane fractions of corneal fibroblasts. These antibodies, which have been described previously, and 25 more monoclonal antibodies developed subsequently were screened on cryostat sections of normal corneas and corneas with macular dystrophy using indirect peroxidase-conjugated or fluorescein-conjugated immunohistochemical staining techniques. Based on the distribution of the antigens recognized by these antibodies, the antibodies were grouped into: Group 1, those which reacted only with corneal stromal cells; Group 2, those which reacted with corneal stromal matrix; Group 3, those which reacted with corneal stromal and endothelial cells; and Group 4, those which reacted with corneal epithelial, endothelial and stromal cells. While most of the antibodies did not show significant differences in their staining pattern between normal corneas and those with macular dystrophy, a monoclonal antibody designated 8F1-3 (Group 3) recognized an antigen in the abnormal intra- and extracellular deposits in the corneas with macular dystrophy (Fig. 1E, G) when tested by peroxidase-conjugated antibody techniques. Antibody 8F1-3, which recognized an antigen on the stromal cells in normal corneas (Fig. 1B), reacted more strongly with the stromal and endothelial cells (not shown) in corneas with macular dystrophy (Fig. 1G), indicating increased levels of the antigen, recognized by 8F1-3, in these cells. The abnormal deposits were concentrated in the subepithelial regions in the corneas with macular dystrophy (arrows, Fig. 1E, G). The remaining antibodies tested, including those against cell surface-associated antigens of corneal stroma, reacted similarly with both normal corneas and those with macular dystrophy. For example, the staining reaction of an antibody designated M19 (Group 4) with normal and corneas with macular dystrophy is shown in Figure 1C and H, respectively.

Several monoclonal antibodies against corneal keratan sulfate were also tested and found to react with normal corneal stroma (Fig. 1D) but did not react with corneas with macular dystrophy (Fig. 1I). Sulfated regions were involved in the antigenic structure of the epitopes recognized by these antibodies.

**Immunohistochemical Staining of the Cells in Culture**

To study the expression of the antigen of 8F1-3 by corneal fibroblasts grown in culture, cells were grown in chamber slides and then reacted with antibody 8F1-3. Without prior fixation of the cells, this monoclonal antibody reacted weakly with the cells. However, when the cells were prefixed with ethanol:acetic acid (3:1), this monoclonal antibody exhibited a strong staining reaction. Further studies were then carried out to determine whether the antigen was present in the cytoskeletal structures of these cells. The cytoskeletons (prepared by PLP fixation followed by Triton X-100 extraction), when reacted with 8F1-3, exhibited a filamentous pattern of staining (Fig. 2A) which resembled the staining pattern seen for intermediate filaments.

To determine whether the antigen of 8F1-3 is associated with intermediate filaments, an indirect approach was first used. A characteristic feature of intermediate filament-containing cells is their response to microtubule inhibitors such as colchicine; the intermediate filaments withdraw from their cytoplasmic arrays to form a perinuclear birefringent cap. This cap formation was evident in corneal fibroblasts using either 8F1-3 (Fig. 2B) or antivimentin antibody (Fig. 2D), suggesting the possible association of the antigen recognized by 8F1-3 with intermediate filaments.

**Immunoelectron Microscopic Analyses**

An indirect colloidal gold-conjugated immunostaining was employed to localize the antigenic determinants of 8F1-3. The PLP-fixed and Triton X-100-extracted cells of corneal fibroblasts were reacted with 8F1-3 or antivimentin antibody followed by gold-conjugated antimouse IgG. Colloidal gold was found specifically along the filaments when cells were reacted with either of these antibodies (Fig. 3B, C). Similar to vimentin filaments recognized by antivimentin antibodies, those recognized by 8F1-3 were approximately 8–10 nm in diameter; they were therefore identified as the intermediate type of filament. However, the patterns of distribution of gold particles were different for antivimentin and 8F1-3 antibodies. In the case of 8F1-3, gold particles were distributed in

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**Fig. 1.** Indirect immunohistochemical staining of normal corneas and corneas with macular dystrophy. Cryostat sections reacted with monoclonal antibodies 8F1-3 (second row left, normal; bottom left and second row right, macular corneal dystrophy), M 19 (third row left, normal; third row right, macular corneal dystrophy), antikeratan sulfate (fourth row left, normal; fourth row right, macular corneal dystrophy) and control ascites fluid from a mouse bearing SP2/O Ag14 tumor (top left, normal; top right, macular corneal dystrophy). Serial consecutive sections of corneas with macular dystrophy reacted with 8F1-3 and antivimentin antibodies (bottom left and bottom right, respectively). Blue color indicates peroxidase reaction with benzidine dihydrochloride at the site of antibody binding and the pink color is due to the counterstain, safranin O. Note staining reaction with the subepithelial deposits (arrows) in corneas with macular dystrophy when reacted with the antibody 8F1-3 (bottom left and second row right); the same regions showing negligible staining when reacted with antivimentin antibody (bottom right). Bar = 100 μm except top left, second row left and second row right, where bar = 40 μm.
Fig. 2. Indirect immunofluorescent staining of corneal fibroblasts. Fibroblasts reacted with monoclonal antibody 8F1-3 (A) and antivimentin antibody (C) and showed a fibrous network that extends throughout the cytoplasm. Fibroblasts were treated with colchicine for 18 hr prior to fixation, reacted with monoclonal antibody 8F1-3 (B) and antivimentin antibody (D), and showed perinuclear capping of the filaments. Bar = 25 \( \mu m \).

clusters in certain regions along the filaments (Fig. 3B, arrow heads), while in the case of vimentin, gold particles were dispersed evenly along the intermediate filaments (Fig. 3C).

Immunohistochemical Staining of Corneas With Antivimentin

Antivimentin monoclonal antibody (Labsystems, Inc., Chicago, IL) was immunohistochemically tested against corneal sections. Unlike 8F1-3 (Fig. 1E), antivimentin antibody reacted negligibly with the deposits in corneas with macular dystrophy (Fig. 1J), and there were no obvious differences in the staining intensities of the cells in normal and dystrophic corneas. The staining patterns of normal corneas with antivimentin antibody and 8F1-3 were identical.

Immunoblot Analyses of the Antigen

When the cytoskeletal fraction of the corneal fibroblasts grown in culture were analysed by SDS-PAGE followed by immunoblot technique, two polypeptides with M\(_r\) of 45k and 48k reacted with 8F1-3 (Fig. 4, lane C). Antivimentin antibody reacted with the polypeptide with a M\(_r\) of 58k as expected (Fig. 4, lane B). A minor polypeptide with a M\(_r\) of 45k also reacted with antivimentin antibody; it is probably a breakdown product of vimentin.\(^\text{28}\)

Discussion

Macular corneal dystrophy is characterized by distinctive lesions in the corneal stroma. Accumulation of abnormal material within the stromal and endothelial cells, and deposition of a similar substance in the subepithelial regions of the corneas are conspicuous features of macular corneal dystrophy.\(^\text{1-3}\) Based on histochemical studies, this accumulated material has been thought to consist of glycosaminoglycans; however, it is resistant to digestion by enzymes such as hyaluronidase, chondroitin ABC lyase and keratanase.\(^\text{12,29}\) An immunohistochemical approach was used in the present study to characterize the nature of these abnormal lesions, which are predominant in the subepithelial regions. A monoclonal antibody, designated 8F1-3, recognized an antigen which accumulated in the cells and subepithelial deposits in corneas with macular dystrophy. These deposits in the corneas with macular dystrophy are thought to be derived from cellular debris. Compared to 8F1-3, sev-
Fig. 3. Electron micrographs of the cytoskeletons of corneal fibroblasts. Fibroblasts immunoreacted with a monoclonal antibody for negative control (see Materials and Methods) (A), with monoclonal antibody 8F1-3 (B), and with antivimentin antibody (C, see next page); followed by a colloidal gold-conjugated goat antimouse IgG. Note in B (8F1-3) the gold particles bound along the intermediate filaments in clusters and, in C, (antivimentin) evenly along the filaments (arrowheads). Bar = 400 nm.

Numerous other monoclonal antibodies against cell surface-associated and intracellular antigens of corneal stroma reacted very weakly with these deposits, indicating that there was abnormal accumulation of the antigen recognized by 8F1-3 in the cells and cell-derived debris. Light microscopic studies followed by
immunoelectron microscopic analyses indicated that this antigen was associated with intermediate type of filament in the cultured normal corneal fibroblasts. However, immunoelectron microscopic studies revealed that vimentin and the antigen of 8F1-3 have very different distributions on intermediate filaments. The antivimentin used in the present studies had even distribution along the filaments. However, 8F1-3 label, although distributed specifically along the intermediate filaments, was clustered in intermittent regions. This distribution pattern of 8F1-3 resembled that reported for epinemin, a recently described protein that is associated with vimentin filaments in non-neural cells. In response to colchicine treatment, perinuclear capping of the filaments recognized by 8F1-3 was similar to that observed for vimentin filaments. However, immunotransblot analyses of the cytoskeletal-associated components, separated by SDS-PAGE, indicated that 8F1-3 reacted with 45,000 and 48,000 M\(_\text{r}\) polypeptides. In accordance with the literature, antivimentin antibody recognized 58,000 M\(_\text{r}\) polypeptide. This observation indicated that the antigen(s) recognized by 8F1-3 was not vimentin or polypeptides resulting from the proteolytic degradation of vimentin. Further evidence that the antigen recognized by 8F1-3 was a new intermediate filament-associated antigen and not a breakdown product of vimentin was the finding that when antivimentin antibodies and 8F1-3, used in the immunotransblot analyses, were reacted with the corneas with macular dystrophy, the antivimentin antibodies exhibited a negligible reaction, while 8F1-3 exhibited a very strong reaction.

Electron microscopic studies of corneas with macular dystrophy have demonstrated the presence of numerous single membrane-delimited intracytoplasmic vacuoles filled with fibrillar material in the keratocytes. Immunoelectron microscopy to determine whether the antigen of 8F1-3 was associated with this abnormal fibrillar material in the corneas with macular dystrophy was not successful in the present studies, probably due to the denaturation of the antigen by the fixatives used.

The intermediate filaments are the least understood components of the cytoskeletal structure and their possible functional significance remains obscure. However, dramatic rearrangements of these filaments during cellular mitosis suggests that they may be dynamic structures. In the corneal stroma, during mitotically active states, including fetal development and wound healing, significantly higher levels of the antigen, recognized by 8F1-3, have been seen than in the quiescent cells in the adult cornea (unpublished data). Although this finding correlates...
Fig. 4. Immunoblot analyses of the cytoskeletal antigens of corneal fibroblasts. The antigens in the cytoskeletal extracts of corneal fibroblasts were separated on 10% SDS-polyacrylamide gels. The protein bands in the gels were then electrophoretically transferred to nitrocellulose paper, and the immunoreactivity of the antibodies with the transferred proteins was tested using an indirect peroxidase-conjugated antibody technique as described in Materials and Methods. Lane A: staining of protein bands on transblots with amido black. Lanes B, C and D immunoreacted with antivimentin antibody, monoclonal antibody 8F1-3 and a monoclonal antibody designated J19 (control for nonspecific binding, see Materials and Methods), respectively. Lane B: antivimentin antibody reacted with vimentin band (58k Mr polypeptide). Lane C: antibody 8F1-3 reacted with two polypeptides with Mr of 45k and 48k, respectively.

with that observed in the pathological states of corneas with macular dystrophy, its significance is not interpretable.

The components of the intermediate filaments depolymerize and polymerize into filaments under appropriate simulated conditions in vitro. Therefore, it is reasonable to speculate that their synthesis and degradation may play a role in their organization and reorganization during the cell cycle. The abnormal accumulation of the intermediate filament-associated antigen may, therefore, disrupt the normal cell cycle and may cause metabolic abnormalities.

Organ culture studies have demonstrated that corneas with macular dystrophy did not synthesize sulfated proteokeratan sulfates but synthesized an immunologically cross-reacting proteoglycan with unsulfated oligosaccharide chains. Based on these observations, Nakazawa et al have proposed that macular corneal dystrophy is caused by an error in the synthesis of keratan sulfate, possibly involving the specific sulfotransferases responsible for sulfation of the lactosaminoglycan backbone of the chains. In vivo existence of a similar abnormality in proteokeratan sulfates in corneas with macular dystrophy was indicated from our observation that several monoclonal antibodies against keratan sulfates did not react immunohistochemically with corneas from patients with macular dystrophy. Because later studies had shown that sulfated regions were essential for the antigenic structure of the epitopes, nonreactivity of these antibodies demonstrated absence of sulfated keratan sulfate in the corneas with macular dystrophy. Which of the abnormalities in the corneal stroma is a primary event leading to secondary complications remains to be investigated.

Key words: macular corneal dystrophy, monoclonal antibodies, intermediate filaments, cytoskeleton, cornea

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