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Cell-Surface Associated Proteins of Corneal Fibroblasts: Dissection With Monoclonal Antibodies

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It is now generally accepted that the cell surface is involved in the interaction of the cells with the extracellular matrix. To identify and characterize cell-surface-associated components of corneal fibroblasts, several monoclonal antibodies were developed. Hybridomas were developed by fusing mouse myeloma cells SP2/OAg14 with spleen cells from mice immunized with membrane fractions of corneal fibroblasts grown in culture. Twenty-five hybridomas secreting monoclonal antibodies to cell-surface components were selected by an enzyme-linked immunosorbent assay using corneal fibroblasts grown in microtiter plates as the substrate. Immunohistochemical staining demonstrated that the antigenic determinants recognized by these antibodies were not present on corneal epithelial cells, but were present on skin fibroblasts. The antigenic deteminants recognized by two of these antibodies, designated 10D2 and 716, were matrix components of the corneal stroma. Immunochemical characterization of the antigens was carried out by indirect precipitation of the radioactively labeled cellular proteins with the monoclonal antibodies and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the precipitates. Four antibodies were able to precipitate antigens from cell extract in detectable amounts. Antibodies designated 5E2, 9G2, and 10D2 recognized antigens consisting of polypeptides of approximate molecular weights 105K and 110K, while antibody 716 recognized an antigen of 100K molecular weight. However, based on the tissue distribution and cell-surface distribution, these antibodies reacted with different antigenic determinants. The antigen recognized by 716 was also secreted by cells in culture but consisted of 220K and 200K polypeptide chains. It was tentatively identified as cellular fibronectin, based on the reaction of this antigen with polyclonal antibodies to plasma fibronectin.

Key words: monoclonal antibodies, corneal fibroblasts, cell surface, fibronectin

Corneal fibroblasts are responsible for the formation of a transparent corneal stromal matrix consisting mostly of collagenous lamellae and proteoglycans. Although in the adult cornea fibroblasts are quiescent, these cells retain the capacity to grow

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and divide throughout adult life when stimulated, as for example in healing wounds [1,2]. The cell surface and its interactions with the extracellular matrix in various cellular functions have received a great deal of attention in the past few years because they are involved in regulating many cellular processes including cellular differentiation. Presently, little is known about the corneal fibroblast cell-surface components and their possible association with various cellular phenomena such as regulating cell migration, the production and organization of a complex extracellular matrix, cell division, and inhibition of growth and division. Cells grown in tissue culture are known to express many differentiated functions of the cells in vivo [eg, 3–5]. Avian corneal fibroblasts grown in vitro have been shown to differ from skin and heart fibroblasts both biochemically and morphologically [6–8]. Corneal fibroblasts were observed to produce more hyaluronic acid and less chondroitin-6-sulfate than either skin or heart fibroblasts [6–7] Morphologically, monolayers of corneal fibroblasts consisted of randomly oriented polygonal cells, whereas monolayers of skin fibroblasts consisted of long, narrow ragged cells in parallel array and heart cells formed multilayers of criss-crossed cells [8]. In the present studies, we have utilized human corneal and skin fibroblasts grown in vitro to characterize cell-surface-associated components. Hybridoma-derived antibodies have been used to dissect the cell surface of corneal fibroblasts and to compare it with skin fibroblasts.

METHODS

Cells and Membrane Purification

Human corneal fibroblasts were established in culture, as described by Stoesser et al [9]. Human skin fibroblasts (WI38) were obtained from Microbiological Associates, and were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum (DME+S) under 5% CO_2-95% air. Membrane-rich fractions were then isolated by the $ZnCl_2$-method of Warren and Glick [10] as modified by Shin and Carraway [11] from corneal fibroblasts grown to confluence in 50 roller bottles (490 cm^2 surface area/bottle). Membrane fractions were frozen at −70°C in aliquots containing 200 μg of protein. Proteins were measured according to Lowry et al [12].

Hybridoma Production

Balb/C mice were immunized subcutaneously with the membrane fraction in PBS (containing 100 μg protein) with Freund's complete adjuvant. The second and the third injections, consisting of 100 μg of membrane proteins in PBS with and without incomplete adjuvant, respectively, were given intraperitoneally at 4-wk intervals. Three days after the final injection, spleen cells from the mice were prepared for fusion with mouse myeloma cells SP2/OAg14 [13] obtained from the Salk Institute (Cell Distribution Center, San Diego). The cells were fused with polyethylene glycol 1550 (Accurate Laboratory, Hicksville, NY) following the procedure of Galfre et al [14]. From each hybridization, the cells were dispersed into 2 × 96-well microtiter plates in hybridoma medium (Hy) [15] with 20% fetal calf serum. Half the medium in the wells was replaced with HY containing hypoxanthine, aminopterine and thymidine (HAT) [16] for the next three days and once every third day from then onwards. Within 2–3 wks, hybridoma growth was noticed in the microtiter wells. The culture superntants from the hybridomas were screened for the presence of antibody by an enzyme-linked immunosorbent assay (ELISA).

Enzyme-Linked Immunosorbent Assay

The ELISA test was performed using corneal or skin fibroblasts grown in 96 well-microtiter plates as the substrate, using a modification of Engvall and Perlmann's technique [17]. The cells were rinsed three times with phosphate-buffered saline (PBS), and each well was then covered with 100 μl of 10% heat-inactivated rabbit serum (HIRS) in PBS for 1 hr. After removing the HIRS, cells were covered for 1 hr with 100 μl of hybridoma culture supernatant, incubated for 1 hr with peroxidase-conjugated rabbit antimouse IgG (1:80 dilution in HIRS in PBS) from Cappel Laboratory, Cochranville, PA, followed by 100 μl of 5-amino-salicylic acid reagent [18]. The plates were rinsed three times with PBS between each of the above steps. The change in color to brown indicated possible presence of antibodies to cell-surface-associated antigens. Both antibodies of IgG and IgM class were detected since the peroxidase-conjugated antibody that was used cross-reacted with mouse IgM.

The hybridomas secreting antibodies were cloned and recloned by the limiting-dilution technique. One or two of the cloned hybridomas were selected for further characterization of their antibodies. The antibodies were concentrated by ammonium sulfate fractionation of culture supernatants of hybridomas grown in large quantities. Hybridomas also were grown as ascites tumors in Balb/c mice and the ascites fluid was used as the source of high concentration of monoclonal antibodies [19].

Indirect Enzyme-Linked Immunostaining

Histological staining for light microscopy was carried out on cryostat sections of human skin or corneas from donor human eyes (obtained from Medical Eye Bank of Western Pennsylvania). The tissues were frozen in Tissue-Tek II O.C.T. Compound, and cryostat sections (6 μm) of the tissues were transferred to microscope slides. Immunostaining was carried out using an indirect immunoperoxidase-conjugated antibody technique, as described earlier [20]. The same procedure was used for staining cultured cells grown on chamber slides. A reagent containing benzidine dihydrochloride and safranin O [21] was used for the detection of peroxidase-conjugated antibody. After dehydrating through ethanol and xylene, the sections were mounted in permount (Fisher Scientific Co., Pittsburgh, PA) for light microscopic observation.

Determination of Immunoglobulin Class of Hybridoma Antibody

Since the parent myeloma cell SP2/OAg14 does not synthesize any immunoglobulin, it was possible to carry out direct immunodiffusion tests with ammonium sulphate concentrated monoclonal antibodies from hybridoma culture supernatants. Double immunodiffusion was carried out against antimouse κ- and λ-light chains (Miles Laboratory, Elkhart, IN), antimouse IgM (μ-chain specific) and antimouse IgC (γ-chain specific) from Cappel Laboratories as described earlier [20].

Labeling and Immunoprecipitation of Cell-Surface-Associated Antigen

A lactoperoxidase-catalyzed labeling technique was used for preferentially labeling cell-surface proteins with ^{125}I [22]. A confluent culture of corneal fibroblasts or skin fibroblasts in a 25-cm^2 tissue culture dish was labeled for 10 min using 0.5–1 mCi of ^{125}I (carrier-free, 17 Ci/mg in 2 ml of PBS with 100 μl lactoperoxidase (1 mg/ml), 100 μl glucose oxidase (2 U/ml), and 100 μl glucose (50 mM). After washing the plates three times with PBS containing 0.04% tyrosine, the cells in each dish were scraped and extracted with 0.5–1.0 ml of cold extraction buffer (PBS with 0.5%

Nonidet P-40, 15% glycerol, and 2 mM phenylmethylsulphonylfluoride). After incubating the extract for 1 hr on ice, it was centrifuged at 1,000g for 20 min followed by 25,000g for 1 hr. The supernatant was used for immunoprecipitation. To biosynthetically label the cellular proteins, a confluent layer of cells was covered with methionine-free DME with 10% calf serum and 10 μCi/ml of [^{35}S]L-methionine (specific activity 400 Ci/mmol). After 24-hr incubation at 37°C, the medium was removed and the cells were extracted with the extraction buffer, as described earlier.

To 250 μl of the Nonidet P-40 supernatants containing ^{125}I- or ^{35}S-labeled proteins of glycoproteins, 20 μl of preimmune mouse serum was added followed by 200 μl of rabbit antimouse IgG antibody containing 0.05% NP-40, and precipitation was allowed to occur overnight at 4°C to remove nonspecifically precipitating proteins. The antigens were then precipitated with the monoclonal antibody by adding 10 μl of ascites fluid containing specific antibodies. After 2 hr at 4°C, 100 μl of rabbit antimouse IgG was added and further incubated at 4°C overnight. The precipitate was centrifuged, washed five times with the extraction buffer, and the final pellet was dissolved in the SDS sample buffer containing dithiothreitol for SDS-polyacrylamide gel electrophoresis. Samples were electrophoresed on 8% SDS-PAGE slab gels according to Laemmli [23].

Affinity Purification of Antigens

Antigens were purified from the culture filtrate by ammonium sulfate precipitation (40% saturation) followed by affinity purification on antibody-Sepharose CL-4B column. Antigens were eluted with 4 M urea in 10 mM Tris-HCl and dialyzed against 10 mM Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl.

RESULTS

Initial Screening of Hybridomas and Determination of Ig Class

Monoclonal antibodies were generated from hybridomas developed by the hybridization of mouse myeloma cells (SP2/OAg14) with spleen cells of mice which were immunized with membranes isolated from cultured human corneal fibroblasts. A total of 380 hybridomas were produced in two separate fusion experiments. Of these, 25 hybridomas were found to secrete antibodies to cell-surface antigens of corneal fibroblasts as determined by the ELISA technique. After subcloning twice in succession, one clone from each hybridoma was selected for further investigations. Initial screening by ELISA technique indicated that the antibodies from all of these 25 hybridomas also react with human skin fibroblasts.

By double-immunodiffusion technique using specific antibodies against mouse immunoglobulin, 11 of these monoclonal antibodies were found to be IgM type and 14 were IgG type with κ-light chains (the immunoglobulin class of some of the monoclonals is listed in Table I, see below).

Immunohistochemical Staining

The monoclonal antibodies described here were used to study the distribution of their antigenic determinants on corneal and skin fibroblasts, and corneal epithelial cells in primary cultures. All except three of the antibodies were specific to the fibroblast cells and were absent on corneal epithelial cells. The antibodies designated 8C2, 9F1, and 7D1 reacted with both the fibroblasts and the epithelial cells. Based on the pattern of immunostaining, as examined by light microscopy, at least three sets

TABLE I. Characterization of Monoclonal Antibodies

Antibody	Immunostaining of cells grown in monolayers	Tissue staining		Antigens MW (on skin fibroblats and corneal fibroblasts)	Class of antibody	Antigens secreted by corneal fibroblasts, MW of polypeptides
		Cornea	Skin			
10D2	Intense on cellular processes	Stroma	Dermis	105,000 110,000	IgG	ND
716	On fibrous network of cellular processes	Stroma	Dermis	100,000	IgG	200,000 220,000
5E2	More intense at edges of cells			105,000 110,000[a]	IgG	ND[b]
9G2	More at edge of cells			105,000 110,000	IgG	ND
8C2	Uniform on cell surface			ND	IgG	ND
9F1	Uniform on cell surface			ND	IgM	ND
7D1	Uniform on cell surface			ND	IgM	ND

[a]Not present on skin fibroblasts.
[b]Not detectable.

of antigenic determinants were recognized by different monoclonal antibodies. While ten antibodies reacted with the fibrous matrix and cellular processes (eg, 10D2 and 716, Fig. 1a,b), seven antibodies reacted with the cell surface (eg, 8C2, Fig. 1e). The remaining eight antibodies also reacted with the cell surface but more intensely at the edges of the cells (eg, 5E2 and 9G2, Fig. 1c,d). Although all of these antibodies reacted with the cells in culture, only two antibodies (10D2 and 716) reacted with corneal and skin tissue sections. The antigens recognized by the antibodies were found to be present in the corneal stromal matrix and the dermal region of the skin (Fig. 2).

Immunochemical Characterization of the Antigens With the Monoclonal Antibodies

From cell extracts containing either ^{125}I-labeled surface proteins or biosynthetically [^{35}S]methionine-labeled proteins, only four different antigens could be coprecipitated in detectable amounts using the monoclonal antibodies and antimouse IgG antibodies. The SDS-PAGE analysis of the ^{125}I-labeled cell surface antigens in the precipitates is shown in Figure 3. The methionine labeled antigens showed similar patterns on SDS-PAGE (Fig.4). Results of the analysis are summarized in Table I. As can be seen from these analyses, the antigens recognized by two of the monoclonal antibodies (9G2, 10D2) both on skin and corneal fibroblasts have similar molecular weights. However, the antibody 5E2 recognized only one polypeptide on the skin fibroblasts but two polypeptides on corneal fibroblasts.

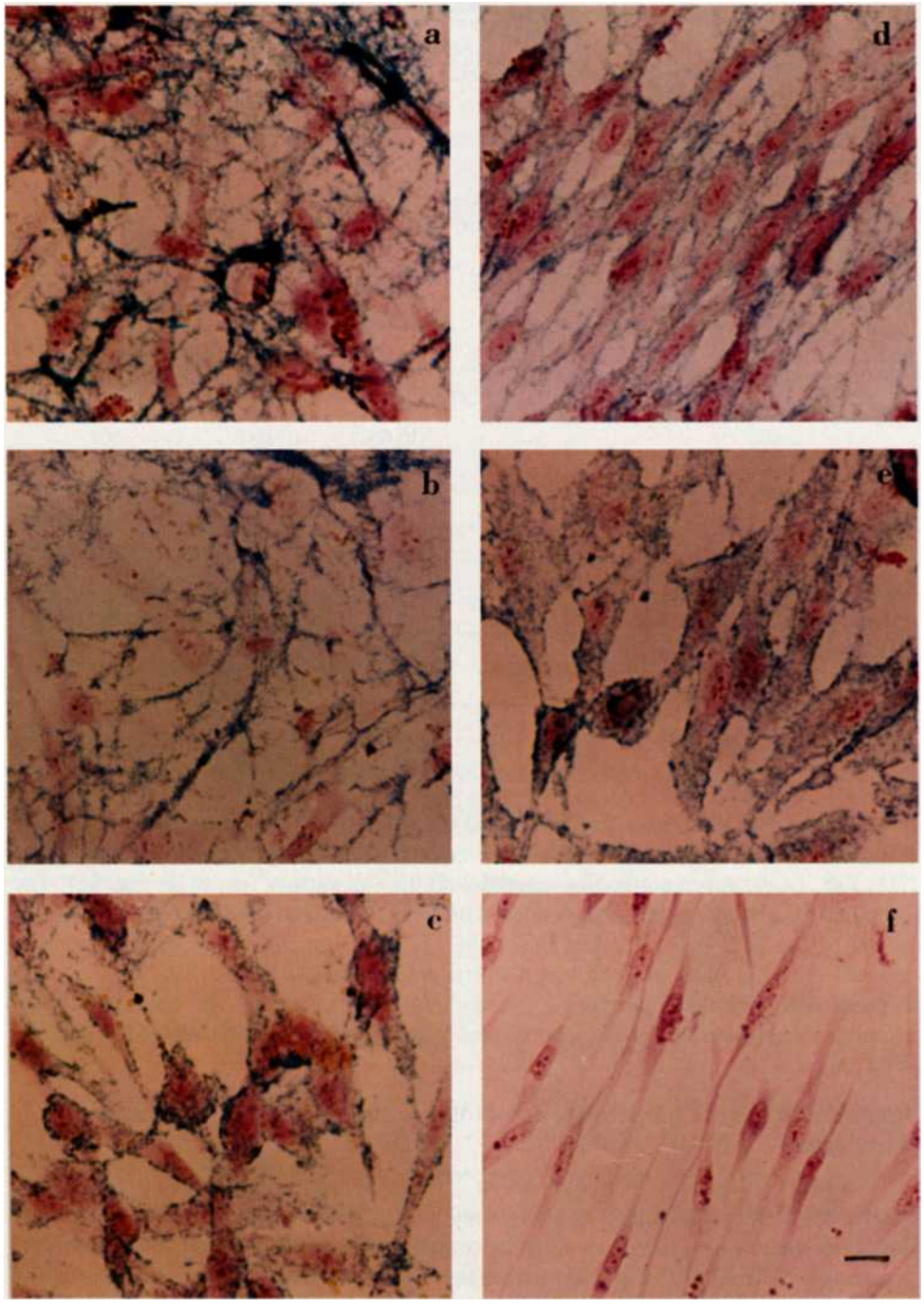

Fig. 1. Indirect immunohistochemical staining of human corneal fibroblasts in culture with monoclonal antibodies: a, 10D2; b, 716; c, 5E2; d, 9G2; e, 8C2; f, ascites fluid from a mouse bearing SP2/0 Ag14 tumor as a control. Blue color indicates peroxidase reaction with benzidine dihydrochloride at the site of antibody binding. The counterstain, safranin O, stained the cytoplasm and nuclei pink. Bar = 20 μm × 250.

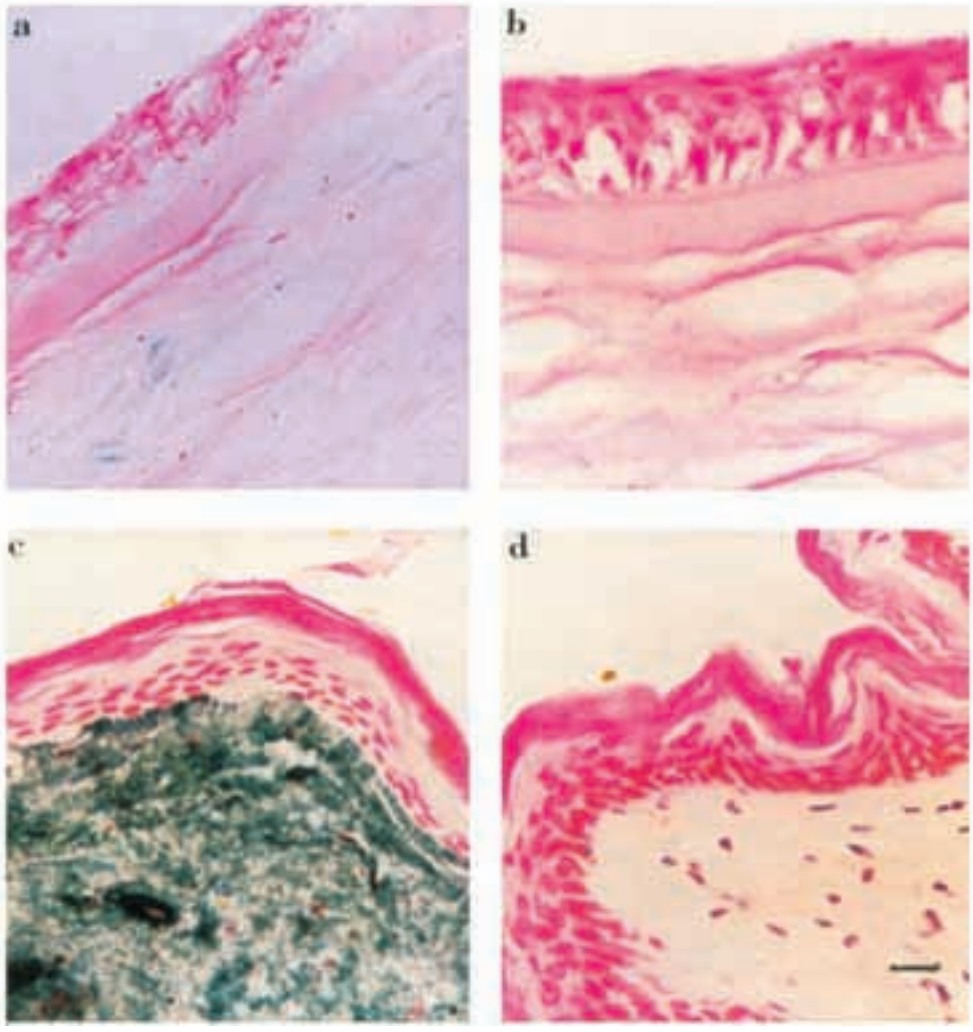

Fig. 2. Indirect immunohistochemical staining of human corneal (a,b) and human skin (c,d) sections with monoclonal antibody 716 (a,c) and with ascites fluid (control) from a mouse bearing SP2/O Ag14 tumor (b and d). Blue color indicates peroxidase reaction with benzidine dihydrochloride at the site of antibody binding, and pink color is due to the counterstain, safranin O. Bar = 20 μm. × 250.

To determine whether any of these antigens recognized by the monoclonal antibodies described here were secreted by cells in culture, culture supernatants containing de novo synthesized ^{35}S-labeled proteins were mixed with the antibodies and the immunoprecipitates were analyzed by SDS-PAGE. The results indicate that only the antigen recognized by the antibody designated 716 was secreted by the cells in detectable amounts (Fig. 4, lane 10). This antigen, as already discussed, is present in the corneal stroma and the dermal regions of the skin. However, while the antigen on the surface of the cells consisted of a polypeptide with a molecular weight of

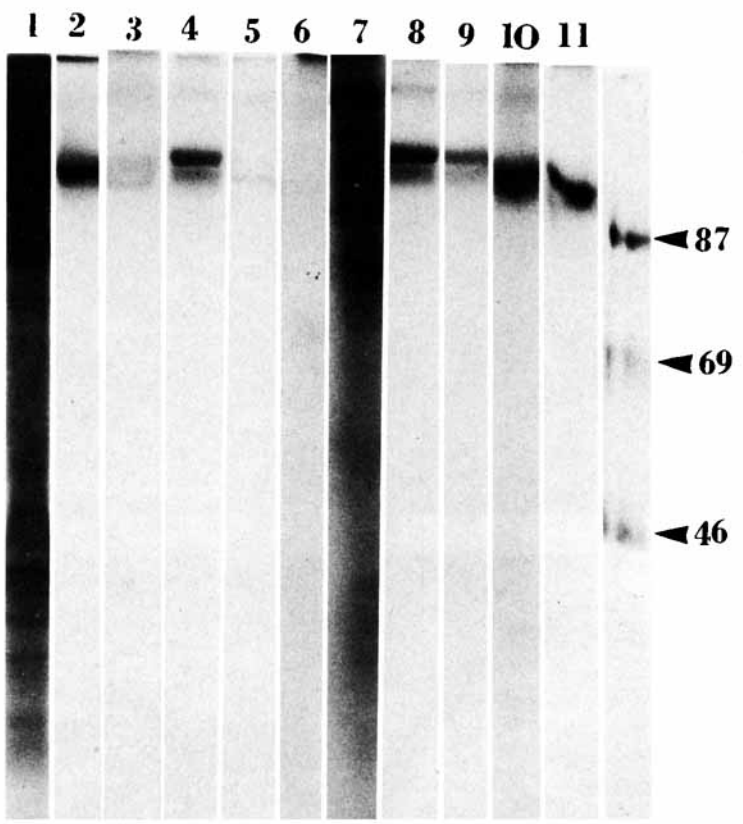

Fig. 3. Autoradiographs of SDS-PAGE-separated proteins in the immunoprecipitates of ^{125}I-labeled surface proteins of skin fibroblasts (lanes 1–6 cut from a single gel) and corneal fibroblats (lanes 7–11 cut from a single gel). Immunoprecipitates were obtained using the following antibodies: lanes 2 and 8, 5E2; 3 and 9, 9G2; 4 and 10, 10D2; 5 and 11, 716; and 6, ascites fluid control. Lanes 1 and 7 are ^{125}I-labeled surface protein of skin and corneal fibroblasts, respectively, in the cell-extracts. Approximate molecular weights of the protein bands were calculated separately for each gel.

100K, the antigen was found in the culture supernatant as disulfide-linked dimer composed of polypeptides with molecular weights of 200K and 220K (Fig.4). This antigen from the culture supernatants migrated as a 400K polypeptide in SDS-PAGE under nonreducing conditions. It is possible that the detergent-extractable antigen 716 in the cell extracts is derived from 220K or 200K polypeptides by proteolytic cleavage. The size of this antigen secreted by cells suggested that this antigen may be a cellular fibronectin [see review 24]. Since plasma fibronectin is antigenically very similar to cellular fibronectin [25,26], the reactivity of antibody 716 was tested against plasma fibronectin using ELISA technique (Fig. 5a). This antibody did not cross-react with human plasma fibronectin. However, polyclonal antibodies (Collaborative Research, Inc, Lexington, MA) reacted very strongly with affinity purified 716 antigen (Fig. 5b) from the culture supernatants of corneal and skin fibroblasts. Therefore, it was concluded that antibody 716 combined with an antigenic determinant of a cellular fibronectin-related protein.

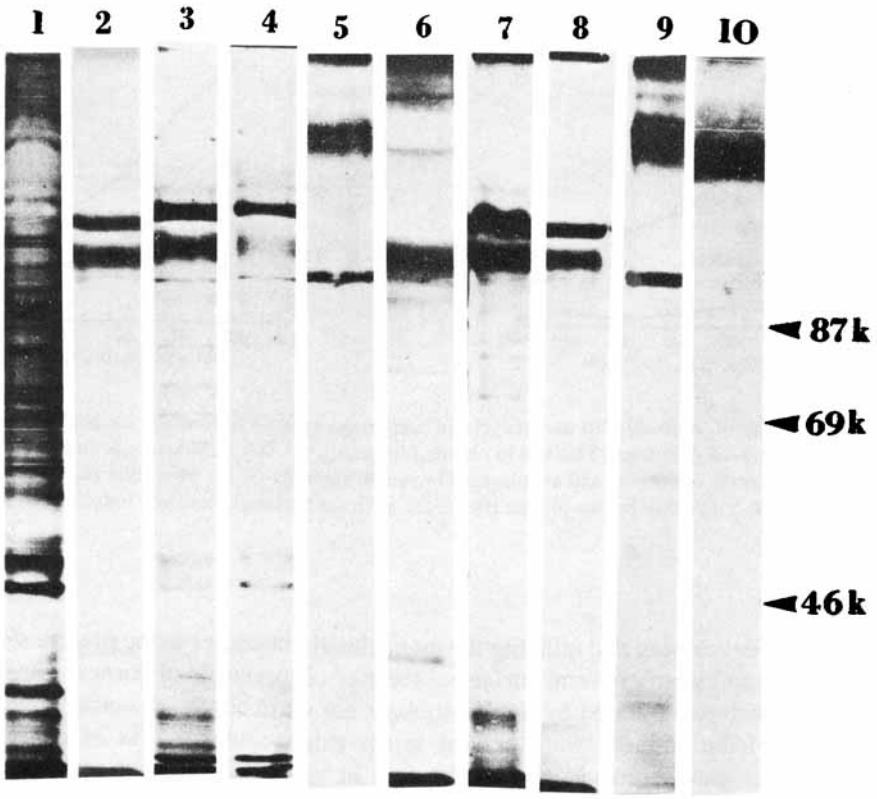

Fig. 4. Autoradiographs of SDS-PAGE-separated proteins in the immunoprecipitates obtained by reacting [^{35}S]methionine-labeled proteins with monoclonal antibodies: Immunoprecipitates in lanes 2–5 were derived from corneal fibroblasts and in lanes 6–9 from skin fibroblasts. Monoclonal antibodies used for the immunoprecipitations correspond with 2 and 6, 5E2; 3 and 7, 9G2; 4 and 8, 10D2; 5 and 9, 716. Lane 1 is the cell extract of corneal fibroblasts containing ^{35}S-labeled de novo synthesized proteins. Lane 10 corresponds with the proteins in the immunoprecipitates obtained by reacting the monoclonal antibody 716 with the culture supernatant of corneal fibroblasts labeled with [^{35}S] methionine. Lanes were cut out from two separate slab gels run identically at the same time. Molecular weights of the peptide bands on each gel were calculated separately for each gel.

DISCUSSION

The interactions of the cell surface with extracellular components are involved in the transmission of information between the outside and the inside of the cell. Corneal fibroblasts are responsible for the formation of a unique transparent connective tissue matrix. One of the components of this matrix which is specific to the corneal stroma is a glycosaminoglycan, keratan sulfate I [27,28]. However, there are other principal components of the corneal stromal matrix, including type I collagen and fibronectin, which are common to other connective tissues of the body. It is possible that the cell-surface of corneal fibroblasts has a unique make up, along with the cell-surface domains shared by fibroblasts of different origin or other cell types in

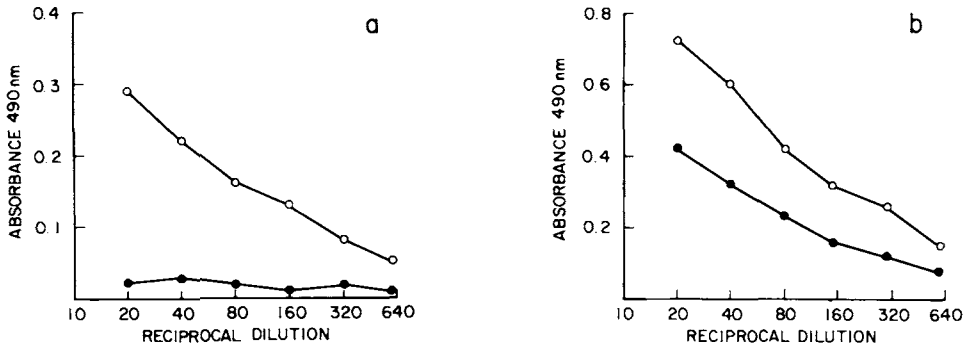

Fig. 5. Binding of antibody 716 and polyclonal antihuman plasma fibronectin antibody to affinity-purified cellular protein (antigen 716) and to plasma fibronectin. ELISA technique was employed for the titrations of antibody 716 (•---•) and antiplasma fibronectin antibody (°---°). Microtiter plates for ELISA were coated with 5 μg/ml of human plasma fibronetin (a) (from Bethesda Research Lab, Inc.) or antigen 716 (b).

the body. By developing and utilizing the monoclonal antibodies in the present studies, we were able to identify several surface-associated components of corneal fibroblasts in culture which were shared by skin fibroblasts but were absent on corneal epithelial cells. Two of the antigens were present in the extracellular matrix of the corneal stroma. Other antigens could not be detected in vivo on quiescent cells. Cells in culture are often thought to behave like dividing cells in vivo under special conditions such as in the repair of wounded tissue. Therefore, it is possible that those antigens which are absent in the quiescent cells may be present on dividing cells either in fetal corneas or in healing tissues.

One of the antibodies, designated 716, which reacted with the corneal stromal matrix, was found to react with cellular fibronectin but not with plasma fibronectin. This antibody will be a valuable tool in immunohistochemical and immunochemical studies in differentiating plasma fibronectin from cellular fibronectin. A monoclonal antibody exhibiting specificity for cellular fibronectin has been recently described by Atherton and Hynes [29].

Immunochemical studies indicated that the antibody designated 5E2 detected a difference between corneal and skin fibroblasts. While only one polypeptide with an approximate molecular weight of 105K was detected on skin fibroblasts, two polypeptides of molecular weights of 110K and 105K were detected on corneal fibroblasts. Studies are in progress to determine whether the antigenic determinants recognized by any of the antibodies described in this communication are associated with the regulation of cell morphology, cell growth, cell division, or the interactions of corneal fibroblasts with matrix components.

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