Fibronectin production by cultured human trabecular meshwork cells. DAVID M. WORTHEN AND PATRICK H. CLEVELAND.

Postmortem explants of human trabecular meshwork from 23 eyebank eyes had a growth rate of 32% (71/ 160) cultures, with 8% (12) showing abundant cellular spread. One hundred percent confluency was achieved with eyes from donors of 11 to 40 years of age. One of the cultures grew profusely and we were able to study it through eight passages in culture. This culture had the morphologic characteristics described by Polansky et al. for cultured human trabecular meshwork cells. A sensitive radiorinunoassay for fibronectin in tissue culture supernatant showed an increase in fibronectin secretion over a 96 hr period, with a characteristic shift-down response occurring between 24 and 48 hr postmedium change. Three of four cultures showed the presence of laminin, a basement membrane protein, establishing that the cells are not fibroblasts. (INVEST OPHTHALMOL VIS SCI 23:265-269, 1982.)

Biopsy samples of the trabecular meshwork taken from patients with glaucoma and from age-matched controls have demonstrated the appearance of a more abundant extracellular osmophilic glycoprotein in glaucomatous eyes. Recently, Rodrigues et al. demonstrated that this material consists at least in part of fibronectin, laminin, and type IV collagen. Furthermore, this material was present in greater abundance in trabeculectomy specimens from glaucomatous eyes than from age-matched controls. Alvarado and Polansky have also noted the production of fibronectin by trabecular cells cultured from postmortem eyes.

Fibronectin is a glycoprotein secreted by cells onto the surfaces, into the extracellular matrix, and into the blood. It is a component of basement membrane, along with type IV collagen and laminin. It has a high affinity for denatured collagen, proteoglycans, myofibrils, elastin, fibrin, and certain bacterial cell walls. It functions as a cell-to-cell or cell-to-substrate junction and cement. It is also an opsin to aid phagocytosis because of that binding function. Circulating fibronectin in the bloodstream aids in the repair of burns and defense against infections.

Laminin is a glycoprotein with two or three polypeptide chain subunits. It is found in normal basement membrane and is antigenically distinct from fibronectin and type IV collagen. It has also been demonstrated in cultures of human corneal endothelial cells and epithelial cells, but it is not produced by fibroblasts. We have developed a method for measuring fibronectin and detecting laminin secreted in tissue culture supernatants of human trabecular meshwork cells taken from eye-bank eyes.

Materials and methods. Human eyes obtained by the San Diego Eye Bank between 1 and 7 hr after death were cultured 4 hr to 5 days later. Trabecular tissue was carefully dissected under a stereomicroscope within a laminar flow hood in sterile 60 by 15 mm Petri dishes and was rinsed with Dulbecco’s Modified Eagle’s Medium (DME) containing 0.2M HEPES buffer, pH 7.4 (Microbiological Associates, Los Angeles, Calif.). Dissection was accomplished by creating a flap at the periphery of the scleral rim; the flap was then folded back to expose the trabecular meshwork. Strips of trabecular material were removed and placed in the central wells of Linbro 24-well tissue culture plates (Flow Laboratories, Inc., Rockville, Md.) that had been pretreated with 0.2% gelatin (swine skin type I, Sigma Chemical Co., St. Louis, Mo.). Explants were anchored to the bottom of the well with sterile glass coverslips. One milliliter of tissue culture medium was then added to the wells. The tissue culture medium had the following composition: DME containing 20% fetal bovine serum (Microbiological Associates), 2 mM L-glutamine (Gibco, Santa Maria, Calif.), 50 μg/ml gentamicin, 2.5 μg/ml Fungizone (Gibco), and 5% dextran (J. T. Baker Chemical Co., Phillipsburg, N.J.), pH = 7.4. The explants were then placed in a humidified incubator at 37° C with 7.5% CO₂ and were left undisturbed for 5 to 7 days. When the explants reached confluency, the cells were removed with a 0.25% trypsin-EDTA solution (Gibco) and passaged to 30 cm² tissue culture flasks pretreated with 0.2% gelatin, and part of the cell suspension was aliquoted into the wells of a microtiter plate (Falcon Plastics, Oxnard, Calif.) pretreated with gelatin. Cultures successfully subcultured in the flasks were trypsinized and passaged into 30 cm² flasks and microtiter plates pretreated with gelatin. Cells grown in microtiter wells were utilized for fibronectin secretion studies. When these cells reached confluency, the culture medium was changed with medium containing 5% fetal bovine serum (FBS).

Fibronectin was iodinated by the chloramine-T method described by Dorval et al. and was stored at -70° C until used.
Add 20 μl of anti-fibronectin
Add 20 μl of 125I-labeled fibronectin
Filter and wash 3x
Dry filter and remove

Fig. 1. Flow diagram of the competition radioimmunofiltration assay for fibronectin.

Fibronectin produced by cultured human trabecular meshwork was assayed by competitive radioimmunoassay using an immunofiltration manifold (V & P Enterprises, San Diego, Calif.). A flow diagram of the assay is depicted in Fig. 1. Fibronectin purified in our laboratory by gelatin affinity column chromatography was used to generate a standard curve. Rabbit anti-human fibronectin serum was obtained from Cappel Laboratories (Cochraneville, Pa.). Goat anti-rabbit immunobeads were obtained from Bio-Rad Laboratories (Richmond, Calif.). In brief, the assay was performed by the addition of 50 μl of tissue culture supernatant dilutions to replicate wells of a VP-107 plate, followed by addition of 20 μl of 125I-labeled fibronectin (50,000 counts per minute/20 μl), 20 μl of anti-fibronectin, and finally 20 μl of immunobeads (goat anti-rabbit IgG). The plate was covered with sealing tape and allowed to incubate at 22°C for 18 hr. The wells were filtered and washed three times with 300 μl of gel buffer and allowed to dry for 5 min. The filter paper discs were then removed and placed in plastic carrier tubes, and the amount of 125I-fibronectin bound to the anti-fibronectin was determined in a Searle automated gamma-counter equipped with a z-module computer to determine the concentration of fibronectin in the tissue culture supernatant.

The presence of laminin in the tissue culture supernatants was determined with an enzyme-linked immunoassay described by Rennard et al. (sheep anti-laminin was generously provided by Drs. P. G. Robey and C. R. Martin of the National Institute of Dental Research, Bethesda, Md.).

Results. Twenty-three eyebank eyes were used to begin 160 cultures. Seventy-one (32%) showed growth and 12 (8%) showed abundant cellular spread. The donors were 11 to 40 years of age. Of those with abundant cellular spread and that underwent passage, seven grew through two passage levels, three grew through three passage levels, and one (24B2) grew through the eighth passage.

The morphologic appearance of the cells in culture was distinct from fibroblasts and corneal endothelial cells and resembled that described by Polansky et al. (see Fig. 2, A). The morphologic appearance of these cells changed with the cell density of the culture (Fig. 2, B), and at confluence most of the cells had the same morphologic appearance regardless of the passage level.

The production of fibronectin in the tissue culture supernatants from four passage levels of trabecular meshwork culture 24B2 is depicted in Fig. 3. Control studies to determine whether our anti-fibronectin serum would detect bovine fibronectin in the tissue culture media indicated that this serum was essentially specific, demonstrating 0.0004% crossreactivity between bovine and human fibronectin. Before these supernatants were sampled, the cultures were confluent and the culture medium was changed at time 0 with 5% FBS in DMEM. The kinetics of fibronectin secretion into medium by all four passage levels demonstrated a characteristic “shift-down” response between 24 and 48 hr after the medium was changed, followed by a “shift-up” between 48 and 96 hr.

Discussion. Our results confirm findings by Polansky et al. and Grierson et al. that human trabecular meshwork cells can be established and passaged in culture. Fibroblasts can contaminate and overgrow explant cultures; however, our trabecular meshwork cultures demonstrated a morphologic appearance distinct from fibroblasts and...
they produced laminin, thus proving they are not fibroblasts. It is important to note that we were able to culture and passage trabecular meshwork cells that had the same growth pattern and morphologic appearance as those described by Polansky et al. However, in our study we were able to achieve this without the specific addition of fibroblast growth factor (FGF) to culture medium. Polansky et al. suggested that although trabecular meshwork cells can be grown in 10% FBS, increasing the concentration to 15% caused change in cellular morphology. In this study 20% FBS was used without changing the characteristic trabecular meshwork cell morphology. We have found that neither purified nor unpurified FGF was necessary for the culture of trabecular meshwork cells from patients up to the age of 40 years. The ability to substitute 20% FBS for FGF makes the culture of human trabecular meshwork cells economically more feasible, since commercial FGF is quite expensive.

Our sensitive radioimmunoassay method for quantitating fibronectin levels allows us to perform kinetic studies on the production, secretion,
and regulation of fibronectin. These cells continued to produce fibronectin after seven passages in culture, and the regulatory mechanisms controlling the secretion of fibronectin remained the same, i.e., the shift-down response. Furthermore, the rate of fibronectin secretion was similar with all passage levels. The classic kinetic shift-down/shift-up response demonstrated in Fig. 3 suggests that the kinetics of soluble fibronectin secretion are related to the nutritional environment of the cell. Consequently, studies on the production of fibronectin must take the nutritional effects on the dynamics of fibronectin secretion into consideration.

From the Veterans Administration Medical Center and the University of California, San Diego, Calif., and Johns Hopkins University Medical School, Baltimore, Md. Submitted for publication Nov. 2, 1981. Reprint requests: David M. Worthen, M.D., Office of Academic Affairs (14), Veterans Administration 810, Vermont Avenue, N.W. Washington, D.C. 20420.

Key words: human trabecular meshwork, tissue culture, fibronectin, laminin

REFERENCES
A technique for obtaining sheets of intact rabbit corneal epithelium. ILENE K. GIP-SON AND STEVEN M. GRILL.

We have developed a technique for obtaining sheets of intact epithelium from rabbit corneas. Nine-millimeter corneal buttons are removed and placed in culture medium containing 1.2 U/ml Dispase II, a bacterial neutral protease. The posterior half of the stroma is removed with forceps. The anterior half is incubated in the Dispase medium for 1 hr at 35°C. The epithelial sheet is then removed by gentle probing with forceps between the epithelium and the stroma. Sheets so obtained have intact basal cells and desmosomes, and the free cell surface of basal cells send out cytoplasmic blebs. The action of the enzyme appears to be at the level of hemidesmosome basement membrane attachment. Polarity of the sheets is easily determined because the cut edges of the sheet curl inward toward the apical surface. These sheets provide excellent viable epithelium for studies of epithelial adhesion and synthesis and pure epithelium for culture. (INVEST OPHTHALMOL VIS SCI 23:269-273, 1982.)

The availability of large, viable sheets of intact corneal epithelium facilitates studies of epithelial adhesion and synthesis and of epithelial-mesenchymal interaction. Such sheets obtained quickly also provide a source of pure epithelium for culture. Previous investigators have prepared epithelial sheets from chick embryos by incubating corneas in trypsin and/or collagenase or EDTA solutions for short periods of time.1, 2 The ease with which one can obtain embryonic chick corneal epithelium probably relates to the absence of hemidesmosomes at these early stages of development.1 Adult mammalian corneal and epithelial epithelium have also been isolated with trypsin3 or collagenase4 solutions or have been obtained by abrasion or keratectomy to produce less adherent epithelium.5 These procedures involve either long incubation periods6 or corneal trauma.4, 5

We report here a simple, rapid procedure for obtaining large sheets of intact corneal epithelium from rabbits. The technique is based on the use of the neutral protease Dispase II to loosen cultured epidermal epithelial sheets from culture dishes.6 Dispase, a bacterial neutral protease from Bacillus polymyxna, has also been used to disperse mammalian cells in tissue culture.7, 8

Methods. New Zealand white rabbits were

Fig. 1. a, Technique for detaching rabbit corneal epithelium from stroma. After 1 hr incubation of cornea in culture medium containing Dispase, a jeweler’s forceps is used to securely hold cut edge of remaining anterior half of stroma. A second jeweler’s forceps held in a (closed) clamped position is used as a probe to gently nudge away and separate the epithelium from the stroma. b, Micrograph of whole rabbit corneal epithelial sheet that was removed from stroma by incubation with the bacterial neutral protease Dispase II. (×7.)