tant is incomplete and is not a consequence of complete failure to distribute opsin to an appropriate site.

Key words: rods mice, opsin, antibodies, immunocytochemistry, electron microscopy

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From the Technion Faculty of Medicine, Haifa, Israel* and Department of Pathology, VA Medical Center, West Haven, Connecticut and Yale Medical School, New Haven, Connecticut. This research was supported by NIH grants EY-845, EY-3239 and the Veterans Administration. Submitted for publication: September 24, 1985. Reprint requests: Dr. Izhak Nir, Technion Faculty of Medicine, P.O. Box 9649, Haifa, Israel.

References

**Immunocytochemical Localization of Fibronectin to the Retinal Pigment Epithelium of the Rat**

Richard M. Pino

Fibronectin is an anionic asialoglycoprotein that is found on a variety of cell types. This study was undertaken in an attempt to localize fibronectin to the rat retinal pigment epithelium with ultrastructural immunocytochemistry. Using Fab-HRP conjugates specific for fibronectin, reaction product was localized on the surface of the apical processes and within the cell to GERL. After treatment of tissue by the biotin-avidin method employing ferritin–avidin, ferritin particles marked the apical processes in a quasi-regular distribution. Tufts of particles were separated by a linear distance of 65–85 nm. Fibronectin was not localized to rod outer segments. Invest Ophthalmol Vis Sci 27:840–844, 1986

Rod outer segments (ROS) are phagocytized and degraded by the retinal pigment epithelium (RPE) in the process of photoreceptor renewal. It is probable that membrane receptors are involved in this process. Thus, a knowledge of membrane-associated substances of the RPE may be important to the understanding of the internalization of photoreceptors in normal animals and the dysfunction seen in retinal degeneration in which RPE phagocytosis of ROS is markedly reduced.

Anionic (negative) domains of glycoproteins and glycolipids of the RPEs glycocalyx have been considered as possible recognition molecules of ROS. Although high levels of anionic sites have been demonstrated on the RPE surface with cationic ferritin, the chemical composition of these sites is not fully known. The binding of cationic ferritin was not altered by prior digestion with a number of enzymes that had been used to remove anionic sites other locations. Subsequent work using lectin-affinity cytochemistry with ferritin markers revealed the presence of sialic acid on the RPE. However, the number of sialic acid sites identified was less than the total number of anionic sites marked by cationic ferritin.

Fibronectin (FN) is an anionic (pl 5.5–6.3) asialoglycoprotein that is found in plasma and on the surfaces of many cell types. Two of the many functions attributed to FN are cell–cell attachment and the opsonization of particles prior to phagocytosis. Recently, chick RPE has been shown to synthesize FN in vitro. FN, as assayed by biochemical methods, has not been
Fig. 1. Electron micrographs of rat retinal pigment epithelium (RPE) treated with an Fab-HRP conjugate specific for fibronectin. Reaction product (arrows) indicative of the localization of fibronectin is uniformly distributed on the apical processes of the RPE (×36,400). Inset. Fibronectin is localized to GERL (arrows) adjacent to the Golgi stacks (G) (×45,700).

identified as a component of the bovine RPE. The present cytochemical study was undertaken to determine if FN is present on the RPE of the rat.

Materials and Methods. Animals: Wistar-Furth male rats (150–250 grams; Harlan-Sprague Dawley; Walkersville, MD) were used in this study. Rats were used in conformance to the ARVO Resolution on the Use of Animals in Research.

Reagents: The IgG fraction of goat anti-human FN serum was purchased from Cappel Laboratories (West Chester, PA). Biotinylated rabbit anti-goat IgG and ferritin–avidin D were obtained from Vector Laboratories (Burlingame, CA), papain from Worthington Enzymes (Freehold, NJ), human fibronectin from Bethesda Research Laboratories (Gaithersburg, MD) and the remaining reagents from Sigma Chemical Co. (St. Louis, MO).

Preparation of Fab-HRP Immunoconjugates: Fab fragments were prepared from the IgG fraction of goat anti-FN and coupled to horseradish peroxidase (HRP) by the general methods as described previously.7

Tissue Preparation: Under ether anesthesia, rat eyes were enucleated and placed in Hanks' balanced salt solution. At the limbus, the anterior segment was dissected from the posterior eye cup and the neural retina was removed from the RPE.1,3 The neural retina and RPE were fixed by immersion (4°C, 1 hr) in 1.25% glutaraldehyde-1% formaldehyde (freshly prepared from p-formaldehyde) in 0.1 M Sorensen's phosphate buffer, pH 7.3. After an overnight buffer wash in 0.2 M phosphate buffer, pH 7.3, the eye cups and neural retina were rinsed (15 min) in 0.15 M glycine in 7.5% sucrose and diced into small pieces.

Fab-HRP Labeling: Eye cup tissue was cut into 40-μm sections with a TC-2 tissue sectioner (Sorvall; Newton, CT). These sections were immersed in 25% sucrose-10% glycerin, frozen in a dry ice–ethanol bath, quickly thawed,7 rinsed in phosphate buffered saline, pH 7.3 containing 2% sucrose (PBS), and treated with the anti-FN Fab-HRP conjugate diluted 1:1 with PBS. After 24–48 hr, the tissue was washed extensively in PBS (3–24 hr) and reacted in the following medium.
**Fig. 2.** Electron micrograph of rat retinal pigment epithelium treated by the biotin-avidin method to localize fibronectin. Clusters of ferritin-avidin particles (arrows) separated by 65-85 nm mark the apical processes (×28,600). Inset. Higher magnification micrograph demonstrating clusters of ferritin-avidin particles (×58,500).

Adjusted to pH 7.0 to reveal conjugate HRP activity: 15 mg 3,3'-diaminobenzidine tetrahydrochloride, 10 ml 0.05 M Tris-HCl, and 0.01% H₂O₂ (final concentration).³

**Biotin-Avidin Method:** Pieces of eye cups and neural retina were treated as follows: (1) the IgG fraction of goat anti-human FN diluted 1:1 with PBS (3 hr), (2) PBS (3 × 20 min), (3) biotinylated anti-goat IgG diluted 1:30 with PBS (2 hr), (4) PBS (3 × 20 min), (5) ferritin-avidin D (0.1 mg/ml PBS) (3 hr). Preliminary studies indicated that the primary antibody used at the 1:1 dilution yielded the most reproducible results.

**Cytochemical Controls:** Controls consisted of incubating human FN with either the Fab-HRP conjugates or with the IgG fraction of anti-human FN prior to the treatment of the tissue.

**Final Processing:** Following the cytochemical incubations, all tissue was postfixed in 2% aqueous osmium tetroxide, dehydrated in 2,2-dimethoxypropane, and embedded in EM-Bed 812 (EM Sciences; Fort Washington, PA).³,⁷ Thin sections were obtained and examined unstained or stained with lead citrate.

**Results.** Reaction product was localized to the apical surface of the RPE in a uniform distribution with the Fab-HRP conjugate (Fig. 1). Intracellular reaction product was localized to GERL (Fig. 1, inset). (GERL is a system of smooth endoplasmic reticulum located near the trans aspect of the Golgi stacks and, in some cells, may perform a function similar to those Golgi elements.⁸ In the RPE, lysosomes and melanosomes are formed from GERL.⁸) Immunostaining by the biotin-avidin method revealed tufts of ferritin particles on the RPE in a quasi-regular arrangement (Fig. 2). These tufts were separated by a linear distance of 65-85 nm.

There was no localization seen in control tissue incubated in either Fab-HRP immunoconjugates or IgG fractions adsorbed with FN (Fig. 3). A few immunopositive sites were localized to the surface of ROS (Fig. 4) in some experiments.

**Discussion.** The results of this immunocytochemical study show that FN is present on the apical surface of the RPE. The absence of FN in the interphotoreceptor matrix indicates that the FN on the RPE is a cellular rather than an adsorbed protein. Furthermore, the localization to GERL shows that the FN is synthesized.
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by the RPE and extends previous findings by other investigators using biochemical methods. In contrast to the heavy labeling of the RPE, only an occasional, sparse, localization was present on the ROS. This could be due to small quantities of FN on ROS membranes. It is likely, however, that the material localized to the ROS was RPE derived and remained attached to the ROS during removal of the neural retina from the underlying tissue.

FN is an elongated, flexible protein that can expand or contract in its local environment. The tufts of ferritin-avidin particles associated with the RPE surface may be indicative of a large number of antibody molecules bound to this elongated protein in addition to the amplification afforded by the biotin-avidin method. The nonrandom distribution is similar to the pattern of staining observed after cationic ferritin labeling at a low pH. The latter has been attributed to sialic acid residues. Since FN is an asialoglycoprotein, the FN sites on the RPE would be distinct from those due to sialic acid. The uniform staining produced by Fab-HRP conjugates may have been the result of single, rather than multiple, binding of the immunoconjugate to antigenic sites and the diffusion of reaction product associated with the cytochemical method.

Two functions ascribed to FN which are pertinent to RPE function are cell-cell attachment and opsonization of particles prior to phagocytosis. FN can interact with some glycosaminoglycans and gangliosides. The RPE FN could interact with the glycocalyx of the ROS, possibly in concert with molecules of the interphotoreceptor matrix, and assist in the attachment of the neural retina to the RPE. This interaction may also include the cytoskeletal elements of the RPE as found for other cell types. Although plasma FN is usually associated with opsonization, cell surface FN has been shown to be required for macrophage function and may be necessary for the normal function of the RPE.

Key words: fibronectin, eye, immunocytochemistry, retinal pigment epithelium, ultrastructure

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Interphotoreceptor Retinoid-Binding Protein in Retinal Rod Cells and Pineal Gland

Mertlyn M. Rodrigues,* Joseph Hackett,* Reginald Gaskins,* Barbara Wiggert,† Ling Lee,† Michael Redmond, and Gerald J. Chader†

Immunoelectron microscopic staining demonstrates Interphotoreceptor Retinoid-Binding Protein (IRBP) in monkey rod cell cytoplasm with virtually none in cone cells. The pineal also contains significant amounts of IRBP demonstrating a similarity of pinealocytes to rod but not cone photoreceptors. Invest Ophthal Mol Vis Sci 27:844-850, 1986

Rods and cones are specialized photoreceptor cells of the retina which mainly function in scotopic (low-light) and photopic (bright-light) vision respectively. Even though a rhodopsin-like photopigment is found as low on the phylogenetic scale as Chlamydomonas, the origins and divergence of the two photoreceptors are not well understood. Likewise, the development and function of the pineal gland in relationship to the visual system is not clear, particularly with regard to the similar nature or origins of pinealocytes and retinal rod or cone cells.

We and others have identified a large, soluble glycoprotein, the Interphotoreceptor Retinoid-Binding Protein (IRBP) that may function as a vitamin A transport vehicle between the neural retina and the retinal pigment epithelium (RPE). It is primarily an extracellular protein of the retinal interphotoreceptor matrix (IPM).1 IRBP is the major soluble protein of the IPM, the only retinoid-binding protein in the sub-retinal space and binds endogenous or exogenously added retinol in a light-dependent manner.2 The main site of cellular concentration and/or origin of this important protein is yet unclear. We have reported previously that the concentration of IRBP is greatly decreased in inherited human retinal degenerations, corresponding to the primary loss of rod photoreceptor cells.3 The synthesis of IRBP by neural retina but not by RPE has been demonstrated in organ cultures of monkey and human retina.4,5 In toto, these studies provide only indirect evidence for the production of IRBP by rod photoreceptor cells. Using immunocytochemical techniques at the light and electron microscopic level, we now report on the selective presence of IRBP in rod photoreceptor cells. Using immunocytochemical techniques at the light and electron microscopic level, we now report on the selective presence of IRBP in rod photoreceptor cells of the primate retina and that little if any IRBP is found in or associated with cone photoreceptor or Müller (glial) cells. Moreover, we also detect IRBP in the pineal gland, demonstrating a new biochemical link between this organ and the retina.

Materials and Methods. Primary antisera to purified monkey IRBP was raised in rabbits and affinity purified using glutaraldehyde crosslinked IRBP-Sepharose immunosorbent.6 The final protein concentration of this preparation was 0.8 mg/ml. Absorption of the antibody was accomplished by adding an excess of purified monkey IRBP to the antibody, incubating at 4°C for 24 hr and removing the antibody–antigen precipitate by centrifugation.

Rhesus monkey (Macaca mulatta) eyes were obtained from 1–3-year-old animals. Immunocytochemical staining of their retinas was performed by light microscopy using indirect immunofluorescent staining and immunoperoxidase staining (avidin–biotin complex, ABC).7 Retinas were either fresh frozen or were fixed in 0.5–1.0% phosphate-buffered glutaraldehyde for up to 2 hr, followed by freezing in liquid nitrogen. Frozen sections (6-μ thick) were fixed in ace-