Immunocytochemical characterisation of proteins secreted by retinal pigment epithelium in retinas of normal and Royal College of Surgeons dystrophic rats

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ABSTRACT

In a previous study, an antigen consisting of proteins secreted by retinal pigment epithelial (RPE) cells was injected into a sheep and the specificity of the resulting antiserum was shown by Western blotting and its effects on retinal development were determined in vitro and in vivo. In the present study, the distribution of these secreted proteins was determined by light microscopy immunocytochemistry in cultured neonatal rat RPE cells and retinas of normal and Royal College of Surgeons (RCS) dystrophic rats and cerebrum of normal adult rats. Immunolabelling for these RPE-secreted proteins was detected in cytoplasmic vesicles surrounding nuclei and within processes of cultured normal and transformed rat RPE. In retinas of late postnatal and adult rats, dense immunostaining was found in the cytoplasm of RPE cells and ganglion cell bodies. In addition to RPE and ganglion cells, scattered photoreceptors within the thin outer nuclear layer and small structures within the debris zone were also densely immunoreactive in retinas of 2-mo-old RCS dystrophic rats. The numbers of immunostained ganglion cells appeared to decrease in retinas of older RCS rats, although the immunoreactivity within the RPE appeared to increase in density. No other neuron within the retina, i.e. bipolar, amacrine or horizontal, was immunoreactive for RPE-secreted proteins. In the cerebral cortex of adult rats, immunoreactivity for RPE-secreted proteins was primarily detected within large perikarya of pyramidal neurons and smaller granule neurons. In conclusion, we report an immunocytochemical analysis of an antiserum raised against secreted proteins of rat RPE. This antiserum recognised proteins within secretory-like vesicles of cultured neonatal normal and transformed rat RPE and showed a specificity for RPE and ganglion cells in normal rat retinas, that appeared to be developmentally regulated, and neuron perikarya in adult rat cerebrum.

Key words: Cerebral cortex; photoreceptor cells.

INTRODUCTION

The development of the retina is thought to be, at least partially, regulated by the retinal pigment epithelium (RPE). This hypothesis is supported by the fact that the RPE exists as a monolayer and is positioned immediately adjacent to and differentiates prior to the sensory retina (Braekevelt & Hollenberg, 1970; Young, 1985). A number of studies have demonstrated that RPE or its conditioned media are responsible for photoreceptor maturation (Hollyfield & Witkovsky, 1974; Sheedlo & Turner, 1995, 1996a, b; Sheedlo et al. 1995a), including outer segment development (Spoerri et al. 1988; Stiemke et al. 1994; Lin et al. 1996). RPE cells also appear to play a role in the proliferation of retinal progenitor cells and the eventual organisation of the retinal layers (Liu et al. 1988; Vollmer & Layer, 1986). The influence of the RPE on retinal development, survival and disease processes, may involve secreted factors. In this regard, RPE cells have been shown to synthesise/secrete several proteins in vitro, such as basic fibroblast growth factor (FGF-2), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β) (Schweigerer et al. 1987; Campochiaro et al. 1988; Bost et al. 1992;
Martin et al. 1992; Takagi et al. 1994). Although there is little evidence of specific novel RPE-factor secretion in vivo, the in vitro studies described above provide the necessary stimulus to investigate such a possibility. In support of this hypothesis, a 50 kDa novel pigment epithelium-derived factor (PEDF) was recently shown to cause differentiation in retinoblastoma cells (Tombran-Tink & Johnson, 1989; Tombran-Tink et al. 1992) and found to be a member of the serine protease inhibitor family (Steele et al. 1992).

Photoreceptor cells in retinas of RCS rats begin to degenerate by apoptosis, a form of programmed cell death (Papermaster & Nir, 1994) by the second postnatal week. By 3–4 mo, most photoreceptor cells have been lost in RCS retinas (LaVail, 1981).

Degeneration of photoreceptor cells in retinas of RCS rats is thought to be directly or indirectly due to dysfunctional RPE cells that have been shown to be defective in phagocytosis of shed rod photoreceptor outer segments (Mullen & LaVail, 1976).

In searching for trophic factors that affect the development of the neural retina, one goal is to explore their possible biological role(s) and therapeutic application that may directly influence the survival and continued development of retinal cells, such as photoreceptors, in healthy and diseased retinas. For example, FGF-2 has been shown to temporarily affect photoreceptor protection in the RCS rat model of inherited retinal dystrophy (Faktorovich et al. 1990) and against light damage in the Fischer rat (Faktorovich et al. 1992). In addition, FGF-2 protected the inner retina from ischaemic injury in rats (Unoki & LaVail, 1994). Recently, factors secreted into a defined medium by a transformed neonatal rat RPE cell line were shown to affect ganglion cell neurite growth, retinal cell survival, development and progenitor cell proliferation and differentiation (Sheedlo & Turner, 1996a, b). Consequently, we have directed our attention towards the isolation and characterisation of proteins secreted by neonatal rat RPE cells that may be responsible for the above described cellular activity in vitro. One step in this regard would be the production of an antiserum against secreted proteins of RPE cells that could then be used to determine its distribution in the neural retina and its effects following vitreal administration.

We have shown that the antiserum and an IgG antibody isolated from this antiserum caused retardation of retinal development in vitro and in vivo, particularly on photoreceptor cell development (Sheedlo et al. 1998). In addition, this antiserum recognised RPE-secreted proteins in a narrow molecular range of 60–70 kDa (Sheedlo et al. 1998). In the present study, an antiserum that cross-reacts with RPE-secreted proteins was used to determine the distribution of these proteins in cultured neonatal rat RPE cells, retinas of normal and RCS dystrophic rats and in the cerebral cortex of normal adult rats.

MATERIALS AND METHODS

Animals

Pigmented Long Evans were purchased from Harlan Sprague Dawley (Indianapolis, IN). Pink-eyed Royal College of Surgeons (RCS) dystrophic rats and nonpigmented Fischer rats were obtained from National Institutes of Health, Bethesda, MD. The animals were housed in an American Association for Accreditation of Laboratory Animal Care (AAALAC) approved facility at the University of North Texas Health Science Center at Fort Worth, TX and handled in accordance with National Institutes of Health and AAALAC guidelines.

RPE cell isolation, secreted protein collection and antiserum production

RPE cells were isolated from 6–8-d-old Long Evans rats using a dispase (Collaborative Research Inc., Bedford, MA) technique (Chang et al. 1991; Young et al. 1994). The isolated RPE cells were grown to confluence in Dulbecco’s minimum essential medium supplemented with F12 nutrient (DMEM/F12, Gibco, Grand Island, NY), 10% fetal bovine serum (FBS, Sigma, St Louis, MO), gentamicin (Sigma) and kanamycin (Sigma). One culture of these cells from 6-d-old rats became spontaneously transformed after the second passage, is currently at 110th passage and are designated tnrRPE (Sheedlo et al. 1996a, b, 1997).

Confluent cultures of normal and transformed neonatal rat RPE cells were grown for 2 d in 5% CO$_2$ at 37 °C in DMEM/F12+ antibiotics. The medium was collected, centrifuged, filter sterilised (0.45 μm, Gelman Sciences, Ann Arbor, MI) and stored at −70 °C (Sheedlo et al. 1992, 1995a; Sheedlo & Turner, 1995). The antigen for antiserum production was secreted proteins, conditioned medium (CM), of transformed RPE cell cultures. CM of cultured tnrRPE cells at 34th passage was concentrated to 1.5 mg protein in a 1.1 ml total volume using centricon-3 concentrators (Amicon Inc., Beverly, MA). This antigen mixed with Freund’s complete adjuvant was injected intradermally, intramuscularly and subcutaneously into a sheep and ~ 100 ml of serum was collected 4 wk later. The injection and
collection were performed at Ferrell Farms Inc. Research Farm (McCloud, OK), which is a USDA registered facility (no. 73-R-101). Preimmune serum was collected by venipuncture from this sheep prior to injection of the antigen (Sheedlo et al. 1998).

**Immunocytochemistry**

Normal and transformed neonatal rat RPE cells were cultured overnight on coverslips, rinsed, then fixed with 4% paraformaldehyde in 0.01 sodium phosphate-buffered saline (PBS), pH 7.4 for 10 min. As a blocker, these cultures were first incubated with 1% normal rabbit serum for 60 min, then with the antiserum diluted 1:500 or 1:1000 in PBS +1% normal rabbit serum (NRS) +0.5% Triton X-100 (Sigma) overnight at 4°C. After PBS rinses, the cultures were incubated with a rabbit antisheep antibody conjugated with fluorescein isothiocyanate (Vector Laboratories, Burlingame, CA) diluted 1:225 for 60 min. After PBS rinses, the cultures were mounted with VectaShield mounting medium (Vector Lab.). The immunostained cultures were examined and photographed with a Nikon Microphot-FXA light microscope equipped with fluorescent optics (Sheedlo & Turner, 1996a, b; Sheedlo et al. 1997).

Retinal sections from 12 and 45-d-old non-pigmented Sprague-Dawley, 12-mo-old Fischer and 10-d-old to 9-mo-old pink-eyed RCS rats were deparaffinised, rehydrated and treated with 0.3% H$_2$O$_2$ to quench endogenous peroxidase activity. After treatment with 1% normal rabbit serum for 60 min, the sections were incubated with antiserum or preimmune serum diluted 1:500 or 1:1000 in PBS +1% NRS +0.5% Triton X-100 (diluent) and, the next day, with a rabbit antisheep horseradish peroxidase conjugate (Vector Lab.) diluted 1:225 in the above described diluent. After 18 h, the sections were treated with 0.025% diaminobenzidine + 0.01% H$_2$O$_2$ in Tris-HCl, pH 7.6 for 2 min. After dehydration and clearing in xylene, the sections were mounted in Permout (Fisher, Fair Lawn, NJ) and photographed using Nomarski optics (Sheedlo & Siegel, 1986; Sheedlo et al. 1989a).

Adult Wistar rats (Spartan Research) were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer.
The cerebra were sectioned coronally, fixed an additional 6 h, then embedded in paraffin. Sections (5 µm) of cerebra were mounted onto glass slides and treated immunocytochemically for RPE-secreted proteins as described above for retinal tissue.

**RESULTS**

RPE-secreted proteins were localised in cultured neonatal rat RPE cells by immunofluorescence. Normal neonatal rat RPE cells in primary cultures showed immunostained cytoplasmic vesicles (Fig. 1A), even in those cells immediately adjacent to heavily pigmented RPE cells (Fig. 1B). In addition, cultured transformed neonatal rat RPE cells at 57th passage exhibited dense immunostaining for the RPE-secreted proteins within cytoplasmic vesicles (Fig. 1C). The tnRPE cells in areas of low density formed processes also contained immunostained vesicles (Fig. 1D). Cell cultures incubated in the preimmune serum at similar concentrations showed no specific staining (not shown).

![Image of immunocytochemical localisation of RPE-secreted proteins in normal nonpigmented rat retinas.](image)

**Fig. 2.** Immunocytochemical localisation of RPE-secreted proteins in normal nonpigmented rat retinas. Retinas of normal rats were exposed to antiserum and processed for peroxidase immunostaining. Little immunoreactivity for RPE-secreted proteins was observed in retinas of 12-d-old Sprague-Dawley rats, primarily in ganglion cells (arrowheads) (A). However, in retinas of 45-d-old Sprague Dawley rats, dense immunolabelling was apparent in the RPE cell layer (arrows) and ganglion cells (arrowheads) (B). In retinas of 12-mo-old Fischer rats, dense immunoreactivity was also observed in the RPE (arrows) and ganglion cells (arrowheads) (C). When preimmune serum replaced the antiserum, no specific immunostaining pattern was shown in retinas of 12-mo-old Fischer rats (D). Viewed at high magnification, immunoreactivity was clearly observed in RPE (arrows) (E) and ganglion cells (arrowheads) (F) of 45-d-old Sprague-Dawley rat retinas. ONL, outer nuclear layer; INL, inner nuclear layer; OS, outer segments; IS, inner segments; IPL, inner plexiform layer. Bar, 100 µm (A–D); 50 µm (E, F).
Immunocytochemical localisation of RPE-secreted proteins in retinas of adult pink-eyed RCS dystrophic rats. In retinas of 2-mo-old RCS rats immunostaining for the RPE-secreted proteins was dense in the RPE (arrows) and ganglion cells (arrowheads) (A). Note also the presence of immunolabelling in scattered putative photoreceptors (arrows) and ganglion cells (arrowheads) (B). The scattered cells with dark nuclei were photoreceptors (large arrows), between the debris zone and INL, in retinas of 2-mo-old RCS rats as shown here in a toluidine-blue stained thick plastic section. The RPE are indicated by the small arrows (C). At high magnification, the staining in the RPE (small arrows) and putative photoreceptors (large arrows) was clearly revealed. Note also that nuclei (arrowheads) of RPE did not immunolabel for these proteins, although the debris zone (DZ) appeared to exhibit a light, diffuse immunoreactivity (D). In retinas of 3-mo-old RCS rats, immunostained putative photoreceptors were few in number, especially when compared with that seen in retinas of 2-mo-old RCS rats, although the RPE (arrows) and ganglion cells (arrowheads) continued to show dense immunoreactivity (E). INL, inner nuclear layer; DZ, debris zone. Bar, 100 µm (A, E); 50 µm (B, C); 25 µm (D).

Immunolocalisation of RPE-secreted proteins was also demonstrated in rodent retinal sections. Immunostaining for RPE-secreted proteins was light in retinas of 12-d-old Sprague-Dawley rats, specifically in the ganglion cells, but was extremely light in the RPE (Fig. 2A). However, dense immunoreactivity was detected within RPE cells of 45-d-old nonpigmented Sprague-Dawley rats, as well as in cell bodies of ganglion cells (Fig. 2B). The density and pattern of immunostaining in retinas of 12-mo-old nonpigmented Fischer rats was quite similar to that observed in retinas of 45-d-old Sprague-Dawley rats (Fig. 2C). When the preimmune sheep serum was substituted for the antiserum, no specific immunolabelling was observed in any of the retinas used in this study (Fig. 2D). When viewed at high magnification, dense immunostaining for the RPE-secreted proteins was clearly detectable in the RPE (Fig. 2E) and the cytoplasm of ganglion cells (Fig. 2F) of 45-d-old Sprague-Dawley rats.

Immunostaining for the RPE-secreted proteins was dense in the RPE and ganglion cells of 2-mo-old RCS rats, as also shown in normal adult retinas (Fig. 3A). However, in contrast to the normal retina, dense immunoreactivity was also observed within the remaining photoreceptors in the thin outer nuclear layer (ONL) in retinas of 2-mo-old RCS rats. Interestingly, densely immunostained bodies were also detected within the debris zone. These structures may represent dislodged photoreceptor cells which are known to migrate into this region following extensive retinal degeneration (Fig. 3B). The irregular distribution of these immunostained cells in the ONL would indicate that they may be a subset of surviving rod photoreceptors, as shown in toluidine blue-stained sections (Fig. 3C). At high magnification, dense immunostaining for the RPE-secreted proteins was clearly apparent in the RPE and associated with bodies in the debris zone (Fig. 3D). In retinas of 3-mo-old RCS rats, immunoreactivity was still observed in RPE and ganglion cells, although immunolabelled photoreceptors were not detected (Fig. 3E), as these latter
Fig. 4. Immunocytochemical characterisation of an antiserum against RPE-secreted proteins in retinas of adult pink-eyed RCS dystrophic rats. In retinas of 4-mo-old RCS rats dense immunoreactivity was observed in the RPE (arrows) and ganglion cells (arrowheads) (A). However, the RPE of 6-mo-old RCS rats exhibited a discontinuous pattern of immunolabelling (arrows) (B), while some densely-labelled, enlarged RPE-like cells appeared to be detached from Bruch membrane (arrowheads) (C). In retinas of 9-mo-old RCS rats, immunoreactivity was still dense in the RPE and, in some areas, an immunolabelled structure (arrow), possibly a neovascular profile with surrounding RPE cells, extended between the RPE and the inner nuclear layer (INL) (D). Note also that few ganglion cells (arrowheads) showed immunostaining. Bar, 100 µm (A, D); 50 µm (B); 25 µm (C).
cells are almost completely degenerated by this time in these retinas.

Retinas of 4-mo-old RCS rats showed a similar distribution and density of immunostaining for RPE-secreted proteins as seen in 3-mo-old RCS rats (Fig. 4A). However, retinas of 6-mo-old RCS rats had patchy regions of the RPE that showed no immunoreactivity for the RPE-secreted proteins. Some immunostained RPE cells appeared enlarged and detached from Bruch’s membrane (Fig. 4 B, C). In addition, in retinas of 9-mo-old RCS rats, immunostained structures, resembling neovascular profiles, projected from the RPE into the inner nuclear layer (Fig. 4D). It appeared that fewer ganglion cells were immunolabelled for RPE-secreted proteins in retinas of 9-mo-old RCS rats than seen at earlier times or in normal adult retinas (compare Fig. 4D with Figs 2C, 3 A, 4A).

In order to determine if proteins recognised by the RPE-secreted protein antiserum were associated with cells and/or cellular elements within other regions of the central nervous system other than the rat retina, the cerebral cortex of adult rats was analysed immunocytochemically. Throughout all layers of the cerebral cortex of adult Wistar rats immunoreactivity for RPE-secreted proteins was detected in perikarya of large neurons, which resembled pyramidal neurons (Fig. 5A). In addition, round profiles, possibly small granules neurons, within the molecular layer of the cerebral cortex were densely immunolabelled for RPE-secreted proteins (Fig. 5 B).

**DISCUSSION**

The antiserum against RPE-secreted proteins described in this study primarily recognised only 3 proteins in rat RPE-CM as determined by Western blotting (Sheedlo et al. 1998). These proteins were also found within secretory-like vesicles in cultured rat RPE cells. Fetal human RPE-CM was shown to contain pigment epithelial-derived factor (PEDF), a 50 kDa trophic factor which stimulated differentiation of a retinoblastoma cell line (Tombran-Tink & Johnson, 1989; Tombran-Tink et al. 1992). However, the message for this factor was recently found to be downregulated as the retina ages (Tombran-Tink et al. 1995). In contrast, proteins recognised by this antiserum gradually increased in staining intensity with age and was most intense in the adult retina. In addition, PEDF was localised using a polyclonal antibody within nuclei and cytoskeletal elements of cultured RPE cells. Neither nuclei nor cytoskeletal components appeared to be immunolabelled for RPE-secreted proteins in any cell or tissue examined in this study. Therefore, for these reasons we conclude that
the RPE-secreted proteins recognised by our antiserum are distinctly different from the PEDF molecule.

From our in vitro results, immunolocalisation of RPE-secreted proteins was anticipated in the RPE of intact retinas, but its presence in ganglion cells was an unexpected finding. In contrast to the peripheral nervous system, neurotrophic factors in the CNS are synthesised predomnately by neurons (Thoenen, 1995). Therefore, the localisation of RPE-secreted proteins in ganglion cell bodies may not be unexpected if it indeed relates to a trophic factor. This prospect appears entirely feasible since ganglion cells rapidly respond by extensive axon regeneration in RPE-CM supplemented retinal explants (Sheedlo & Turner, 1996a, b). The RPE-secreted proteins appear to be developmentally regulated since immunoreactivity for these proteins was most intense in adult tissues, but significantly weaker in neonatal retinas. Similarly, a 61 kDa polypeptide epitope recognised by a monoclonal antibody RET-PE10 is a late-appearing marker of RPE differentiation and was maintained throughout adult life (Neill et al. 1993), but is not found in ganglion cells. A 65 kDa RPE protein has been recently reported, but this protein is not found in cultured RPE cells, is not glycosylated, and thus is not a secreted protein (Hamel et al. 1993).

One of the most intriguing aspects of the regulation of the RPE-secreted proteins recognised by our antiserum is its activity in RCS retinas. In retinas of 2-mo-old RCS rats, a time of extensive retinal degeneration, the intensity of immunoreactivity appeared more pronounced than in normal retinas, especially in the region of the retina normally occupied by surviving photoreceptors. Previous work showed that this region is occupied by opsin-immunostained photoreceptors (Sheedlo et al. 1989a, b). However, immunoreactivity for the RPE-secreted proteins was not detected in immature or fully-differentiated photoreceptors of normal adult rat retinas. The reason for the accumulation of RPE-secreted proteins in dying photoreceptor cells is unknown.

By 9 mo immunostaining for RPE-secreted proteins continued to be dense, but patchy, within RPE cells and it appeared that fewer ganglion cells were immunolabelled. It is well known that RPE cells of RCS rats are defective in phagocytosis of outer segments (Dowling & Sidman, 1962) and possibly other membrane-mediated events and, at later stages, migrate from Bruch’s membrane (Caldwell et al. 1984). An explanation for the apparent increase in the RPE-secreted proteins in RCS remains to be determined. Several proteins have been localised in RCS retinas including glial fibrillary acidic protein (Eisenfeld et al. 1984; Li et al. 1993), opsin, Na, K-ATPase (Sheedlo et al. 1989a, b), carbonic anhydrase and cellular retinaldehyde binding protein (Sheedlo et al. 1995b). None of these proteins or other proteins localised within RCS retinas have a distribution remotely similar to that shown for RPE-secreted proteins recognised by our antiserum.

Interestingly, immunoreactivity for RPE-secreted proteins was found in neuronal perikarya of the adult rat cerebral cortex, which demonstrated that these proteins may not be unique to the retinal ganglion cells and RPE. In this regard, we have shown previously that cultures of neonatal rat astrocytes secrete proteins that have a similar in vitro effect on photoreceptor cells and Müller cells as that shown for RPE-cell factors (Sheedlo et al. 1995a). Thus, astrocyte-secreted factors may play a role in brain development similar to that of RPE-secreted factors in the retina.

A rat RPE-secreted protein was recently isolated and determined to be a member of a trypsin protease inhibitor family using Edman degradation. This protein showed cross-reactivity to the antiserum used in this study by Western blot analysis. Additionally, this rat protein in neonatal rat RPE cells and neural retina has 3 isoforms as determined by reverse transcriptase-polymerase chain reaction (RT-PCR) (Turner et al. 1998).

In conclusion, RPE-secreted proteins recognised by an antiserum were found in RPE and ganglion cells, but not other neurons, in mature normal and degenerating retinas, photoreceptor cells in adult RCS retinas and cultured neonatal rat RPE cells. The cerebral cortex of adult rats showed immunoreactivity in pyramidal and granule neurons. This antiserum will assist in the isolation and ultimate purification of RPE-secreted proteins for biochemical and amino acid sequence analyses.

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REFERENCES


BRAIKEVELT CR, HOLLENBERG MJ (1970) The development of the


