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Extracellular Release of Tissue Plasminogen Activator Is Increased with the Phagocytic Activity of the Retinal Pigment Epithelium

Brenda J. Tripathi, James K. Park, and Ramesh C. Tripathi

Primary confluent cultures of retinal pigment epithelium (RPE) cells harvested from porcine eyes were challenged with latex spheres. The extracellular release of tissue plasminogen activator (t-PA) that was associated with phagocytosis of the beads by the RPE was measured by using enzyme-linked immunosorbent and chromogenic substrate assays. In the presence of serum and as compared to the controls, the phagocytosis of latex spheres produced a 5.5-fold increase in the amount of t-PA released into the ambient medium. Cell lysis was not a significant feature in either treated or control groups. Because the RPE in vivo is exposed to serum through the fenestrated choriocapillaris, and because phagocytosis by the RPE outer segments shed by the photoreceptors contributes to the maintenance of the functional integrity of the neural retina, we suggest that the release of t-PA associated with the phagocytic activity of RPE cells is an important physiologic event. Invest Ophthalmol Vis Sci 30:2470–2473, 1989

The retinal pigment epithelium (RPE) and the photoreceptor cells of the retina are intimately related morphologically and physiologically through the interdigitation of the apical processes of the RPE cells with the outer segments of the rods and cones. One of the major functions of the RPE is the daily phagocytosis of the discs shed by the outer photoreceptor tips during the course of renewal of the photoreceptor membrane.[1] Among the various events associated with phagocytosis in general, one event that has been documented is the extracellular release of lysosomal enzymes, collagenase, and neutral serine proteases by phagocytes such as macrophages, neutrophils, monocytes, and RPE cells.[2–7] These enzymes degrade extracellular macromolecules.[4] It is known that an increase in the activity of a plasminogen activator of undetermined type is associated with the phagocytic activity of macrophages, and that RPE cells possess various macrophagelike characteristics.[8] Therefore, we measured the tissue plasminogen activator (t-PA) that is released into the conditioned culture medium by RPE cells when they are challenged with latex spheres, an organic marker.

From the Department of Ophthalmology and Visual Science, University of Chicago, Chicago, Illinois.

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Reprint requests: Ramesh C. Tripathi, MD, PhD, Eye Research Laboratories, University of Chicago, 939 East 57th Street, Chicago, IL 60637.

Materials and Methods

Cell Culture

Fresh porcine eyes were obtained from a local meat packing firm within 1 hr after the death of the animals. We established cultures of RPE cells by using a modification of the method of Edwards.[9] The eyes were stored at 4°C for 10 hr after enucleation to facilitate separation of the neural retina from the RPE. Under sterile conditions, we excised the anterior segment of the eye by making a circumferential incision at the ora serrata. The vitreous and the neural retina were gently pulled away with forceps, and the exposed RPE was rinsed twice with Hank's balanced salt solution. A sufficient amount of trypsin–EDTA solution (Gibco, Grand Island, NY) to cover two thirds of the RPE area was added and pipetted against the RPE for about 4 min. The cell suspension was then pipetted out and diluted with an equal volume of culture medium consisting of minimum essential medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and a 1% solution of penicillin–streptomycin at a pH of 7.4. The cells were centrifuged, resuspended in culture medium, counted, and plated at a density of approximately 10,000 cells/cm^2 in 100 × 20 mm tissue culture dishes (Becton Dickinson, Lincoln Park, NJ). The culture medium was changed every 2 days.

Latex Bead Preparation

To 100 μl of latex bead suspension (Dow Chemicals, Midland, MI) we added a 1% penicillin–streptomycin solution. After 1 hr of incubation at 25°C, the beads were centrifuged at 200 *g* for 20 min. The supernatant was discarded, and the pellet was resuspended in 10 ml sterile distilled–deionized water and recentrifuged. Finally, the pellet of latex beads was resuspended in 3 ml of culture medium to yield a final concentration of 4×10^7 particles/ml.

Phagocytosis Experiments

Two milliliters of culture medium containing latex beads were added to confluent RPE monolayers. Phagocytosis of latex particles by RPE was monitored by phase-contrast microscopy and time-lapse videomicrography. After 24 hr, during which time more than 90% of the RPE cells were observed to have ingested latex spheres, the culture medium was replaced with fresh medium; medium was replaced daily thereafter. Control cultures received medium without the addition of latex beads. Each sample of conditioned medium was assayed for t-PA levels during a 6-day period. Upon termination of each experiment, the cultured RPE cells were harvested with 0.25% trypsin and counted in a hemocytometer. Because the RPE in vivo is normally exposed to serum products which leak through the fenestrated endothelial lining of the choriocapillaris, two separate experiments were performed. In one of these, culture medium supplemented with 10% fetal calf serum was used, and in the other, serum-free medium was used.

Enzyme-Linked Immunosorbent Assay

The amount of t-PA was quantitated by an enzyme-linked immunosorbent assay (American Diagnostics, New York, NY). To each well of a 96-well microtiter plate (Costar, Cambridge, MA), we added a coating that consisted of 200 μl of goat anti-human t-PA prepared from a 2 mg/ml 0.1 M $NaHCO_3$ stock solution diluted to 1:800. The plates were incubated at 25°C for 3 hr in a moist chamber. The coating was discarded, and wells were washed with saline–Tween-20. Subsequently, 200 μl of the conditioned-medium samples and human t-PA standards were incubated at 25°C in a moist chamber for 18 hr. The samples were removed, and the wells were washed four times with saline–Tween-20. To each well, 200 μl of peroxidase-conjugated anti-t-PA IgG (prepared from a stock solution as a 1:2000 dilution with PBS–Tween) was added and allowed to incubate for 3 hr at 25°C. The conjugate was discarded, and the wells were washed four times with saline–Tween-20. Finally, 200 μl of substrate, 0.4 mg/ml orthophenylenediamine, and 0.01% hydrogen peroxide dissolved in citrate–phosphate buffer, pH 5.0, were added to each well, and the plates were incubated for 30 min in the dark at 25°C. The enzymatic reaction was terminated by the addition of 50 μl of 4.5 M sulfuric acid per well. Absorbances were read on an ELISA reader (Bionetics, Charleston, SC) at 492 nm.

Chromogenic Substrate Assay

Functional PA in the conditioned medium was measured by a chromogenic substrate assay (Helena Laboratories, Beaumont, TX). One-milliliter samples of conditioned medium and of t-PA standards were acidified with 0.5 ml acetate buffer, pH 3.9 and 150 μl of 20% acetic acid. An equal volume of 0.05 M Tris buffer, pH 8.3, containing 0.01% Tween-80 was added to the acidified sample. One hundred microliters of this prepared sample, 100 μl of plasminogen–S-2251 solution (one volume 3.8-mM S-2251, one volume 10-CU/ml plasminogen, and three volumes Tris buffer), and 50 μl of 3.3 mg/ml fibrin fragment solution were mixed and incubated at 37°C for 2.5 hr. Absorbances were read at 405 nm.

Results

The average basal rate of release of t-PA by unchallenged RPE cells in serum-free medium was 0.66 ± 0.04 ng/24 hr/10^6 cells. Cultured RPE cells that were exposed to latex particles in serum-free medium elaborated t-PA at an average rate of 1.30 ± 0.07 ng/24 hr/10^6 cells, beginning within 24 hr after the introduction of latex particles and continuing at this rate during the remainder of the 6-day observation period (Fig. 1). The difference in the rates of t-PA

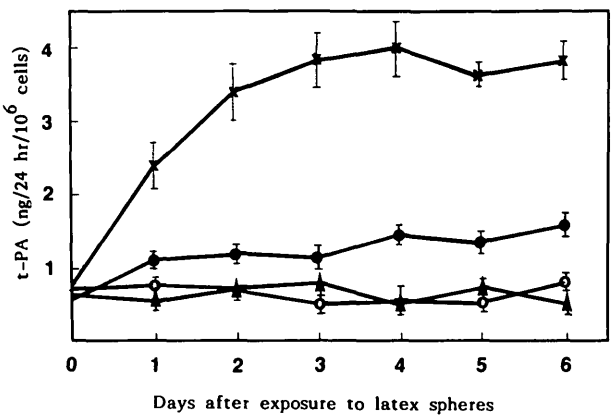

Fig. 1. Measurement of t-PA by ELISA in conditioned medium of cultured RPE cells associated with phagocytosis of latex spheres, with or without serum. Data are presented as mean ± SEM (n = 3). ○, control, without serum; ▲, control, with serum; ●, latex-treated, without serum; ×, latex-treated, with serum.

release by control cultures and by cultures exposed to latex spheres was statistically significant ($P < 0.005$).

Control cultures of RPE maintained in a medium supplemented with 10% fetal calf serum elaborated t-PA at a basal rate of 0.69 ± 0.04 ng/24 hr/10^6 cells. However, RPE cultures exposed to latex spheres in medium containing 10% fetal calf serum demonstrated a significant increase in the extracellular release of t-PA antigen (3.72 ± 0.1 ng/24 hr/10^6 cells, $P < 0.005$) beginning 2 days after the introduction of latex particles (Fig. 1). Concurrently, the PA activity increased from a basal rate of 0.75 IU/ml to 1.75 IU/ml (Fig. 2). Both rates were sustained throughout the additional 4 days of observation. Staining with trypan blue showed no significant cell damage either in the control cultures or in those exposed to latex particles, with or without the addition of serum.

Discussion

Our observation that the secretion of t-PA was increased upon ingestion of latex spheres by RPE in vitro suggests involvement of this serine protease in the process of phagocytosis. This is consistent with earlier findings that a plasminogen activator of undetermined type was released by phagocytosing macrophages, as were other neutral proteases by various other phagocytes.[2,3,7,10] Our finding that exposure of RPE to a combination of serum and latex spheres induced a 5.5-fold increase in t-PA release, as compared to a 2-fold increase with latex spheres alone and as compared to no increase with serum alone, implies a synergistic effect on t-PA release by RPE cultures when phagocytosis of latex spheres occurs in the presence of serum. As revealed by the trypan blue exclusion test, cell lysis could not account for the differences in the rate of extracellular release of t-PA.

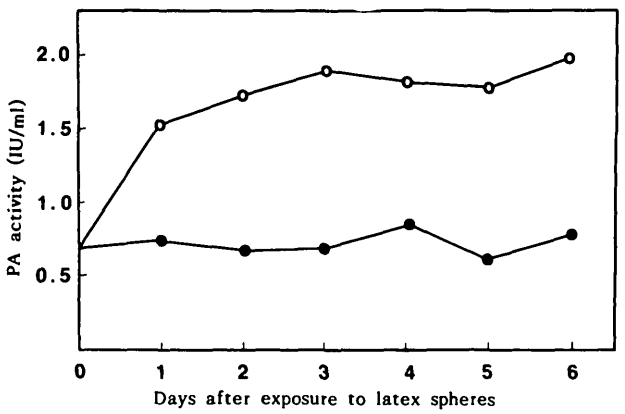

Fig. 2. Chromogenic substrate assay of PA released by RPE cultures associated with phagocytic activity of latex spheres. ●, control cultures exposed to serum alone; ○, cultures exposed to latex-spheres in the presence of serum.

In view of the different specificities of the chromogenic substrate assay and the ELISA method, as well as their independent units of measurement, the results obtained from these two techniques are not directly comparable. The ELISA method detects total t-PA, whereas the chromogenic substrate assay primarily detects free, functionally active PA.[11] Because serum components, plasmin, and PA-inhibitors, which bind t-PA, were present in vitro, it is possible that a higher amount of PA was available than was actually measured by the functional assay. Plasminogen activator–inhibitors are secreted by,[12] and have been detected in, RPE cells.[13]

The appearance of serine proteases within or on the surface of some phagocytes is considered to be a necessary prerequisite for phagocytosis to take place.[14,15] The enzymes released by the phagocytes also have been implicated in mediating the degradation of extracellular macromolecules.[4] Plasminogen, an abundant extracellular zymogen, is converted by t-PA into plasmin, which, either directly or indirectly, promotes the degradation of extracellular matrix components.[16] Plasminogen activators are believed to regulate extracellular proteolysis, including connective-tissue turnover and remodeling, and cell movement and migration.[17] The interphotoreceptor matrix (IPRM) consists of glycoproteins,[18] including fibronectin,[19,20] and of proteoglycans.[21] During renewal of the outer segments, the apical cell processes of the RPE move through the IPRM as the microvilli engulf packets of outer segments. Therefore, it is possible that, in vivo, t-PA is secreted and then degrades various components of the IPRM to facilitate this process. A similar degradation of the extracellular matrix permits the movement and migration of other cells, such as trophoblasts.[16]

The secreted t-PA may also be involved in the degradation of the photoreceptor outer segments. It has been proposed that because cultured RPE cells release acid hydrolases into the culture medium when challenged in vitro with a preparation of the outer segments, some digestion of the shed photoreceptor discs occurs prior to ingestion by the RPE.[22] Trypsin, a broad-spectrum neutral protease which is similar to plasmin and which is homologous in many respects to t-PA and to other serine proteases, has been shown not only to degrade the cone extracellular matrix region of the IPRM,[23] but also to be capable of cleaving rhodopsin.[24] Whether t-PA has a similar role remains to be investigated.

The presence of t-PA within the RPE cells has been shown immunohistochemically.[25] Recently, t-PA was detected also in subretinal fluid from human patients with retinal detachment, and the t-PA–plasmin system was proposed to be involved in proliferative vi-

treoretinopathy.[26] The origin of the t-PA, however, was not investigated. Our present findings that RPE elaborates t-PA in vitro implicates these cells as a possible source of the t-PA that was found in the subretinal fluid.

Key words: enzyme-linked immunosorbent assay, functional assay, interphotoreceptor matrix, tissue culture, t-PA inhibitors

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