Shwu-Jiuan Sheu
Taiji Sakamoto
Roman Osusky
Hsin-Min Wang
Thomas E. Ogden
Stephen J. Ryan
David R. Hinton
Rayudu Gopalakrishna

Abstract • Background: Transforming growth factor-β (TGF-β) plays an important role in the pathogenesis of many ocular diseases, including proliferative vitreoretinopathy. We examined the effect of TGF-β on the phagocytosis of rod outer segments by retinal pigment epithelium (RPE), which is a major function of RPE, and investigated the dependence of this effect on the protein kinase C (PKC) pathway. • Methods: Phagocytic uptake of fluoresceinated bovine rod outer segments was determined by flow cytometry. RPE cells were treated with TGF-β1 or TGF-β2 and their effects on phagocytosis were examined. The effects of various PKC inhibitors (calphostin C, staurosporine, and extended exposure to phorbol 12-myristate 13-acetate, PMA) and a stimulator (brief exposure to PMA) on RPE phagocytosis was evaluated. • Results: Both TGF-β1 and TGF-β2 up-regulated RPE phagocytosis and PMA abolished the up-regulating effect of TGF-β. In contrast, PKC inhibition by staurosporine and calphostin C resulted in increased phagocytosis. A combination of TGF-β and PKC inhibitor treatment did not produce any additive effect on phagocytosis. • Conclusion: We concluded that TGF-β up-regulates human RPE phagocytosis, but that this effect is counteracted by PKC activation. It is possible that this TGF-β-induced effect is due, in part, to a negative modulation of the PKC-dependent pathway.

Introduction

Transforming growth factor-β (TGF-β) comprises a family of multifunctional cytokines, three of which are found in normal ocular tissues of mammals [27, 37]. TGF-β may be involved in diseases such as proliferative vitreoretinopathy (PVR); it has been implicated in pre-retinal proliferation, in retinal pigment epithelium (RPE)-mediated gel contraction, and in intraocular fibrosis [8, 12, 38, 46], and elevated levels of TGF-β in vitreous of eyes with PVR have been reported [8]. By virtue of their position at the blood-retinal barrier, the RPE cells may be crucial for the initiation and propagation of ocular inflammation and proliferative diseases, including PVR [1, 7, 28]. Phagocytosis is a major function of RPE cells and is essential to maintain homeostasis of the microenvironment in the eye. Because TGF-β has been found to modulate phagocytosis of other cell types [15, 31], we wished to test the effect of TGF-β on RPE cell phagocytosis.

RPE cell phagocytosis has been shown previously to be down-regulated by increased protein kinase C (PKC) activity [18]. To determine whether the observed effect of TGF-β on phagocytosis might also involve the PKC
pathway, we studied these effects in the presence of PKC activator and inhibitors.

**Materials and methods**

**Isolation and culture of human RPE cells**

Primary RPE cell cultures were established using a modification of the method of Del Monte and Maumenee [10]. Briefly, the anterior segment, vitreous and neurosensory retina were surgically removed from postmortem eyes obtained from the Lions Doheny Eye Bank. The sagittally bisected posterior segments were rinsed twice with minimal essential medium (MEM; Irvine Scientific, Santa Ana, Calif.). The RPE cells were collected by mechanical scraping with a No. 10 surgical blade and then seeded in six-well culture plates (Falcon Plastics, Oxnard, Calif.) coated with laminin (Sigma, St. Louis, Mo.). The cells were incubated with growth medium [GM; MEM containing 10% fetal calf serum, 1% nonessential amino acid and 1% glutamine/penicillin/streptomycin (Irvine Scientific)] at 37°C in a humidified atmosphere of 95% air/5% CO2. Cells in passages 4–9 were used for these experiments. Identity of RPE cells was confirmed by immunohistochemical staining of cytokeratin [fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-pancytokeratin antibody (Sigma)]. Cells were plated in 24-well tissue culture plates (Falcon) for 5–7 days, until they reached confluence.

**Isolation and labelling of rod outer segments**

Bovine rod outer segments (ROS) were isolated from frozen bovine retina (Pel-Freeze, Mogars, Ark.) according to the method of Papamaster [35]. The ROS were suspended and incubated in the dark for 1 h at 21°C in several milliliters of Hanks balanced salt solution (HBSS; Irvine Scientific) containing 10 μM FITC/ml (Sigma). The FITC-ROS were separated from free FITC by centrifugation at 800 g. The supernatant was discarded and the FITC-ROS pellet was rinsed with HBSS, recentrifuged, and the supernatant again discarded [4]. The FITC-ROS pellet was then suspended in GM. The inoculum level (4 × 10⁶ ROS/ml) was determined by means of a hemocytometer.

**Feeding of ROS**

Each well of confluent RPE explants was inoculated with 700 μl of GM containing 2.8 × 10⁶ ROS and incubated at 37°C for 3 h. Both the ROS and RPE cells were acclimated to the temperature of the incubator for 10 min prior to being combined.

**Stimulation of confluent RPE cells with TGF-β**

Confluent RPE cells were preincubated with human TGF-β1 (0.1, 1.0, or 10 ng/ml; Collaborative Biomed, Bedford, Mass.) and recombinant human TGF-β2 (0.1, 1.0, or 10 ng/ml; Genzyme, Cambridge, Mass.) prepared in GM for 24, 48 or 72 h. Both the ROS and RPE cells were acclimated to the temperature of the incubator for 10 min prior to being combined.

**Treatment of RPE cells with the PKC activator/inhibitor**

Confluent RPE cells were preincubated with 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma) for 15 min, 60 min or 24 h. In other experiments cells were treated with 1 or 10 nM staurosporine (Boehringer-Mannheim, Indianapolis, Ind.) for 1 h with 200 nM calphostin C (LC Service Corp., Woburn, Mass.) for 1 h under light, or with 200 nM calphostin C for 1 h under light followed by 100 nM PMA for 15 min. In addition, cells preincubated with TGF-β1 (1.0 ng/ml for 72 h) were washed three times with PBS, followed by either the PKC activator, 100 nM PMA (15 min) or PKC inhibitors, 10 nM staurosporine (1 h) and 200 nM calphostin C (1 h under light). The limited concentration (<0.5%) of ethanol added to the cells as a vehicle for PKC activator and inhibitors has no effect on RPE phagocytosis.

**Flow cytometry**

The samples were prepared following the protocol described in the literature [29, 47]. The cells were detached using 0.05% trypsin with ethylenediamine tetraacetic acid (Irvine Scientific) for 2–4 min. The external adhering FITC-ROS were largely removed by this procedure [4, 29, 36, 47], as confirmed by fluorescence microscopic examination. Cells were recovered by centrifugation at 1500 rpm for 5 min. The resulting cell pellet was resuspended in PBS and assessed immediately by flow cytometry.

Fluoresceinated ROS uptake was measured using a fluorescence-activated cell sorter (FACStar Plus, Becton Dickinson, Mountain View, Calif.). A 5-W argon laser tuned to 488 nm at 200 mW was used to excite the fluorescein-labelled ROS. Fluorescence emission was collected using a selective 530 ± 15 nm band pass filter. Forward and side light scatter was used to gate the desired scattered events (RPE cells) from dead cells, debris and free FITC-ROS. There were both negative and positive controls in each experiment. The negative control consisted of untreated RPE cells only, while the positive control was untreated RPE cells challenged with fluoresceinated ROS. The data were printed out as a histogram. With the curve from the negative control, we set the gate for each experiment to obtain the percentage of positive phagocytosing cells. Each flow cytometry run consisted of 5000 scattering events. The data were presented as phagocytic index, as modified from previous reports [19, 36, 47], which was calculated as follows: mean fluorescence (total fluorescence/number of cells sorted) multiplied by the percentage of phagocytosing cells. Results were shown as percentage of untreated positive control.

**Statistical methods**

All experiments were performed four times. Analysis of variance was used to evaluate differences for each experimental group. Pairwise comparisons were determined by the least significant difference (LSD) test. Only preplanned comparisons were made to ensure the overall type I error (α = 0.05).

**Results**

**Effect of TGF-β on RPE cell phagocytosis**

TGF-β has been studied widely in various types of cells, including macrophages, B cells, leukemia cells and retinal pigment epithelial cells. Most of the effective doses fell between 0.1 and 10 ng/ml; the effect of TGF-β on cell function was cumulative with time [2, 15, 21, 22, 31, 34, 38, 45]. We selected 0.1, 1.0 and 10 ng/ml for dose-response studies and 24, 48 and 72 h for time-response studies on the effect of TGF-β on human RPE phagocytosis. As shown in Figs. 1 and 2, both TGF-β1 and TGF-β2 increased phagocytosis up to twice that of un-