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LABORATORY INVESTIGATION

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Transforming growth factor-β regulates human retinal pigment epithelial cell phagocytosis by influencing a protein kinase C-dependent pathway

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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: Phagocytotic 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 produced 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 preretinal 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% CO_2. 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 Papermaster [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 (4×10^6 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 2.8×10^6 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. The cells were washed three times with phosphate-buffered saline (PBS) before being fed with ROS.

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-β (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 ($a = 0.05$).

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-

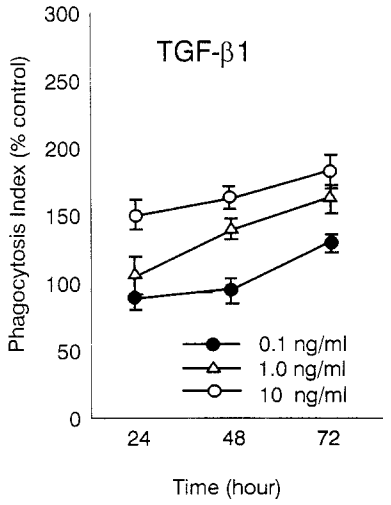

Fig. 1 Up-regulation effect of TGF-β1 on human RPE phagocytosis. Results are expressed as percentage of control obtained with GM alone. Error bars represent SEM ($n=4$). The phagocytosing index is: mean fluorescence (total fluorescence/number of cells sorted) multiplied by percentage of phagocytosing cells

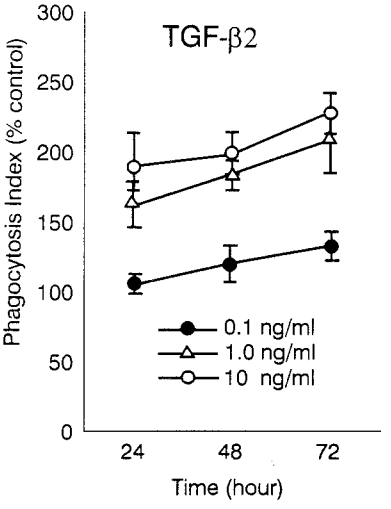

Fig. 2 Up-regulating effect of TGF-β2 on human RPE phagocytosis. Results are expressed as percentage of control obtained with GM alone. Error bars represent SEM ($n=4$)

treated controls. The stimulating effect of TGF-β at a concentration of 0.1 ng/ml was not obvious over the first 48 h. At concentrations of 1.0 and 10 ng/ml, however, the stimulatory effect was seen at 24 h and increased through 72 h. At this point, there was a significant difference between 0.1 and 1.0 ng/ml (TGF-β1, $P=0.0001$; TGF-β2, $P=0.0001$) but not between 1.0 and 10 ng/ml (TGF-β1, $P=0.1516$; TGF-β2, $P=0.056$). Thus, the combination of 1.0 ng/ml and 72 h incubation time was chosen for further experiments. The results also showed that TGF-β2 has a greater effect on RPE phagocytosis than does TGF-β1 ($P=0.0493$) (Figs. 1, 2, 4).

Next, we were interested to find out whether the PKC pathway is involved in mediating the TGF-β effect on RPE cell phagocytosis. Initially, the effects of PKC activators or inhibitors on RPE cell phagocytosis were evaluated to determine the involvement of PKC pathway in this process. This was followed by testing the effect of these PKC modulators on TGF-β-stimulated phagocytosis to gain insight into the mechanism of TGF-β action.

Effect of PKC modulators on RPE cell phagocytosis (basal)

We selected PMA, a known activator of PKC, to test the hypothesis that PKC is involved in RPE phagocytosis. Pretreatment of RPE cells with 100 nM PMA for a brief time (15 min) prior to quantitating ROS phagocytosis resulted in a dramatic (80%) decrease in phagocytosis compared with that of untreated controls (Fig. 3). In contrast, the PMA inhibiting effect was eliminated by prolonging the PMA pretreatment for a longer time (24 h). Thus, the brief PMA treatment, which is known to activate PKC, led to a decrease in phagocytosis, whereas prolonged treatment with PMA, which is known to down-regulate PKC [23], resulted in a lack of PMA inhibitory effect on phagocytosis. This supports the suggestion that PKC activation is a negative modulator of phagocytosis [18].

We selected two structurally unrelated PKC inhibitors to determine whether inhibition of PKC could increase phagocytosis. Calphostin C was selected because it has been reported to be a very specific inhibitor for PKC [25], and has been shown also to irreversibly inactivate PKC by oxidation [13]. Therefore, a single brief exposure of cells to this inhibitor is sufficient to block the PKC effects for a prolonged time (hours); continued exposure to this drug, which could result in unwanted side effects, is not required. Staurosporine was selected for use in these experiments because it is the most potent inhibitor of PKC, although it is somewhat less specific than some other inhibitors [43]. RPE cells were pretreated with 200 nM calphostin C for 1 h, then washed and the ROS phagocytosis determined. As shown in Fig. 3, calphostin C pretreatment increased phagocytosis to 206% that of the untreated controls ($P=0.0001$). RPE cells were pretreated with 10 nM staurosporine for 1 h, and then phagocytosis was measured while the staurosporine remained in the medium. This inhibitor also increased phagocytosis, to 197% that of the untreated controls ($P=0.0001$) (Fig. 3). The finding that phagocytosis was increased by two structurally unrelated inhibitors of PKC suggests that PKC is involved in phagocytosis.

Because PMA treatment for a short time (15 min) decreased phagocytosis, probably because of PKC acti-

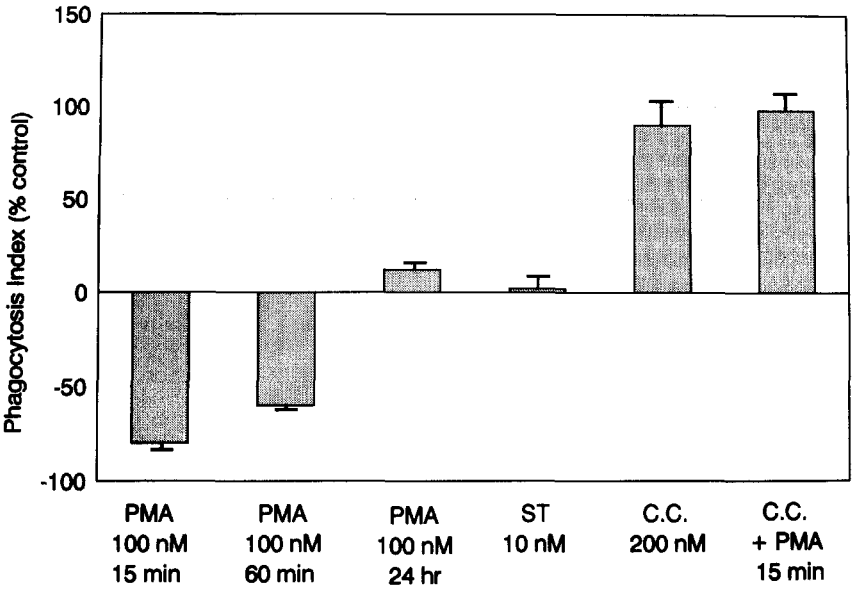

Fig. 3 Effect of PKC activator and inhibitors on human RPE phagocytosis. PMA inhibited phagocytosis after brief incubation, but lost this effect after 24 h. Both staurosporine (10 nM) and calphostin C (200 nM) increased phagocytosis. Calphostin C treatment followed by short time treatment with PMA caused less inhibiting effect that did treatment with PMA alone. *ST* staurosporine, 1 h; *C.C.* calphostin C, 200 nM, 1 h under light; *C.C.*+PMA calphostin C, 200 nM, 1 h under light, followed by PMA, 100 nM, 15 min. Results are expressed as percentage of control obtained with GM alone. Error bars represent SEM ($n=4$)

vation, we investigated whether the inhibition of PKC by calphostin C would block the PMA inhibitory effect. RPE cells were treated initially with 200 nM of calphostin C for 1 h, followed by PMA for 15 min, after which phagocytosis was determined. While the PMA treatment alone decreased phagocytosis by 80%, calphostin C pretreatment resulted in a PMA-induced decrease in phagocytosis of only 30% (Fig. 3). This suggests that a PKC inhibitor can counteract the PKC activator effect on phagocytosis and supports the theory that PKC activation may down-regulate phagocytosis.

Effect of PKC modulators on TGF-β-stimulated phagocytosis

Since PKC activation may decrease phagocytosis, we examined whether PKC activation by PMA could block TGF-β-stimulation of phagocytosis. RPE cells treated with 1.0 ng/ml TGF-β for 72 h were further treated with PMA for 15 min prior to measuring ROS phagocytosis. This pretreatment with PMA abolished the stimulatory effect of TGF-β2 and reduced it for TGF-β1 (Fig. 4). Phagocytosis was still enhanced using TGF-β1 and PMA.

To determine the effect of PKC inhibition on TGF-β action, we tested whether the effects of TGF-β and PKC inhibitors were synergistic. When the TGF-β-treated cells were further treated with either calphostin C or staurosporine, the increase in phagocytosis was no greater than that observed with TGF-β alone (Fig. 4). If the TGF-β-induced increase in phagocytosis were mediated by a PKC-independent mechanism, the combination of TGF-β and PKC inhibitors should produce an additive effect on phagocytosis. The lack of any such additive effect on phagocytosis by the combination of TGF-β and PKC inhibitors suggests that PKC inhibition may play a part in the TGF-β-mediated up-regulation of phagocytosis.

Discussion

Although much work has been done to study the effect of TGF-β on cellular function, to the best of our knowledge this is the first report that TGF-β increases phagocytosis of ROS in cultured human RPE cells. This effect was seen at concentrations comparable to those used in other investigations [2, 15, 21, 22, 31, 34, 38, 45]. We also found that TGF-β2 had a greater effect than did TGF-β1. Many studies have revealed that most of the immunoreactive TGF-β in ocular fluid is TGF-β2 [8, 9, 11, 44]. One possible explanation for the difference between the effects of these two isoforms of TGF-β is that the RPE cell membrane has a higher affinity for TGF-β2 than for TGF-β1, thereby rendering TGF-β2 more effective in up-regulating phagocytosis.

There is little information on the nature of the intracellular signal elicited by the cell surface receptor for TGF-β, although PKC, phosphatidylinositol, guanine nucleotide-binding proteins (G proteins) and c-AMP have been implicated as second messenger systems for TGF-β-mediated biological activity [2, 20, 21]. In some cell types, TGF-β has been reported to increase PKC activity in its signal transduction [30, 32, 39]; however, in human B cells and TGF-β-resistant gastric carcinoma cells, TGF-β was found to decrease PKC activity [2, 21]. In RPE cells, the signal transduction mechanisms involved in TGF-β action are not known. Accordingly, we studied the effect of a PKC activator and

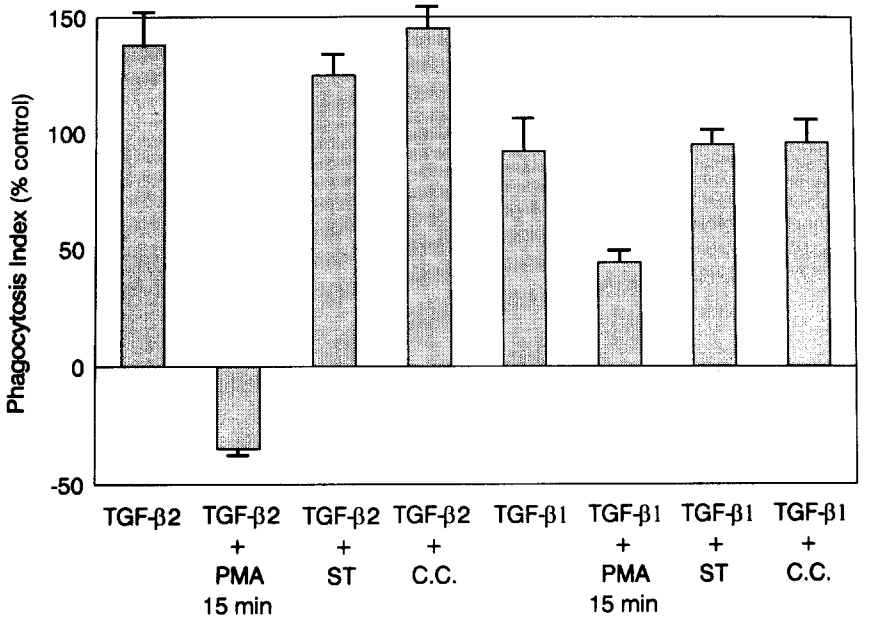

Fig. 4 Interaction of PKC activator/inhibitor with TGF-β (1.0 ng/ml, 72 h). Cells treated with TGF-β followed by brief exposure to PMA (100 nM, 15 min) lost the up-regulating effect of TGF-β. In contrast, neither staurosporine (*ST*) nor calphostin C (*C.C.*) affected the ability of TGF-β to stimulate phagocytosis. *TGF-β*+PMA: TGF-β, 1.0 ng/ml, 72 h followed by 100 nM PMA for 15 min; *TGF-β*+ST: TGF-β, 1.0 ng/ml, 72 h followed by 10 nM staurosporine for 1 h; *TGF-β*+C.C.: TGF-β, 1.0 ng/ml, 72 h followed by 200 nM calphostin C for 1 h under light. Results are expressed as percentage of control obtained with GM alone. Error bars represent SEM ($n=4$). TGF-β1 vs TGF-β1+ST, $P=0.8832$; TGF-β1 vs TGF-β1+C.C., $P=0.8822$; TGF-β2 vs TGF-β2+ST, $P=0.4085$; TGF-β2 vs TGF-β2+C.C., $P=0.8724$

inhibitors on ROS phagocytosis by human RPE cells and their effect on TGF-β-stimulated phagocytosis, in an attempt to determine whether the PKC pathway is involved in TGF-β-mediated up-regulation of human RPE phagocytosis.

In this study, PKC activation by brief treatment with PMA resulted in a decrease in phagocytosis by human RPE cells. Nevertheless, on continuing this PMA treatment for a prolonged time (24 h), this inhibition of phagocytosis disappeared. This may be due to a dual effect of PMA on PKC. PMA causes initial short-term activation of PKC, but subsequently results in depletion of PKC, due to degradation of the enzyme over time [23]. This is consistent with a previous study by Hall et al. [18], which showed that activation of PKC by PMA rapidly inhibited the phagocytosis of ROS by cultured rat RPE cells and that staurosporine could antagonize this effect. In our experiments, treating the human RPE cells with an inactivator (calphostin C) specific for PKC, prior to PMA, lessened significantly the inhibiting effect of PMA. The potent but less specific PKC blocker staurosporine [43] increased phagocytosis. In addition, we found that the more specific PKC inactivator, calphostin C, which can irreversibly inactivate PKC [13], also increased phagocytosis, adding strength to our conclusion that RPE phagocytosis is enhanced by PKC inactivation and suppressed by PKC activation. These results further support the hypothesis that the PKC pathway may negatively modulate RPE phagocytosis [18].

Several studies have suggested that RPE phagocytosis is a receptor-mediated event, and human RPE cells express on their apical surface a mannose receptor that is similar to the macrophage mannose receptor [4, 5, 16, 17, 26, 42]. Roubey et al. [40] reported that, in human neutrophils, binding appeared to be primarily a function of the quantity or density of CR3 expressed on the cell surface, while the ingestion phases of phagocytosis required a qualitative activation of CR3. Binding and ingestion may respond differently to stimuli. Staurosporine inhibited PMA-induced phagocytosis but not EC3bi binding. A similar finding reported by Hall et al. [18] was that increased PKC activity turned off ROS ingestion, but not binding, in cultured rat RPE cells. Since PKC has been found to functionally down-regulate the receptor of epidermal growth factor (EGF) as well as the T3/T-cell antigen receptor complex of human T lymphocytes, preventing further stimulation by antigen to proliferation [6, 23, 41], we postulate that PKC might change the character of the receptor to negatively modulate ingestion of ROS by human RPE cells.

In our study, the TGF-β-induced up-regulation of phagocytosis in human RPE cells could be almost completely abolished by brief pretreatment of cells with PMA, which is known to activate PKC. Since the PKC inhibitors calphostin C and staurosporine increased phagocytosis, TGF-β-stimulated phagocytosis may in-

volve a down-regulation of the PKC-mediated pathway. Lack of an additive effect in stimulating phagocytosis by a combination of TGF-β and PKC inhibitors further suggests that negative modulation of the PKC-dependent pathway may be involved in the action of TGF-β.

Studies on phagocytic cells other than RPE (monocytes, neutrophils, macrophages and gingival fibroblasts) showed that PKC activation stimulates, rather than suppresses, phagocytosis [3, 14, 24, 33, 48], while TGF-β was found to decrease phagocytosis by mouse myelomonocytic leukemia cells and neutrophils [15, 31]. The discrepancy between these reports may be due to a difference in receptors, second messenger systems, phagocytic mechanisms or distribution of PKC isoenzymes in different cell types.

Characterization of the RPE PKC isoenzyme profile and direct measurement of PKC isoenzyme activities from cell membrane, cytosol and phagosome will be required to directly demonstrate the extent of involvement of the PKC pathway in TGF-β-induced modulation of RPE phagocytosis. An understanding of cytokine effects on RPE function may ultimately form the basis for intervention in diseases involving altered RPE function or proliferation.

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