Promotion of Adhesion and Migration of RPE Cells to Provisional Extracellular Matrices by TNF-α

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PURPOSE. Adhesion and migration of retinal pigment epithelial (RPE) cells to provisional extracellular matrices (ECM) is important in the development of epiretinal membranes found in proliferative vitreoretinopathy (PVR). Tumor necrosis factor alpha (TNF-α) is found in PVR membranes and regulates many functions of RPE cells. In this study, the effects of TNF-α on adhesion and migration of RPE cells to various components of ECM were examined and elucidation of the mechanism of the response was attempted.

METHODS. Mitogen activated protein kinase (ERK1/2; MAPK) activation was measured by immunoblot. RPE cells pretreated with TNF-α (10 ng/ml) or TNF-α + PD98059 (a specific inhibitor of MAPK, 30 μM) for 24 hours were compared with control RPE. Attachment was measured by modified MTT assay on fibronectin and collagen types I and IV. Spreading was measured by staining with fluo3-AM and confocal laser scanning microscopy. Migration of RPE cells on substrates was determined by Boyden chamber assay using PDGF-BB (20 ng/ml) as a chemotactic factor. Integrin expression was determined by flow cytometry and RT-PCR.

RESULTS. TNF-α rapidly activated MAPK and increased the extent of attachment, spreading and migration on fibronectin and collagen type I (P < 0.01) but not on collagen type IV. TNF-stimulated RPE cells showed increased mRNA and surface protein expression for α1 and α5 integrin (P < 0.01) but not α3 integrin subunit. Neutralizing the anti-α1 antibody inhibited migration on collagen type I, whereas α5 antibody inhibited fibronectin-induced migration. Treatment with both TNF and PD98059 reduced attachment and migration on provisional ECM and reduced the upregulated integrin expression to control levels.

CONCLUSIONS. After treatment with TNF-α, there is increased expression of specific integrins associated with increased adhesion and migration on provisional ECM (fibronectin and collagen type I). This effect is mediated, at least in part, by activation of MAPK signaling pathway. (Invest Ophthalmol Vis Sci. 2000;41:4324–4332)

Proliferative vitreoretinopathy (PVR) is a well-recognized complication of serious ocular trauma and rhegmatogenous retinal detachment.1 After chorio-retinal injuries, the entry of serum components and inflammatory cells into the subretinal space may expose retinal pigment epithelial (RPE) cells to a variety of cytokines, resulting in RPE cell activation and separation from the monolayer. Activated RPE cells proliferate and migrate through a provisional extracellular matrix (ECM) within the subretinal space and through retinal holes to form pathologic membranes on both surfaces of the neural retina.2 Contraction of the pathologic epiretinal membrane may lead to retinal detachment and blindness.3 In the PVR epiretinal membrane, RPE cells predominate, but fibroblasts, macrophages, and glial cells are also found.9 An understanding of the mechanisms involved in the migration of RPE cells from the monolayer through the ECM and how this is regulated by the cytokine environment would provide insight into the pathophysiology of PVR.

The provisional ECM found in early stages of PVR is synthesized locally by several cell types including RPE cells and is composed primarily of fibronectin and collagen type I.1,4 In contrast, normally quiescent RPE cells of the intact monolayer sit on a basement membrane layer that is primarily composed of type IV collagen and laminin on the inner surface of Bruch’s membrane.5 RPE cells interact with the ECM by way of integrins. Alterations in integrin expression have been shown in a variety of proliferative disorders and states including cancer,6 wound healing,7,8 and angiogenesis.9 Increased expression of certain integrins including α1, α2, α4, α5, α6, β1, β2, and β310,11 subunits have been detected in specimens of human PVR membranes.

Regulation of integrin expression is strongly influenced by cytokines, including those found in PVR membranes.3 One of the most prominent of these cytokines is tumor necrosis factor-alpha (TNF-α) whose mRNA and proteins are widely expressed in PVR membranes.12 TNF-α is often derived from activated macrophages although in PVR membranes RPE and glial cells may also be a source.15

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TNF-α stimulates cells by activating one or both of the two known surface receptors designated as TNF-α receptor (TNFR) type I (p55) and type II (p75). We have previously demonstrated the presence of p55 mRNA and protein and the relative absence of p75 in primary cultured RPE cells. Activation of the TNFR leads to a cascade of events resulting in the activation of protein kinase A, protein kinase C, mitogen-activated protein kinase (MAPK), and ceramide-dependent protein kinase pathways. One of the major subfamilies of the MAPK family is the extracellular regulating kinases (ERK1 and ERK2). We have previously shown that these MAPK are critical in the proliferation and migration response of RPE to growth factors such as platelet-derived growth factor (PDGF). Because the MAPK signaling pathway has been shown to be especially critical in the interaction of activated cells with ECM, we were interested to determine its role in mediating RPE/ECM interaction in response to TNF-α.

We hypothesized that TNF-α plays an important role in altering the attachment and migration of RPE cells to favor provisional ECM molecules through changes in integrin expression. Such interaction is hypothesized to occur, in part, through MAPK signaling as part of generalized activation response of RPE cells.

**Materials and Methods**

**Cells and Cytokines**

RPE cells were isolated from human eyes obtained from the Doheny Lions Eye Bank and cultured in Dulbecco’s minimal Eagle’s medium (DMEM; Fisher Scientific, Pittsburgh, PA) with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma, St. Louis, MO), and 10% heat inactivated fetal bovine serum (FBS; Irvine Scientific, Santa Ana, CA) as previously described. Second- to fifth-passage cells grown to confluence for 48 to 72 hours were used for these experiments. Cells were pretreated 24 hours with TNF-α (10 ng/ml; Boehringer Mannheim, Indianapolis, IN) in DMEM with 0.4% FBS in the absence or presence of the MAPK-specific inhibitor PD98059 (30 µM; New England Biolabs, Beverly, MA).

**Statistical Analysis**

All experiments were repeated three times and compared using the paired Student’s t-test; the level of confidence for statistical significance was determined to be \( P < 0.01 \).

**Immunoblot Assay**

Confluent cells grown in six-well plates were starved for 48 hours in DMEM with 0.1% bovine serum albumin and then incubated for 10 minutes with 10% FBS or with serum-free medium containing TNF-α (10 ng/ml), TNF + PD98059 (30 µM), or TNF + calphostin C (100 nM). Cells were lysed, supernatants were collected, and proteins were resolved on Tris-HCl 7.5% polyacrylamide gels (Ready Gel; Bio-Rad, Hercules, CA) at 120 V. The proteins were transferred to PVDF blotting membrane (Millipore, Bedford, MA), and the membranes were probed with polyclonal antibody specific for the dually phosphorylated forms of p42/44 MAPK (at amino acids Thr202 and Tyr204, 1:3000 dilution; New England Biolabs) followed by chemiluminescent detection (Amersham Pharmacia Biotech, Cleveland, OH).

**Adherence Assay**

**Attachment.** The attachment assay was carried out using fibronectin-coated or collagen type I- or IV-coated 96-well plates (Becton Dickinson Labware, Bedford, MA). Confluent RPE cells (10^5/ml) were trypsinized and resuspended in DMEM with 0.4% FBS. One hundred microliters of cell suspension (10^4 cells) was added to each well and allowed to attach for 60 minutes. The cells were washed gently with PBS twice, and fresh medium (150 µl) was added to each well with MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide, 5 mg/ml, 20 µl; Sigma). After 5 hours of incubation, the supernatants were decanted, and the formazan precipitates were solubilized by the addition of 150 µl of 100% DMSO (Sigma) and placed on a plate shaker for 10 minutes. Absorbance at 550 nm was determined on a Dynatech MR 600 microplate reader (Chantilly, VA). Living cell number was proportional to the absorbance of MTT at 550 nm.

**Spreading.** The spreading assay was performed on eight-well chamber slides coated with fibronectin or collagen type I or IV (Becton Dickinson Labware). RPE cells were plated on the chamber slides and attached for 30 minutes in a 37°C incubator. The slides were washed with PBS, then stained with 6 μm Fluoro-AM (Molecular Probes, Eugene, OR) in fresh medium for 35 minutes at 37°C and for 15 minutes at room temperature. The dye stains the cell cytoplasm and facilitates estimation of cell spreading. Cells were washed with PBS, and the slides were mounted. A confocal scanning laser microscope (Zeiss, Thornwood, NY) was used to determine the surface area of cells at the substrate–cell interface by optical section. Quantitation was performed by measuring the contact area of each cell using microplan II software (Donsanto Corp, Natick, MA).

**Attachment Strength.** A modified, centrifugal force–based adhesion assay was used. RPE cells were plated to fibronectin or collagen type I or IV in coated, 96-well plates at 10^4/well, attached for 60 minutes, and then gently washed with PBS. Plates were inverted and centrifuged in the swinging bucket microtiter plate holder of the centrifuge at 500g, 1000g, and 2000g for 2 minutes, respectively. The remaining cells then were incubated for 5 hours at 37°C with fresh medium containing MTT, and absorbance at 550 nm was measured as described above.

**Migration Assay**

Migration was measured by using a modified Boyden chamber assay in 24-well plates in which inserts were coated with fibronectin or collagen type I or IV (2 µg/cm²), and platelet-derived growth factor (PDGF, 20 ng/ml, Boehringer Mannheim) was used as the chemoattractant in DMEM containing 0.4% FBS. After 5 hours’ incubation, the inserts were washed three times with PBS, fixed with cold (4°C) methanol for 10 minutes, and counterstained with hematoxylin for 20 minutes. The number of migrated cells was counted using phase-contrast microscopy (×320). Four randomly chosen fields were counted per insert.

**Antiadhesion monoclonal antibodies (α1, α3, α5; Chemicon, Temecula, CA) were used in the assay. After cells were trypsinized and resuspended in the medium, antibodies were added (1:100) and incubated for 30 minutes at 37°C. An irrelevant antibody served as control. The migration assay was done as above.
grown in serum-free media showed weak activation of ERK1/2 (Fig. 1). RPE cells grown in serum-free media demonstrated only weak ERK1/2 phosphorylation (Fig. 1, lanes 1 and 2). Treatment with 10% FBS as a positive control resulted in strong MAPK activation (Fig. 1, lane 3). After 10 minutes of TNF stimulation, prominent phosphorylation of ERK1/2 (Fig. 1, lanes 4 and 5) was present, which was completely inhibited by pretreatment with PD98059 (lanes 8 and 9). Pretreatment with Calphostin C resulted in only partial inhibition of the TNF response (lane 6, 7).

Collagen and Fibronectin Receptor Expression

Flow Cytometry. Trypsinized RPE cells were resuspended in PBS with 5% goat serum (for reduction of nonspecific staining) for 10 minutes at room temperature. Anti-integrin monoclonal antibodies (1/100 dilution, Chemicon) were added to the solution along with 0.4% FBS. After incubation for 1 hour at 4°C, RPE cells were washed with PBS. Secondary antibodies (anti-mouse IgG-R-phycocerythrin conjugate; Sigma) were added for 50 minutes at 4°C and washed with PBS. After washing, RPE cells were then fixed with 1% paraformaldehyde for 10 minutes. Five thousand cells were used for flow cytometry analysis and mean channel fluorescence was determined with a Becton Dickinson FACScan using Consort 3.0 software.

Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR). Poly(A) + RNA was isolated from control, TNF-treated for 24 hours, and PD98059 + TNF-treated RPE cells using a Fast Track Kit (Invitrogen, San Diego, CA), and 35 cycles of PCR were performed as previously described.15 The oligonucleotide sequences for α1 primers are as follows: 5’GCTCTCTTCTCTGCGTTG3’, and 5’TGGATTCTCGGTAGTTTGCG3’; for α5, 5’CTTCAACTGTTATACCCGAA3’ and 5’TCCAGCTCTGTCTGTCG3’; and for αs, 5’GATCGAATTCGGCATCTTCAGGGCT TTGTACACA3’ and 5’TGGTTCTCCGTTAGT-TCCTTTCTTGCTGTGT3’. PCR products were diluted in denaturing solution (0.4 N NaOH, 25 mM EDTA), neutralized with Tris-HCl (1.0 M; pH 8.0), and transferred to 0.45-μm Nytran membranes (Schleicher & Schuell, Keene, NH) using a MiniNet I dot blot apparatus (Schleicher & Schuell). Membranes were hybridized at 58°C overnight with [32P]ATP-labeled internal oligonucleotide probes, α1: 5’TGTGTTCTGACGTGACGCCCCACATTTCAAGT3’, α5: 5’ACTCAGTCGCCCCACA- AGGATGCTGAGGG3’, αs: 5’ACTTTCTCGATGCTGACGT- GACTTCTTTA3’, and β-actin: 5’CACGGCATGTCACCCGTTGAG3’. Membranes were washed three times with 2× SSC at room temperature, exposed to phosphor screens (Molecular Dynamics, Sunnyvale, CA), and scanned using a phosphorimaging scanner (Molecular Dynamics). Radioactive signals of integrin cDNA were quantified and normalized to the housekeeping β-actin values to adjust for differences in loading.30

Quantification of PCR Products. PCR products were isolated with phenol:chloroform extraction and ethanol precipitation and then resuspended in water. The amount of each PCR product was determined using a Bio-Rad microplate reader. The OD reading was proportional to the absorbance at 550 nm. TNF-α increases attachment, spreading, and attachment strength

RPE cell attachment was measured on collagen type I, collagen type IV, and fibronectin (Fig. 2). After 60 minutes’ incubation, the RPE cells showed similar degree of attachment to provisional ECM (fibronectin and collagen type I) as well as to the normal basement membrane collagen type IV. When RPE cells were pretreated with TNF-α, there was a marked statistically significant (P < 0.01) increase in substrate attachment to either fibronectin or collagen type; however, no significant changes in attachment to collagen type IV were detected. Pretreatment of TNF-stimulated RPE cells with PD98059 significantly decreased the attachment on all three substrates (P < 0.01) compared with TNF-stimulation alone and resulted in levels of attachment that were not statistically different from cells without TNF stimulation.

To further characterize changes in attachment, strength of attachment was measured on fibronectin and collagen types I and IV by using a centrifugal force–based adhesion assay (Fig. 3). In this assay, increasing centrifugal force results in decreased attachment. To isolate the effect of attachment strength from the baseline extent of attachment, we determined the number of cells that remained attached at each centrifugal force level. We observed that attachment of RPE cells to collagen I was significantly increased by 10% FBS treatment (P < 0.01) compared with TNF-stimulation alone and resulted in levels of attachment that were not statistically different from cells without TNF stimulation.
centrifugal point as a percentage of cells attached at zero gravity on each substrate. TNF-treated cells showed a significant ($P < 0.01$) increase in the strength of binding at 1000g on fibronectin and collagen type I compared to control RPE cells at 1000g of centrifugal force compared to control RPE cells ($A$ and $B$, $P < 0.01$). No significant change was seen on collagen type IV ($C$).

Spreading of RPE was measured on fibronectin and collagen types I and IV (Fig. 4). After 60 minutes of attachment, no significant difference in the spreading of control RPE cells on all three ECM-coated chamber slides was found. TNF-α–treated RPE cells showed significantly increased cell spreading compared with control cells when grown on fibronectin and collagen type I ($P < 0.01$). Quantification was performed by measuring the contact area of each cell using microplan II software (Donsato Corp.). No increased spreading of TNF-pretreated RPE was found when they were grown on collagen type IV (Fig. 4A). Previous reports suggested that spreading of fibroblasts is mediated through protein kinase C (PKC). Pretreatment with the PKC inhibitor calphostin C markedly inhibited TNF-induced spreading on fibronectin (Fig. 4B). PKC activation may then result in downstream activation of ERK1/2 MAPK pathway or may phosphorylate other non-MAPK targets. Pretreatment of RPE cells with TNF + PD98059 did not decrease the TNF-increased spreading on fibronectin (Fig. 4B), suggesting that spreading is regulated by the PKC pathway independent of MAPK activation.

![Figure 3](image_url)  
**Figure 3.** TNF-α increases attachment strength on fibronectin and collagen I. The attachment strength on fibronectin and collagen types I and IV was measured by centrifugal force-based adhesion assay. The number of cells attached at each centrifugal force point was compared with the number of similarly treated cells attached at zero gravity, and the value was expressed as a percentage. TNF-α (10 ng/ml) pretreated RPE cells showed significantly increased strength of attachment to fibronectin and collagen type I at 1000g of centrifugal force compared to control RPE cells ($A$ and $B$, $P < 0.01$). No significant change was seen on collagen type IV ($C$).

![Figure 4](image_url)  
**Figure 4.** TNF-α increases spreading. Flou3-AM (6 μm/ml) was used to stain RPE cell cytoplasm and to facilitate quantitation of cell spreading on fibronectin, collagen type I, and collagen type IV. The cell spreading of control RPE showed only slight variation on all three substrates. Spreading of TNF-pretreated RPE cells was significantly increased on fibronectin ($P < 0.01$) and collagen type I ($P < 0.01$) compared with control RPE cells, but no change was seen on collagen type IV (A). A demonstration of confocal images of Flou3-AM–stained RPE grown on fibronectin is shown (B). TNF-stimulated cells (b) showed significant increased spreading compared to control cells (a). Pretreatment of TNF-stimulated cells with PD98059 (20 μm) resulted in no significant difference from TNF alone (c). Pretreatment with the PKC inhibitor calphostin C (100 μm) markedly inhibited TNF-induced spreading (d).
TNF-\(\alpha\) Increases Migration

PDGF is a potent chemotactic factor for RPE cells and is commonly found in PVR membranes.\(^3\) For control cells, migration is significantly increased when PDGF is added to the lower compartment of a Boyden chamber in which the intervening membrane is coated with collagen type I, collagen type IV, or fibronectin (\(P < 0.01\), Fig. 5). When RPE are treated with TNF-\(\alpha\) for 24 hours before seeding them in the upper chamber, there was only a modest increase in the number of migrating cells through the three ECMs in the absence of chemotactic stimulus by PDGF. In contrast, after 24 hours' pretreatment of RPE with TNF-\(\alpha\), the migration induced by PDGF placed in the lower compartment of the Boyden chamber was markedly increased compared with control RPE cells grown on fibronectin (\(P < 0.01\)) and collagen type I (\(P < 0.01\)). This effect of TNF pretreatment on PDGF chemotaxis was not seen when cells were grown on collagen type IV (Fig. 5). Pretreatment of RPE cells with TNF + PD98059 significantly inhibited (\(P < 0.01\)) the TNF enhancement of migration in response to PDGF on fibronectin and collagen type I and resulted in levels of migration that were not significantly different from PDGF alone.

Integrin Expression

Flow Cytometry. Integrins are critical for cell-substrate interactions and may be differentially regulated by TNF-\(\alpha\). Flow cytometry was used to measure expression of integrins with distinct substrate affinities including collagen 1 (\(\alpha 1\)), collagen IV (\(\alpha 3\)), and fibronectin (\(\alpha 5\)). After pretreatment of RPE cells with TNF-\(\alpha\) for 24 hours, cells exhibited significantly increased surface staining for \(\alpha 1\) (\(P < 0.01\)) and \(\alpha 5\) (\(P < 0.01\)) integrin subunits (Fig. 6) but no significant change for \(\alpha 2\) and \(\alpha 3\) (data not shown). TNF-induced \(\alpha 1\) and \(\alpha 5\) integrin expression was suppressed to control levels by pretreatment with PD98059 (\(P < 0.001\), Fig. 6).

Semiquantitative RT-PCR. The \(\alpha 1\), \(\alpha 3\), and \(\alpha 5\) integrin mRNA expression by control and TNF-stimulated RPE (Fig. 7A)
was examined by RT-PCR. TNF-α significantly increased α5 and α1 integrin expression (Fig. 7A, lanes 3 and 9), whereas PD98059 inhibited this TNF effect (Fig. 7A, lanes 2 and 8).

Integrin Blocking Assay. To support the contention that TNF-α pretreatment increased RPE migration by regulating expression of integrin receptors, we used antiadhesion antibodies against α5 integrin (migration on fibronectin), α1 integrin (migration on collagen type I), and α3 integrin (migration on collagen type IV) (Fig. 8). Migration of RPE cells was markedly inhibited by α5 antibody on fibronectin, α1 antibody on collagen type I, and α3 antibody on collagen type IV. There was no inhibition by an irrelevant antibody (data not shown). After treatment with TNF-α, the inhibition of migration on fibronectin by antibody against α5 integrin was most prominent, suggesting that the increased migration caused by TNF-α is associated with increased α5 integrin expression.

DISCUSSION

The quiescent RPE cell rests on a collagen type IV and laminin containing basement membrane on the inner aspect of Bruch’s membrane, and it does not normally divide or migrate away from this layer. Although in this report we chose to study collagen type IV as the representative Bruch’s membrane component, it is likely that laminins, as the major noncollagenous components of Bruch’s membrane, also play an important role in RPE adhesion to the collagenous framework of Bruch’s membrane. In PVR, RPE cells are stimulated by inflammatory cytokines and chemotactic factors, such as TNF-α and PDGF, to migrate away from monolayer into a provisional ECM where they participate in epiretinal membrane formation. In the early stage of PVR, provisional ECM components including collagen type I and fibronectin are synthesized and deposited on the retinal surfaces. Movement of an individual cell from a resting ECM substrate to a provisional ECM requires initial cell attachment to the new matrix, followed by cell spreading, stable attachment, and then migration. In the absence of TNF-α, control RPE do not demonstrate any significant pref-
erence for provisional ECM, providing little incentive for migration from the monolayer. In the presence of TNF-α, however, RPE cells attach more strongly and spread more prominently than untreated control cells on provisional ECMs, but this effect does not occur when cells are grown on collagen type IV. These TNF effects are likely to be cell type- and ECM-specific because previous studies have shown that on collagen, TNF stimulates B-cell spreading but inhibits fibroblast spreading.

After RPE cells establish adhesion, migration through a retinal hole into the vitreous is a critical stage of PVR formation. Previously, we reported that PDGF is a strong chemotactic factor for RPE cells in the presence of fibronectin. In this study we provided evidence that pretreatment of RPE with TNF-α results in a significant increase in the ability of PDGF to stimulate RPE cell migration on provisional ECM. This suggests that TNF-α is upregulating a factor important for PDGF-induced migration because short-term treatment of RPE with TNF has little effect on RPE migration (results not shown). Chemotactic migration is a complex phenomenon involving adhesion to ECM, cell motility, and the effects of chemotactic factors. Spreading also plays a critical role in migration with particular relevance to the speed of migration. Multiple mechanisms are involved in control of the migratory response including growth factor receptor activation of downstream signaling pathways, alterations in cytoskeletal elements and motors, and alterations in the expression and activation of integrins. In other cell types, TNF-α stimulation is associated with activation of small GTP-binding proteins, phosphorylation of paxillin and focal adhesion kinase, and reorganization of the actin cytoskeleton.

Integrins are a class of heterodimeric transmembrane proteins that play essential roles in many cellular processes involving cell-ECM and cell-cell interactions. We found that unstimulated RPE cells expressed α1, α3, and α5 integrin subunits and that expression was strongest for α5. The α1 and α3 integrin subunits are primarily used for attachment to collagens, whereas α5 provides attachment to fibronectin. After treatment with TNF-α, the pattern of integrin expression was dramatically modified. The RPE cells showed a significant increase in α1 and α5 subunits but not in α3 subunit. This predicts and supports our finding that TNF-α-treated cells show increased affinity to fibronectin and collagen 1 but not collagen 4, based on the receptor specificity. An increase in integrin expression in other cell types stimulated by TNF-α is associated with increased cell migration in neutrophils, monocytes, and fibroblasts. The subunit partner of the alpha integrins is generally the β1 subunit, which has a unique distribution in the basal domain of RPE cells and participates in RPE cell migration during PVR.

The specific roles of integrins in RPE cell migration were confirmed by blocking experiments with antiadhesion antibodies for α1, α3, and α5. Those antibodies only partially inhibited migration on specific ECMs, which suggests that migration on fibronectin and collagen may be dependent on the simultaneous activity of several integrins or a coexistent nonintegrin adhesion molecule or receptor. The inhibition of migration by antibodies to integrins demonstrates that RPE cell motility is strongly integrin-dependent on the substrates used. In fact, a disintegrin (Arg-Gly-Asp containing peptides from viper venom) was effective in suppressing RPE cell-induced tractional retinal detachment in the rabbit eye.

Because TNF-α plays a critical role in regulating integrin expression, resulting in increased attachment, spreading, and migration to provisional ECM, we investigated the mechanisms by which TNF-α may alter RPE cell surface integrin expression. Although TNF-α has been shown to activate a number of second- and third-messenger pathways in various human cell types, translation of these findings to our model is difficult because signaling mechanisms are highly dependent on the cell type. Because RPE cells predominantly express p55 TNFR, it is a good model to use for investigating signaling mechanisms activated through this receptor. To determine whether MAPK might influence integrin expression, we used an inhibitor (PD98059) that specifically inhibits ERK kinase (MEK). We found that blocking the MAPK pathway by PD98059 inhibited TNF-induced α1 and α5 integrin expression. TNF-α has been shown to activate MAPK by two pathways, a ras-raf-mek pathway and a ras-independent pathway mediated through PKC. In contrast to MAPK inhibition, PKC inhibition using calphostin C did not inhibit TNF-induced integrin expression (data not shown). Although PKC was found to be important in cell spreading, PKC-independent MAPK activation appears to be a more significant pathway in TNF activation of RPE cells. In conjunction with the role of MAPK in adhesion and migration, the MAPK pathway appears to be a central regulating pathway in the relationship of TNF-stimulated RPE to the ECM.

The downstream substrates of activated MAPK are largely unknown but include a variety of nuclear and cytoplasmic targets such as transcription factors, cytoskeletal elements, inflammatory mediators, and other Ser/Thr kinases. Although it is not clear which of these targets is involved in the upregulation of integrins, myosin light chain kinase (MLCK) has been implicated as a possible target of MAPK activation in cell migration.

Thus, TNF-α is a major regulator of RPE activation responses, including cell attachment, spreading, chemotaxis, and migration. This regulation appears to be mediated through differential expression of distinct integrins that determine the matrix attachment of RPE to Bruch’s membrane or provisional matrices during diseases. The MAPK pathway plays a critical role in the regulated expression of these integrins and in the downstream activation of RPE by TNF. These results suggest that both TNF-α and MAPK may be potential therapeutic targets in disorders such as PVR in which cytokine activation of RPE plays a central role.

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