Integrin- α_5 Mediates Epidermal Growth Factor-Induced Retinal Pigment Epithelial Cell Proliferation and Migration

Zhen Chen^a Chang-zheng Chen^a Wen-rong Gong^b Jun-ping Li^{c, d}
Yi-qiao Xing^a

^aDepartment of Ophthalmology, and ^bCentral Laboratory, Renmin Hospital, Wuhan University, Wuhan, China;

^cDepartment of Ophthalmology, VA Medical Center, Salem, Mass., and ^dDepartment of Ophthalmology, University of Virginia, Charlottesville, Va., USA

Key Words

Epidermal growth factor • Retinal pigment epithelial cells • Integrin- α_5 • Proliferation • Migration

Abstract

Proliferation and migration of retinal pigment epithelial (RPE) cells play a crucial role in proliferative vitreoretinopathy (PVR)-related pathology. Cytokines, including EGF, can result in RPE cell activation and cause PVR. In this study, integrin- α_5 expression was first studied in PVR membranes by immunofluorescence. Then the effect of EGF on integrin- α_5 expression was determined by flow cytometry, Western blot analysis and the reverse-transcription polymerase chain reaction (RT-PCR) in the ARPE-19 cell line. Proliferation and migration of ARPE-19 cells were measured by the methylthiazolyldiphenyl-tetrazolium bromide and Boyden chamber assays. We found that a higher level integrin- α_5 was present at the RPE cell surface in PVR compared with normal retina. EGF could dose dependently increase integrin- α_5 mRNA and protein levels in vitro. EGF promoted ARPE-19 cell proliferation and migration. Neutralizing integrin- α_5 by specific anti-integrin- α_5 antibody abolished most of the effects of EGF. The study provided evidence that EGF might influence PVR by promoting integrin- α_5 expression and subsequent proliferation and migration of RPE.

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Introduction

Proliferative vitreoretinopathy (PVR) is a serious complication of major ocular trauma and rhegmatogenous retinal detachment. Retinal pigment epithelial (RPE) cells are the predominant and critical cell type in PVR membranes [1]. Studies on cultured RPE cells have shown that they attach to and spread on individual matrix molecules, such as collagen [2], laminin [3, 4] and fibronectin (FN) [5]. In early stages of PVR, the provisional extracellular matrix (ECM) is synthesized locally by several cell types including RPE cells [6]. RPE cells interact with the ECM, migrate and proliferate.

Integrins mediate interactions between cells and the ECM [7, 8]. Changes in integrin expression have been shown in a variety of proliferative disorders including cancer [9], wound healing [10], and angiogenesis [11]. Integrins are heterodimers consisting of variable α - and β -subunits. The different combinations of these subunits determine the receptor specificity [12]. The $\alpha_5\beta_1$ -integrin mediates specific binding to FN [13] in a number of cell types, including leukemic cells [14], mammary carcinoma cells [15], aortic endothelial cells [16], fibroblasts [17], and RPE cells [18]. Cell spreading and proliferation are inhibited in α_5 -deficient normal rat kidney fibroblasts [19]. Mammary carcinoma cell proliferation is inhibited by antibodies to $\alpha_5\beta_1$ [15].

Table 1. Clinical characteristics of PVR membranes

Patient No.	Age, years	Sex	Diagnosis	Associated condition
1	39	M	PVR epiretinal membrane	retinal detachment after ocular trauma
2	45	M	PVR epiretinal membrane	retinal detachment, huge hole
3	43	M	PVR epiretinal membrane	rhegmatogenous retinal detachment
4	61	M	PVR epiretinal membrane	retinal detachment after ocular trauma
5	41	F	PVR epiretinal membrane	rhegmatogenous retinal detachment
6	60	M	PVR epiretinal membrane	retinal detachment, huge hole
7	55	F	PVR epiretinal membrane	rhegmatogenous retinal detachment
8	31	F	PVR subretinal membrane	retinal detachment after ocular trauma
9	33	M	PVR subretinal membrane	recurrent retinal detachment
10	51	M	PVR epiretinal membrane	recurrent retinal detachment
11	50	M	PVR subretinal membrane	recurrent retinal detachment
12	48	F	PVR epiretinal membrane	retinal detachment after ocular trauma
13	60	F	PVR epiretinal membrane	recurrent retinal detachment
14	41	M	PVR subretinal membrane	rhegmatogenous retinal detachment

Regulation of integrin expression is strongly influenced by cytokines, including those that are present in PVR membranes [20]. Endothelial growth factor (EGF) has been found in the vitreous, subretinal fluid and PVR membranes in PVR patients [21]. In a previous study, EGF was shown to promote RPE cell proliferation in vitro [22]. All these findings indicate that EGF may play an important role in PVR formation. However, the molecular basis for EGF effects in PVR membranes remains to be determined.

In this study, we first examined the expression of integrin- α_5 in PVR membranes and normal retina. Then, the effects of EGF on the expression of integrin- α_5 , cell proliferation and migration were investigated in cultured RPE cells.

Materials and Methods

Immunofluorescence Staining of Human PVR and Control Retina Samples

Retina samples from 14 patients with PVR were collected from January to September of 2008 in the Department of Ophthalmology, Renmin Hospital, Wuhan University. Among them, 9 cases were males (9 eyes), 5 cases were females (5 eyes). The average age was 47 years. Clinical histories are given in table 1. Six retina specimens were obtained from the normal eyes of cornea transplant donors and served as controls. None of the donors had a known history of eye disease. Among them, 4 cases were males (4 eyes), 2 cases were females (2 eyes). Age range: 28–51 years, mean = 40 years. This research was conducted in accordance with the tenets of the Declaration of Helsinki, and was approved by the Ethics Committee of the Wuhan University School of Medicine. In-

formed consent was obtained from all subjects before the research. The specimens were fixed with 4% paraformaldehyde in PBS for 24 h at 4°C. Following incubation in 20% sucrose for 2 h at room temperature and 30% sucrose for 24 h at 4°C, the specimens were embedded at the optimal cutting temperature in a compound containing 3% glycerol and frozen in liquid nitrogen. Sections of 4–6 μ m were cut and stored at –86°C pending use. For staining, sections were first incubated for 20 min at room temperature in 95% ethanol, and then treated with 0.25% potassium permanganate for 15 min at room temperature to reduce autofluorescence arising from lipofuscin present in the RPE cells. Sections were blocked overnight at 4°C in 5% goat serum (Pierce, USA), then incubated for 90 min at 37°C with mouse anti-integrin- α_5 monoclonal antibody (Chemicon, USA), or with normal mouse serum (Sigma) as negative controls, both at 1:100 dilution. Sections were washed and blocked again in 5% goat serum for 60 min at 37°C, and incubated with secondary antibody (anti-mouse IgG-labeled with FITC, Pierce) at 1:100 dilution for 90 min at 37°C. Sections were subjected to sequential excitation with the 488-nm line and examined with a fluorescent microscope (BX-60, Olympus, Japan).

Cell Culture

Human RPE cells, ARPE-19 cell line, were purchased from American Type Culture Collection (ATCC, USA). The cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) (Gibco, USA) with 10% fetal bovine serum (FBS, Gibco). The medium was changed every 3–4 days and cultures were maintained in an incubator at 37°C in 5% CO₂/95% air atmosphere.

Total RNA Isolation and Reverse-Transcription Polymerase Chain Reaction

For testing the effects of EGF, 2×10^5 ARPE-19 cells were incubated in 6-well plates. Cells were first cultured in DMEM/F12 with 0.1% FBS for 48 h. Then the medium was removed and cells were washed three times with PBS, cultured again in DMEM/F12

without serum. At that time point, the EGF (Cytolab, USA, dissolved in DMEM/F12) was administered. The final concentrations of EGF in the wells were 0, 0.1, 1, 10, 20 and 100 ng/ml, respectively.

Tri-Reagent (Sigma) was used to extract total cellular RNA at 12, 24 and 48 h according to the manufacturer's instructions. One microgram of total RNA was used in the synthesis of first-strand complementary DNA (cDNA) with avian myoblastoma virus reverse transcriptase and oligo(dT) primers using standard protocols. The reverse-transcription polymerase chain reaction (RT-PCR) for integrin- α_5 was carried out at 94°C for 30 s, at 60°C for 30 s and finally at 72°C for 30 s in a total of 30 cycles. Reagent boxes (Boshide, China) were used in RT-PCR. In order to assess the level of integrin- α_5 expression semi-quantitatively, β -actin was coamplified as the internal reference. The primers used for integrin- α_5 were: forward 5'-GGCAGCTATGGCGTCCCACTGTGG-3', reverse 5'-GGCATCAGAGGTGGCTGGAGGCTT-3' (171-bp PCR product). The primers used for β -actin were: forward 5'-TGGATGACGATATCGCTGCTCG-3', reverse 5'-GGTGCTCCTCAGGGGCCACACG-3' (456-bp PCR product). Bands were analyzed with the GelDoc2000 gel documentation system and the QuantityOne 1-D image analysis software (Bio-Rad Laboratories). The ratio of integrin- α_5 mRNA to β -actin mRNA was calculated.

Western Blot Analysis of Integrin- α_5 Protein Expression

After 24 h of culture as described above, the ARPE-19 cells were washed twice in PBS, lysed in 200 μ l of ice-cold lysis buffer containing 150 mM NaCl, 2% SDS, 5 mM EDTA, and 20 mM Tris-HCl (pH 7.5) and centrifuged at 4°C for 10 min at 10,000 g. Cell lysates were mixed with sample buffer and heated for 5 min at 95°C. They were then resolved by SDS-PAGE, transferred to nitrocellulose filters by electroblot, and probed with monoclonal antibodies (diluted 1:500, mouse anti-human integrin- α_5 monoclonal antibodies, Chemicon) directed against integrin- α_5 . The primary antibody was detected with a horseradish peroxidase-conjugated goat anti-mouse secondary antibody. Enhanced chemiluminescence substrate was used to detect the secondary antibody according to the manufacturer's instructions, and the membrane was placed against autoradiographic film (Hyperfilm ECL; Amersham, France). Monoclonal antibody directed against GAPDH was used as an internal standard for checking protein loading.

Flow Cytometry Analysis of Integrin- α_5 Protein Expression

The ARPE-19 cells were harvested and suspended in PBS, and cell density was adjusted to 5×10^6 /ml. Two hundred microliters of cell suspension were transferred to a 5-ml sterile tube. The cells were cultured with 5% goat serum for 10 min at room temperature. Then the first antibody (diluted 1:100) was added and the cells were incubated for 1 h at 4°C followed by washing with PBS. Normal mouse serum (diluted 1:100, Sigma) was used for negative controls. The second antibody (anti-mouse IgG FITC conjugate, diluted 1:50, Pierce) was added and cells were incubated under the same conditions for 1 h; incubation was followed by washing with PBS. The cells were fixed for 10 min with 1% paraformaldehyde and observed by flow cytometry (Elite Esp FACScan, Beckman Coulter, USA). 5,000 cells were tested for each of the groups. The data were obtained and analyzed by using FCSEXPRESS 3.0 software. The difference of integrin- α_5 expression was analyzed according to the average cell fluorescence intensity.

ARPE-19 Cell Proliferation Experiment

(Methylthiazolyldiphenyl-Tetrazolium Bromide Assay)

ARPE-19 cells were inoculated in 96-well plates at the density of 5×10^4 /ml; 200 μ l cell suspension was applied to each well. The cells were cultured in DMEM/F12 medium with 0.1% FBS for 48 h. After removing the medium, the cells were washed with PBS three times. Then cells were divided into 4 groups. Empty control group: DMEM/F12 alone; EGF group: DMEM/F12 + 10 ng/ml EGF; α_5 -antibody group: DMEM/F12 + 10 ng/ml EGF + mouse anti-human integrin- α_5 monoclonal antibody (1:100); irrelevant-antibody group: DMEM/F12 + 10 ng/ml EGF + irrelevant antibody (mouse anti-human vimentin monoclonal antibody, 1:100, Chemicon). After 24 h culture as indicated for each group, cells were incubated for another 4 h after adding 20 μ l/well methylthiazolyldiphenyl-tetrazolium bromide (MTT, Sigma). Following removal of the supernatant, 150 μ l dimethylsulfoxide (Sigma) was added into each well and the plate was oscillated for 15 min. Aperture stop absorption (optical density, OD) was measured colorimetrically at 490 nm (EL-800, Biotek, USA).

ARPE-19 Cell Migration Experiment (Boyden Chamber)

The migration of ARPE-19 cells was detected in a Boyden chamber (Millipore, USA). The experimental groups were the same as in the cell proliferation experiment. Cells were harvested by trypsinization. In the cell suspension, cell density was adjusted to 1×10^6 /ml with DMEM/F12. DMEM/F12 with 10% FBS was added into the lower chamber of the Boyden chamber, and then covered with 8- μ m polycarbonate micropore membrane. 800 μ l of cell suspension were added into the upper Boyden chamber and conventionally cultured for 5 h. After 5 h of culture, cells on the upper side of the polycarbonate membrane were wiped off with cotton; the cells on the lower side of the polycarbonate membrane were fixed in formalin and then stained with Giemsa. The 5 random fields of the polycarbonate membrane were observed under a 400 $\times$ magnification optical microscope and cells were counted. These cells represented the cells with increased migration ability.

Statistical Analysis

All experiments were repeated three times. Statistical analysis software SPSS11.5 was used. Values are means $\pm$ SEM. One-way ANOVA was used to analyze the overall data of every group, and the least significant difference (LSD) t test was used to analyze multicomparison of every group at the same time. The level of confidence for statistical significance was determined to be $p < 0.05$.

Results

Integrin- α_5 Expression Was Increased in Human PVR Membranes

We used an immunofluorescence assay to investigate the expression of integrin- α_5 in PVR membranes and normal retina. The expression of integrin- α_5 was detected (green fluorescent signal) in all 14 samples of PVR membrane and in 6 samples of normal retina. However,

Fig. 1. Immunofluorescence of integrin- α_5 in PVR membranes and normal retina. **a** Light-microscopic structure of a PVR membrane. HE. $\times 100$. **b** Mouse anti-integrin- α_5 monoclonal antibody in a PVR membrane showing strong fluorescence signal (arrows). **c** Negative control in a PVR membrane: normal mouse serum immunofluorescence does not show any specific signal. **d** Light-microscopic structure of normal retina. Mono cell layer of RPE (arrows). GLC = Ganglion cell layer; INL = inner nuclear layer; ONL = outer nuclear layer. HE. $\times 200$. **e** Mouse anti-integrin- α_5 monoclonal antibody in normal retina emitting a weak fluorescence signal on the surface of the neighboring Bruch's membrane. **f** Negative control for normal retina: normal mouse serum does not show any specific signal. Scale bar: 10 μ m.

Fig. 2. Effect of EGF on integrin- α_5 mRNA expression in ARPE-19 cells. Semiquantitative PCR: β -actin mRNA was coamplified as internal control and the ratios between integrin- α_5 mRNA and β -actin mRNA were compared. **a** No effect of EGF is detected af-

ter 12 h. **b, c** After 24 h and 48 h, increased integrin- α_5 mRNA is observed with increasing EGF concentrations; the effect peaks at 10–100 ng/ml EGF concentration (* $p < 0.01$ vs. 0, 0.1 and 1 ng/ml concentration EGF groups).

Fig. 3. Integrin- α_5 protein expression induced by EGF. **a** Representative Western blots for integrin- α_5 and GAPDH protein in ARPE-19 cells treated with different concentrations of EGF in 24 h. EGF induced integrin- α_5 protein expression from 1 to 100 ng/ml. The peak effect appears at concentrations ranging from

10 to 100 ng/ml. **b** Integrin- α_5 protein expression induced by different concentrations of EGF was determined by flow cytometry. Integrin- α_5 expression is dramatically increased in the groups treated at concentrations ranging from 10 to 100 ng/ml EGF in 24 h (* $p < 0.01$ vs. 0, 0.1 and 1 ng/ml EGF).

the staining for integrin- α_5 was strongly positive on the surface of RPE cells in the full thickness of the PVR membrane (fig. 1b). The expression of integrin- α_5 was only evident in the RPE monolayer in control samples, in which the staining was primarily concentrated along the basal surface of the cells neighboring Bruch's membrane with weak green fluorescence signals (fig. 1d).

EGF Increased Integrin- α_5 mRNA and Protein Expression in Cultured ARPE-19 Cells

Since we demonstrated increased expression of integrin- α_5 in the presumed RPE cells in PVR membrane in vivo, we next tried to determine whether EGF induces integrin- α_5 expression in RPE cell in vitro. Cultured ARPE-19 cells were treated with EGF and then integrin- α_5 mRNA expression was determined semiquantitatively by using RT-PCR. β -Actin mRNA was coamplified as the internal control and the ratio between integrin- α_5 mRNA and β -actin mRNA was compared. After 12 h, there was no significant effect of EGF on the expression of integrin- α_5 mRNA in ARPE-19 cells (fig. 2a). However, increased expression of integrin- α_5 mRNA was detected after stimulation with EGF for 24 and 48 h (fig. 2b, c). After 24 h, the effect of EGF on integrin- α_5 mRNA expression started at 1 ng/ml of EGF (0.94 ± 0.08) and peaked at 10, 20 and 100 ng/ml (2.04 ± 0.15 , 1.99 ± 0.17 , 2.05 ± 0.48) ($p < 0.01$). Compared with the control group (0.56 ± 0.05), there was no significant effect with 0.1 ng/ml of EGF (0.58 ± 0.06) ($p > 0.05$).

Consistent with the expression of integrin- α_5 mRNA described above, the integrin- α_5 protein level in ARPE-19 cells was also significantly enhanced by EGF treatment for 24 h as evidenced by Western blot analysis (fig.

3a) and flow cytometry (fig. 3b). In flow cytometry, stronger fluorescence intensity of integrin- α_5 was observed in groups treated with 10 ng/ml (3.98 ± 0.67), 20 ng/ml (3.37 ± 0.63) and 100 ng/ml (3.53 ± 0.79) of EGF, compared with the control (1.34 ± 0.22) and the group treated with 0.1 ng/ml (1.41 ± 0.37); the differences were statistically significant ($p < 0.01$).

EGF-Induced Proliferation and Migration of ARPE-19 Cells Was Largely Abolished by Addition of Antibody to Integrin- α_5

EGF increased the proliferation of ARPE-19 cells. Compared with the control group (0.297 ± 0.024), significant proliferation of ARPE-19 cells was found in the EGF group (0.508 ± 0.084) ($p < 0.01$). The use of mouse anti-human integrin- α_5 monoclonal antibody to neutralize integrin- α_5 abolished most of the EGF effects on cell proliferation ($p < 0.01$). However, there was still a statistically significant difference between the α_5 -antibody group (0.380 ± 0.056) and the control group ($p < 0.05$). In the irrelevant-antibody control group (0.486 ± 0.051), the effect of EGF on cell proliferation remained (fig. 4a).

The effects of EGF on migration of ARPE-19 cells were tested by the Boyden chamber assay (fig. 4b, c). When the cells were stimulated with EGF, the number of migration cells was increased in the EGF (25.6 ± 3.8) and irrelevant-antibody (24.4 ± 4.1) groups. Compared with these two groups, the number of migration cells was significantly lower in the control (5.7 ± 1.4) and α_5 -antibody (13.4 ± 2.7) groups ($p < 0.01$). Migrated cells were decreased by integrin- α_5 antibody ($p < 0.01$). However, there was still a statistically significant difference between the control and the α_5 -antibody groups ($p < 0.05$).

Fig. 4. Effects of EGF on ARPE-19 cell proliferation and migration. **a, b** Compared with controls, EGF promotes ARPE-19 cell proliferation and migration (* $p < 0.01$). When integrin- α_5 is blocked by neutralizing antibody, most effects are abolished (* $p < 0.01$). Irrelevant antibody does not influence these ef-

fects. However, there remains a statistically significant difference between the α_5 -antibody group and the control group (** $p < 0.05$). **c** Result of Boyden chamber assay: the migrated ARPE-19 cells are stained blue (arrow). Scale bar: 100 μ m.

Discussion

Previous studies [23, 24] demonstrated a lack of integrin- α_5 expression on the RPE cell surface of human and chicken normal retinal tissue. But recent work revealed that there was some integrin- α_5 expression on the normal retina RPE cell surface [25]. Robbins et al. [26] showed positive integrin- α_5 expression on RPE in PVR. FN, a special ligand of integrin- α_5 , was also found in PVR [27]. These findings suggested that integrin- α_5 /FN may be involved in the formation of PVR [28]. Our results in the normal retina showed the existence of integrin- α_5 on the RPE cell surfaces of normal retinae confining to the vicinity of Bruch's membrane surface. The expression of integrin- α_5 was relatively weak in normal retinal tissue, but strong in PVR membranes. This suggested that integrin- α_5 might play an important role in this condition.

The severity of PVR is closely associated with the role of growth factors [29]. EGF is secreted by epithelial cells. Intracellular tyrosine is activated by the binding of EGF receptor with its ligand – EGF [30]. In vitro, EGF pro-

motes adhesion and migration of human cutaneous squamous carcinoma cells and keratinocytes by inducing the expression of integrin- α_2 [31, 32]. In this study, we demonstrated that EGF induced integrin- α_5 expression at both mRNA and protein levels in the ARPE-19 cells. The most effective concentration was 10 ng/ml; a further increase in concentration had no additional effects.

RPE cells proliferate and form contraction membranes on both surfaces of the neural retina. It is an important pathology of the PVR [33, 34]. In the pathological condition of rhegmatogenous retinal detachment, RPE cells migrate and form multi-layered cells. This is the primary and key process in the development of PVR. Integrin signaling results in actin cytoskeleton polymerization, which in turn regulates cell shape and motility [35]. Without migration, RPE cells will not gain access to the vitreous. It was shown previously that monoclonal antibody inhibition of integrin- β_1 or - α_1 inhibited RPE cell migration [12, 18]. Our study further demonstrated that in the case of EGF-induced ARPE-19 cell proliferation and migration, integrin- α_5 mediated this process. Neutralizing integrin- α_5 by specific antibody could

abolish most of the effects of EGF on cell proliferation and migration. Identification of the role of integrin- α_5 may help to develop selective methods for the inhibition of PVR.

Compared with the integrin- α_5 antibody group, EGF effects on cell proliferation and migration were still visible in the irrelevant-antibody group. This indicates that integrin- α_5 expression was not the sole reason why EGF could cause cell proliferation and migration. Further studies are needed to determine the other pathways through which EGF exerts its effect on the proliferation and migration of the ARPE-19 cells.

Conclusion

Our results indicate that EGF may induce RPE cell proliferation and migration by promoting integrin- α_5 signaling. Medications aimed at blocking this molecule could be a useful adjunct pharmacological therapy in the prevention of PVR.

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