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Role of Hypoxia and Extracellular Matrix-Integrin Binding in the Modulation of Angiogenic Growth Factors Secretion by Retinal Pigmented Epithelial Cells

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Abstract The retinal pigmented epithelium (RPE) is a monolayer of polarized cells located between retinal photoreceptors and blood vessels of the choroid. The basal surface of RPE cells rests on Bruch's membrane, a complex extracellular matrix structure which becomes abnormal in several disease processes, including age-related macular degeneration (AMD). Ruptures or abnormalities in Bruch's membrane are frequently accompanied by choroidal neovascularization. Disturbed interaction of RPE cells with their extracellular matrix (ECM) could play a role in this process. The present study was undertaken to examine the complex interactions between hypoxia, integrin, and ECM in the regulation of RPE functions. Antibody blocking experiments demonstrated that RPE cell adhesion to vitronectin is mediated primarily through $\alpha v\beta 5$ and adhesion to fibronectin occurs through $\alpha 5\beta 1$. RPE adhesion to immobilized laminin demonstrated highest level of non-RGD-mediated adhesion as compared to that with collagen IV or the RGD matrices such as vitronectin ($\alpha v\beta 5$) , fibronectin ($\alpha 5\beta 1$), or thrombospondin ($\alpha 5\beta 1 + \alpha v\beta 5$). Addition of soluble vitronectin, or fibrinogen to RPE cell cultures resulted in a small to moderate increase in VEGF and FGF2 in the media, while each of these growth factors was dramatically increased after addition of thrombospondin 1 (TSP1). In contrast, soluble fibronectin resulted in differential upregulation of VEGF but not FGF2. Similarly, immobilized TSP1 resulted in differential greater upregulation in VEGF but not FGF2 release from RPE as compared to other ECMs under either normoxic or hypoxic conditions. Additionally, Hypoxia resulted in a time-dependent increase in VEGF, but not FGF2 release in the media. RPE cells grown on TSP1-coated plates showed increased VEGF and FGF2 in their media compared to cells grown on plates coated with type IV collagen, laminin, vitronectin, or fibronectin. The TSP1-induced increase in secretion of growth factors was partially blocked by anti-$\alpha 5\beta 1$, anti-$\alpha v\beta 3$, and anti-$\alpha v\beta 5$ antibodies indicating that it may be mediated in part by TSP1 binding to those integrins. These data suggest that alterations in oxygen levels (hypoxia/ischemia) and ECM of RPE cells, a prominent feature of AMD, can cause increased secretion of angiogenic growth factors that might contribute to the development of choroidal neovascularization. These data also suggest the potential modulatory role of VEGF release from RPE by ECM and $\alpha v\beta 5$ and $\alpha 5\beta 1$ integrins. J. Cell. Biochem. 74:135–143, 1999. © 1999 Wiley-Liss, Inc.

Key words: hypoxia; integrins; matrix proteins; retinal pigmented epithelial cells; VEGF; FGF2; thrombospondin; choroidal neovascularization; age-related macular degeneration

Epithelial cells are greatly influenced by their extracellular matrix (ECM) and abnormal ECM can result in altered structure and function and contribute to disease states [Roskelley and Bissell, 1995]. The retinal pigmented epithelium (RPE) is a monolayer of highly specialized cells interposed between retinal photoreceptors and choroidal blood vessels. The apical surface of RPE cells inter-digitates with photoreceptor outer segments and the basal surface sits on Bruch's membrane, a complex 5-layered ECM structure. The innermost layer of Bruch's membrane is the basal lamina of the RPE which contains laminin, fibronectin, type IV collagen, and various proteoglycans [Campochiaro et al., 1986; Hewitt et al., 1989; Newsome et al., 1987].

Age-related macular degeneration (AMD) is the most common cause of severe visual loss in patients over the age of 60 in developed countries [Group, 1991]. It is identified clinically by

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the presence of drusen—yellow or white spots in the retina—which are collections of abnormal ECM material along Bruch's membrane. Other signs of AMD are RPE detachments (areas where the RPE is elevated and separated from Bruch's membrane) and areas of hypo- or hyper-pigmentation in the macula due to drop out or proliferation of RPE cells. Focal loss of photoreceptors also occurs and along with the gradual loss of RPE cells, constitutes the atrophic portion of the disease in which there is a slow decrease in central vision. Occurrences of choroidal neovascularization are superimposed on the slow degeneration and are often associated with accumulation of sub-retinal fluid, subretinal bleeding, and scarring resulting in sudden and severe loss of central vision.

The pathogenesis of retinal and RPE atrophy in patients with AMD is not known and it is possible that AMD is actually a group of diseases with a similar phenotype. Sorsby's fundus dystrophy is an autosomal dominant inherited disease that shares some phenotypic characteristics with AMD, including extensive ECM deposits along Bruch's membrane, RPE, and photoreceptor degeneration, and a high incidence of choroidal neovascularization [Capon et al., 1988, 1989; Hoskin et al., 1981; Sorsby et al., 1949]. Affected persons in two pedigrees with Sorsby's fundus dystrophy have a mutation in the tissue inhibitor of metalloproteinase-3 (TIMP-3) gene [Weber et al., 1994], whose product participates in regulation of ECM turnover [Apte et al., 1995]. This suggests that disordered ECM metabolism can lead to a phenotype like that seen in AMD. The reason for the occurrence of choroidal neovascularization in patients with AMD is also unknown. The pathogenesis of retinal neovascularization is better understood and it is useful to examine similarities and differences that exist between retinal and choroidal neovascularization. Retinal neovascularization originates from retinal vessels and grows along the inner surface of the retina, while choroidal neovascularization originates from choroidal vessels and grows through Bruch's membrane into the sub-RPE and subretinal space along the outer surface of the retina. There is strong evidence indicating that retinal hypoxia, resulting in increased production of vascular endothelial growth factor (VEGF), plays a role in the development of retinal neovascularization [Miller et al., 1994; Okamoto et al., 1997; Pierce et al., 1995] and that insulin-like growth factor I might also contribute [Smith et al., 1997]. Additionally, increased levels of VEGF and FGF2 have been demonstrated in RPE cells in association with choroidal neovascularization [Amin et al., 1994; Kvanta et al., 1996; Lopez et al., 1996; Ogata et al., 1996; Yi et al., 1997; Zhang et al., 1993], but the cause for these increases is uncertain. Hypoxia of the outer retina and RPE has not been demonstrated in patients with AMD, but there are some pathologic changes in choroidal vessels that suggest that it is a possible implication [Spraul et al., 1996]. The most consistent pathologic finding in advanced AMD in which there is choroidal neovascularization, is accumulation of abnormal ECM resulting in diffuse thickening of Bruch's membrane [Green and Enger, 1993]. This and the presence of focal areas of thickening due to drusen, suggest that there is disordered metabolism of the ECM. We hypothesize that diffuse thickening of Bruch's membrane and other abnormalities of the ECM in AMD eyes affect the structure and function of RPE cells. The abnormal ECM may block normal signaling from the ECM which could affect RPE adhesion and survival thereby leading to degeneration, and stimulating production of angiogenic factors by RPE cells which could contribute to the development of choroidal neovascularization.

Integrins, cell surface receptors that bind ECM molecules, mediate many of the effects of ECM on cells [Hynes, 1987, 1992]. In this study, we have sought to begin to test our hypothesis by determining if ECM components that bind to those integrins alter adhesion and/or production of VEGF and FGF2 by RPE cells. Additionally, we have also investigated the effect of hypoxia on levels of VEGF and FGF2 in the media of RPE cells.

MATERIALS AND METHODS

Reagents

Monoclonal antibodies directed against human $\alpha v\beta 5$ and $\alpha 5$ integrins were obtained from Becton Dickinson (Bedford, MA). A monoclonal antibody directed against $\alpha v\beta 3$ was obtained from Chemicon International Inc. (Temecula, CA). SQ885 is a small molecule anti-αv integrin ligand synthesized at DuPont Pharmaceuticals (Wilmington, DE).

Cell Culture

Human RPE cells were cultured from postmortem eyes obtained from the Old Dominion Eye Bank (Richmond, VA) using a procedure that has been previously described [Campochiaro et al., 1986]. The ARPE-19 RPE cell line that has differentiated characteristics, including spontaneous expression of CRALBP and formation of a polarized monolayer with high trans-epithelial resistance when grown of porous filters [Dunn et al., 1996], was provided by Leonard Hjelmeland, Ph.D. (Davis, CA). RPE cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 5 or 10% fetal bovine serum (FBS, Upstate Biotechnology, Inc., Lake Placid, NY). Cultures were demonstrated to be pure populations of RPE cells by immunocyto-chemical staining for cytokeratins [Leschey et al., 1990].

RPE Adhesion to ECM Proteins

The wells of 96-well plates (Costar 3590) were coated with 100μl of ECM protein (0.5μg collagen type IV, 1.0μg laminin, 5.0μg thrombospondin, 25μg fibrinogen, 25μg vitronectin, or 5.0μg of 120K fibronectin fragment). After an overnight incubation at 4°C, each well was washed twice with 200 μl of phosphate-buffered saline (PBS), followed by PBS with 5% BSA for 1 h at room temperature to block nonspecific binding. RPE cells were detached with 0.005% trypsin-0.1% EDTA, washed, and re-suspended in DMEM at 10^6 cells/ml. Cells were labeled with 2 μmol/l calcein-AM (Molecular Probes #3100, Eugene, OR) for 30 min at 37°C in a humidified incubator. Cells were then washed twice with 40 ml of DMEM and re-suspended in DMEM at 10^6 cells/ml. Cells were pre-incubated with antibodies or media for 15 min at room temperature and 10^5 cells were added to each well of an assay plate, covered with foil to prevent photobleaching, and incubated for 60 min at room temperature on a shaker at very low speed. Unattached cells were gently removed from the wells by washing twice with 200 μl of DMEM, and 100 μl of DMEM was added to each well and fluorescence was read on a Cytofluor II fluorometer (PerSeptive Biosystems, Framingham, MA) at sensitivity 2, excitation wavelength 485 nm, and monitoring wavelength 530 nm.

Growth Factor Secretion by RPE Cells in Response to Various ECM Proteins and/or Hypoxia

To determine the effect of ECM proteins added to the media, confluent monolayers of RPE cells in 48-well plates were washed twice with serum-free DMEM and 1 ml of media containing ECM protein at 1.0μM was added to each well. After 4 h in a 37°C humidified incubator, cell supernatants were collected, centrifuged, and frozen at −70°C until assayed using Quantikine immunoassay kits for human FGF2 or human VEGF (R&D systems, Inc., Minneapolis, MN).

To determine the effect of attachment to ECM proteins in the presence or absence of hypoxia, RPE cells were detached with 0.005% trypsin-0.1% EDTA, washed, and seeded at 3×10^5 cells per well in 48-well plates (Costar) coated with 10μg/well of one of the following human ECM proteins: type IV collagen (Life Technologies, Gaithersburg, MD), laminin (Life Technologies), vitronectin (Becton Dickinson, Bedford, MA), fibronectin (Sigma), or TSP1 (Haematologic Technologies Inc., Essex Junction, VT). Plates were placed in humidified incubators under normal (20% O2) or hypoxic (1% O2) conditions and cell supernatants from triplicate wells were collected after 48 h, centrifuged, and stored at −70°C until assayed for FGF2 or VEGF using Quantikine immunoassay kits. In some experiments, after the cells were attached, 0.1 mM of monoclonal antibody directed against αvβ5, αvβ3, or α5 was added to triplicate wells. To determine the time course of growth factor secretion in response to hypoxia, RPE cells were plated in wells coated with 10μg of type IV collagen, placed in 1% O2, and the media from triplicate wells were harvested and FGF2 and VEGF were measured by immunoassay.

RESULTS

Integrin-mediated adhesion of RPE cells to various ECM proteins RPE cells showed avid adhesion to laminin and vitronectin, moderate adhesion to fibronectin, and poor adhesion to collagen-type IV and TSP1 (Fig. 1A,B). Antibody blocking experiments show that integrin αvβ5 and to a lesser extent α5β1, but not αvβ3, play an important role in the adhesion of RPE cells to vitronectin (Table I). Adhesion of RPE

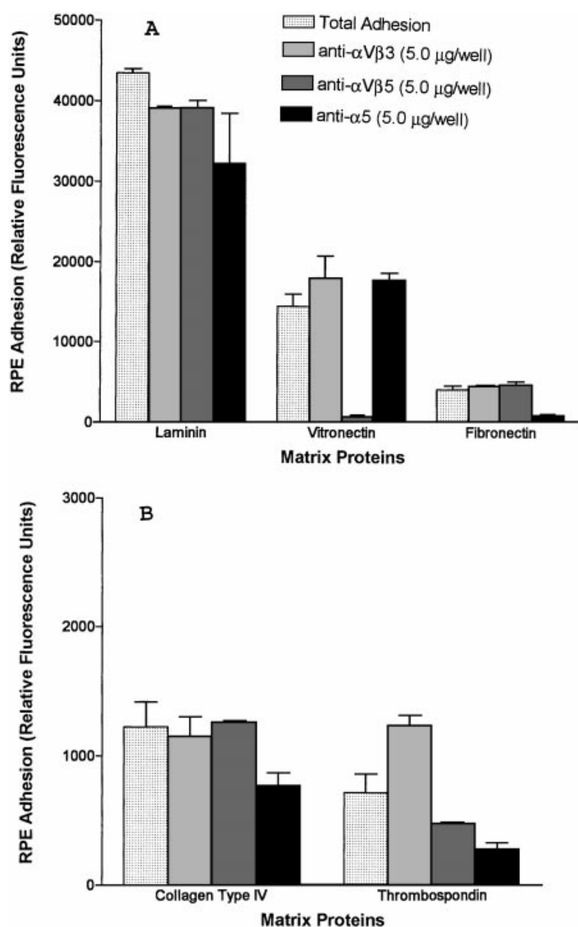

Fig. 1. Inhibition of RPE cell adhesion to extracellular matrix proteins by anti-integrin antibodies. Adhesion assays were performed using wells coated with one of the listed extracellular matrix proteins in the presence or absence (total adhesion) of the listed antibodies as described in Materials and Methods. Each bar represent mean ± SEM, n = 3. RPE cell adherence is greatest to laminin and is minimally inhibited by the antibodies that were tested. There is moderate adherence to vitronectin that is blocked by anti-αvβ5 and moderate adherence to fibronectin that is blocked by anti-α **(A)**. RPE cells bind relatively less to collagen type IV and TSP1 **(B)**.

cells to fibronectin occurred through α5β1, but not α4β1, αvβ3, or αvβ5. There was no detectable RPE binding to fibrinogen, to which vascular endothelial cells avidly bind via αvβ3 (data not shown).

Alteration of VEGF and FGF2 Levels in the Media of RPE Cells by ECM Proteins

In addition to their effect on attachment, ECM proteins may alter other behaviors of cells. We examined the effect of various ECM proteins on the secretion of FGF2 or VEGF from RPE cells. Under basal conditions, FGF2 was not detectable in the media of RPE cells, but was significantly increased 4 hours after the addition of 1 μM vitronectin, fibronectin, or fibrinogen, and was markedly increased after the addition of TSP1 (Fig. 2A). A low basal level of VEGF secretion was identified and was not altered 4 h after addition of 1 μM fibrinogen, but was modestly increased after addition of 1 mM vitronectin and was dramatically increased after addition of 1 μM TSP1 or fibronectin (Fig. 2B). Therefore, addition of TSP1 to RPE cells caused a significant increase in the media of both VEGF and FGF2, but in absolute terms, the amount of VEGF in the media was much greater, roughly 26 times, the amount of FGF2. Adhesion of RPE cells to fibronectin occurred through α5β1, but not α4β1, αvβ3, or αvβ5.

Hypoxia Increases Levels of VEGF But not FGF2 in the Media of RPE Cells

RPE cells maintained under hypoxic conditions showed a time-dependent linear increase in VEGF in their media, while under normoxic conditions there was an initial rise in VEGF in the media that plateau after 24 h (Fig. 3A). There was significantly more VEGF in the media of hypoxic compared to normoxic RPE cells at 24, 48, and 72 h. In contrast with VEGF, there was no increase in FGF2 over time in the media of hypoxic RPE cells and their was less FGF2 in the media of hypoxic cells compared to normoxic cells at 48 and 72 (Fig. 3B). Compared to RPE cells grown on other ECM substrata, cells grown on a TSP1 substratum show increased secretion of VEGF and under both normal and hypoxic conditions. RPE cells grown on type IV collagen, laminin, vitronectin, or fibro-

TABLE I. Antibody Inhibition of RPE Adhesion to Various Matrix Proteins

	Mean % inhibition of adhesion				
Antibody (5 μg/well)	Collagen type IV	Laminin	Vitronectin	Fibronectin	Thrombospondin
anti-alphaVbeta3	5.8	10.2	0.0	0.0	0.0
anti-alphaVbeta5	0.0	10.0	95.5*	0.0	33.9
anti-alpha5	37.2	25.9	0.0	77.7*	61.4*

$^*P < 0.01$

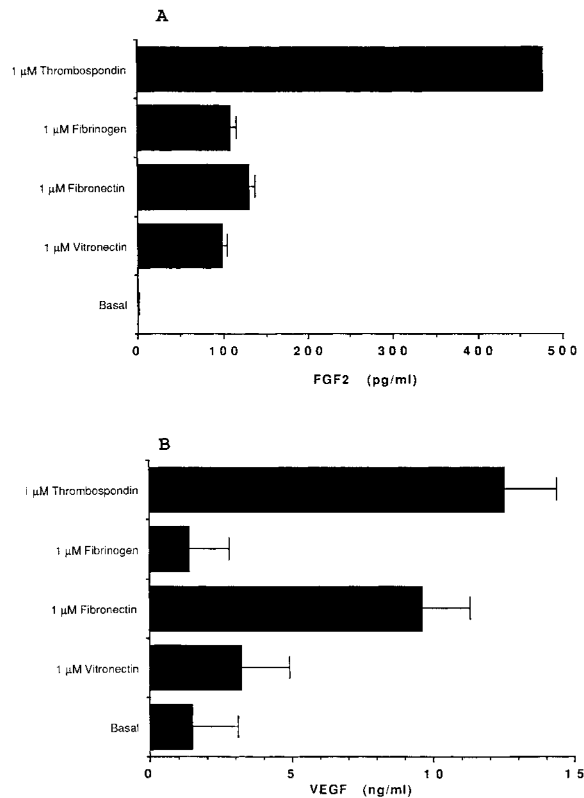

Fig. 2. Measurement of FGF2 **(A)** and VEGF **(B)** in the media of RPE cells after the addition of extracellular matrix proteins. Confluent monolayers of RPE cells were washed with serum-free medium and 1 ml of media containing one of the listed extracellular matrix proteins or media alone (basal) was added to each well. After 4 h at 37°C, cell supernatnats were collected and FGF2 (A) and VEGF (B) were measured by immunoassay. Each bar represent mean ± SEM, n = 3.

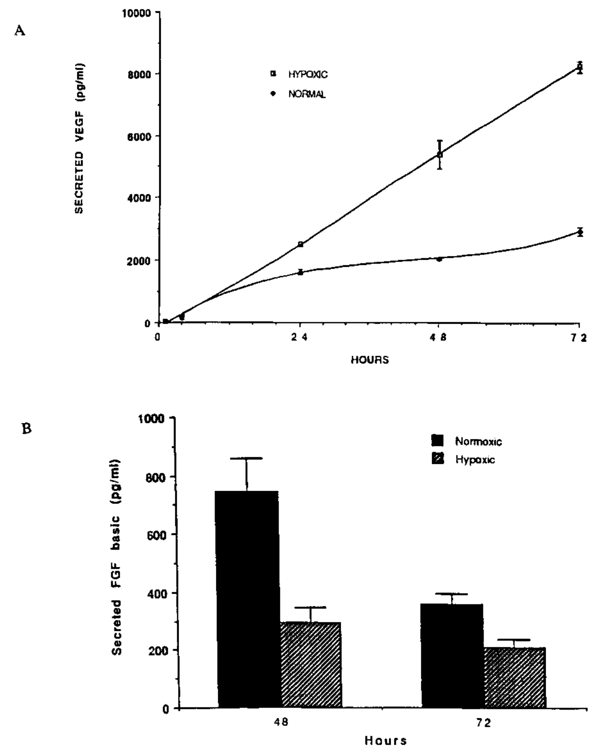

Fig. 3. Hypoxia causes an increase in VEGF and a decrease in FGF2 in the media of RPE cells. Confluent monolayers of RPE cells on a substratum of collagen IV were placed in 1% J (hypoxic) or 20% (normoxic) O2 and immunoassays were done on cell supernatants for VEGF at 0, 24, 48, and 72 h **(A)** and for FGF2 at 48 and 72 h. **(B)** Each bar represent mean + SEM, n = 3. $^*P < 0.01$, Student's *t*-test for difference between hypoxic and normoxic.

nectin, all have about the same amount of VEGF in their media at 48 h after plating, but cells grown on TSP1 have significantly more (Fig. 4A). When RPE cells are grown on the various matrix proteins under hypoxic conditions there is an increase in the amount of VEGF secreted compared to cells grown on the same proteins under normoxic conditions. Cells grown on TSP1 under hypoxic conditions have the greatest amount of VEGF in their media. In contrast, the amount of FGF2 in the media is not significantly increased when cells are grown on TSP1 compared to other matrices, and on all matrices, hypoxia resulted in decreased FGF2 in the media (Fig. 4B).

Antibodies Directed Against α5β1, αvβ3, or αvβ5 Attenuate the TSP1-Induced Increase in VEGF Secretion by RPE Cells

A consistent two-fold increase in VEGF release from RPE was demonstrated under hypoxic relative to normoxic conditions regardless of the matrix protein used (Table IIa,b). The greatest level of VEGF secretion was demonstrated with TSP1 under normoxic or hypoxic conditions as compared to that with other matrix proteins used (Table IIa,b). Addition of monoclonal antibody directed against αvβ3, αvβ5, or α5 to the media of RPE cells attached to a TSP1 substratum caused a small but significant decrease in the amount of VEGF in the media at 48 h (Table IIb). The decrease was more pronounced when small molecule antagonist (SQ885) for αvβ5 was added to the media of hypoxic RPE cells growing on TSP1. SQ885 resulted in a significant inhibition of VEGF release when RPE cells adhered to vitronectin or TSP1 but not to collagen, laminin, or fibronectin (Table II). In contrast, FGF_2 secretion by RPE was not increased under hypoxic condition or in response to the various ECM (Table III).

DISCUSSION

Previous studies have investigated integrins located on RPE cells using antibodies that rec-

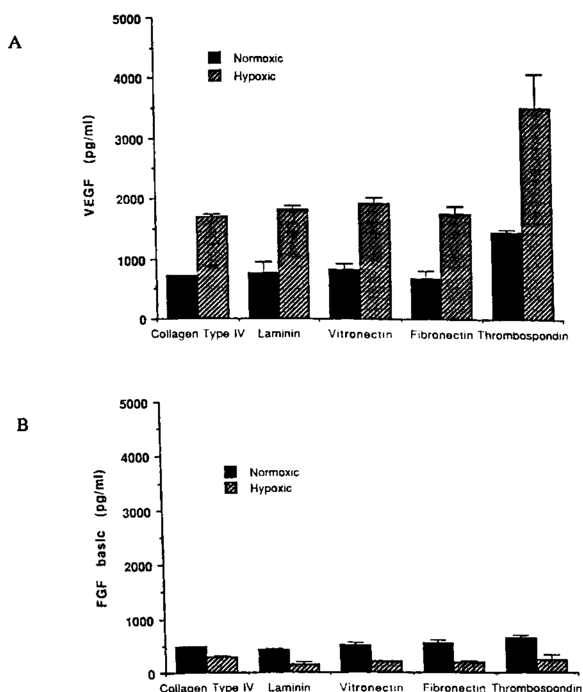

Fig. 4. Measurement of VEGF **(A)** or FGF2 **(B)** in the media of RPE cells grown on substrata of the listed extracellular matrix proteins in a normoxic or hypoxic environment after 24 h. Confluent monolayers of RPE cells grown on a substratum of one of the listed extracellular matrix proteins were placed in 1% (hypoxic) or 20% (normoxic) O2 and immunoassays were done on cell supernatants for VEGF (A) or FGF2 (B) 48 h. Each bar represent mean ± SEM, n = 3. $^*P < 0.01$, Student's *t*-test for difference between hypoxic and normoxic.

ognize integrin monomers and have demonstrated the presence of β1, β2, α2, α4, and α5 [Anderson et al., 1990; Brem et al., 1994; Chen et al., 1997; Hunt et al., 1994; Kupper and Ferguson, 1993; Philp and Nachmias, 1987; Rizzolo et al., 1994]. In monkey RPE, vitronectin receptor was localized to the apical surface and fibronectin receptor was localized to the basolateral surface [Anderson et al., 1995]. Using FACS analysis with antibodies that recognize integrin heterodimers and one antibody that recognizes the α5 monomer, we have demonstrated that cultured RPE cells express several integrins, with levels highest for αvβ5 and α5β1 and intermediate for αvβ3 and α4β (data not shown). FACS provides qualitative distribution of the various integrin receptors. However, the impact of those integrins on RPE functions depend upon their binding affinity and avidity to the respective ECM. RPE cells attach avidly to laminin, presumably through β1 integrin. RPE cells also attach well to vitronectin and moderately well to fibronectin, but attach poorly to collagen type IV and TSP1. Antibody blocking experiments indicate that integrin avb5 and to a lesser extent a5b1, play an important role in the adhesion of RPE cells to vitronectin and attachment to fibronectin is mediated primarily by a5b1. We did not investigate for the presence of αβ1, but previous studies have suggested that it is present on RPE cells and mediates attachment to interstitial collagen and, in pathologic situations, causes contraction of vitreous [Hunt et al., 1994; Kupper and Ferguson, 1993]. Thickening of Bruch's membrane in patients with AMD is due in part to accumulation of excessive type IV collagen. This could perturb the adhesion of RPE cells to its basement membrane and contribute to RPE dysfunction and RPE detachments in AMD. In addition to serving as substrates for attachment, ECM proteins influence the amount of angiogenic growth factors that RPE cells export into their media. Addition of TSP1, fibronectin, or vitronectin to the media of RPE cells increased the amount of VEGF in the media. The largest increase occurred in the presence of TSP1 and RPE cells grown in wells coated with TSP1 had significantly more VEGF in the media than did cells grown in wells coated with collagen type IV, laminin, fibronectin, or vitronectin. The TSP1-induced increase in VEGF secretion is partially blocked by anti-α5 or anti-αvβ3 antibodies, but not anti-αvβ5. This suggests that signaling through αvβ3 and a5b5 could play roles in the TSP1-induced increase in VEGF secretion by RPE cells. The effects are relatively small which could be because the antibodies have limited ability to perturb integrin-TSP1 interactions once they are well established or because other mechanisms are involved and play a larger role. Additional studies are needed to distinguish between these possibilities.

The amount of FGF2 in the media of RPE cells was much less than the amount of VEGF, but it was also increased by the addition of TSP1, fibronectin, or vitronectin. Unlike the situation for VEGF, RPE cells grown in wells coated with TSP1 did not have more FGF2 in the media than did cells grown in wells coated with other ECM proteins. Another difference between VEGF and FGF2 was the effect of hypoxia. Hypoxia increased the secretion of VEGF by RPE cells grown on all matrices, but failed to increase the amount of FGF2 in the media, and in fact caused a decrease. The additive effect of hypoxia and TSP1 on VEGF secre-

TABLE II. VEGF Secreted by RPE Plated on Various Matrix Proteins After 48 Hours

	VEGF (pg/ml) under normoxic conditions (n = 3)				
Anti-integrin	Collagen type IV	Laminin	Vitronectin	Fibronectin	Thrombospondin
Control	726.1 ± 7.6	778.7 ± 175.0	835.6 ± 99.3	694.9 ± 120.8	1459.7 ± 46.9
0.1 μM anti-αvβ5	704.3 ± 42.9	717.6 ± 48.9	788.7 ± 53.2	680.8 ± 42.9	1410.6 ± 108.2
0.1 μM anti-αvβ3	798.3 ± 80.3	753.7 ± 90.6	871.5 ± 87.4	856.4 ± 129.4	1290.3 ± 139.8
0.1 μM anti-α5	660.4 ± 63.7	683.9 ± 70.4	702.7 ± 2.9	655.5 ± 47.0	1149.3 ± 74.6*
10 μM SQ885	787.4 ± 55.3	690.7 ± 64.5	526.8 ± 107.3*	712.4 ± 66.2	892.1 ± 124.4**
	VEGF (pg/ml) under hypoxic conditions (n = 3)				
Anti-integrin	Collagen type IV	Laminin	Vitronectin	Fibronectin	Thrombospondin
Control	1694.3 ± 36.8	1821.3 ± 60.8	1914.7 ± 110	1757.4 ± 120.9	3319 ± 168
0.1 μM anti-αvβ5	1678.8 ± 100.9	1747.0 ± 107.0	1774.8 ± 56**	1714.4 ± 75.1	2608 ± 186**
0.1 μM anti-αvβ3	1624.7 ± 58.1	1781.4 ± 17.6	1894.9 ± 22	1696.0 ± 163.8	3029 ± 320
0.1 μM anti-α5	1635.6 ± 147.5	1751.7 ± 161.0	1823.8 ± 140	1782.6 ± 99.5	2670 ± 243**
10 μM SQ885	1632.3 ± 109.3	1858.7 ± 108.7	1756.3 ± 130*	1758.1 ± 203.0	2571 ± 131**

*$P < 0.05$.
**$P < 0.001$.

TABLE III. FGF Basic Secreted by RPE Plated on Various Matrix Proteins After 48 Hours

	FGF basic (pg/ml) under normoxic conditions (n = 3)				
Anti-integrin	Collagen type IV	Laminin	Vitronectin	Fibronectin	Thrombospondin
Control	497.9 ± 4.4	431.4 ± 9.1	528.2 ± 32.2	552.5 ± 51.9	654.7 ± 52.0
0.1 μM anti-αvβ5	578.8 ± 64.7	411.4 ± 21.5	569.2 ± 25.9	534.2 ± 16.4	597.3 ± 16.3
0.1 μM anti-αvβ3	591.3 ± 42.3	423.9 ± 29.4	608.6 ± 53.6	576.1 ± 7.9	614.5 ± 52.6
0.1 μM anti-α5	609.6 ± 45.3	478.9 ± 61.4	579.8 ± 28.5	652.4 ± 102.8	563.5 ± 31.1
10 μM SQ885	691.6 ± 43.6	508.9 ± 25.1	>3200	1299.7 ± 406.2	2110.4 ± 847
	FGF basic (pg/ml) under hypoxic conditions (n = 3)				
Anti-integrin	Collagen type IV	Laminin	Vitronectin	Fibronectin	Thrombospondin
Control	289.6 ± 29.8	166.8 ± 26.3	215.5 ± 4.3	198.2 ± 24.3	256.9 ± 76.2
0.1 μM anti-αvβ5	303.3 ± 12.2	169.0 ± 19.4	233.8 ± 8.8	256.1 ± 52.3	260.3 ± 12.0
0.1 μM anti-αvβ3	297.3 ± 19.3	195.4 ± 9.5	269.5 ± 11.7	296.0 ± 19.4	294.1 ± 13.1
0.1 μM anti-α5	265.7 ± 9.4	196.0 ± 10.6	234.1 ± 22.1	257.8 ± 24.1	273.7 ± 4.3
10 μM SQ885	295.2 ± 48.3	209.9 ± 8.2	306.2 ± 17.8	262.9 ± 27.2	619.3 ± 229.6

tion by RPE cells and the fact that they have the opposite effects on the amount of FGF2 in the media, suggest that hypoxia and TSP1 utilize different intracellular signaling pathways. These differences are also indicative of differences in the regulation of expression and processing of VEGF and FGF2. Hypoxia increases VEGF mRNA by a combination of upregulation of transcription and increased mRNA stabilization [Aiello et al., 1995; Forsythe et al., 1996; Shima et al., 1995], and since VEGF is a secreted protein, it is not surprising that it is increased in the media of hypoxic RPE cells. In contrast, FGF2 mRNA is not increased by hypoxia [Shima et al., 1995] and the FGF2 gene does not have a signal sequence [Abraham et al., 1986]. However, despite the lack of a signal sequence, FGF2 gets out of cells by a mechanism that has not been clearly defined [Hackett et al., 1997]. Perhaps FGF2 is exported by an energy dependent transport system and depletion of ATP in hypoxic cell results in decreased FGF2 in the media.

Previous studies have suggested that RPE cells may promote the survival of the choriocapillaris, because damage or death of RPE cells results in subsequent atrophy of the choriocapillaris [Korte et al., 1984]. Basal production of VEGF and/or FGF by RPE cells could play a role in this survival-promoting effect. Increases in production above basal levels could stimulate growth of the choriocapillaris and contribute to choroidal neovascularization. This possibility is supported by studies demonstrating

increased expression of VEGF and FGF2 in RPE cells in animal models of choroidal neovascularization [Ogata et al., 1996; Yi et al., 1997; Zhang et al., 1993] and in surgically removed choroidal neovascular membranes from patients [Amin et al., 1994; Kvanta et al., 1996; Lopez et al., 1996]. Alteration of the ECM of the RPE could contribute to the increased expression of VEGF and FGF2 in these situations.

It is interesting that of the ECM proteins tested, TSP1 caused the greatest increase of VEGF and FGF2 in the media of RPE cells. To our knowledge, TSP1-mediated increased export of VEGF and/or FGF2 has not been observed with other cell types, but TSP1-stimulated secretion of PDGF and EGF from mesangial cells has been reported [Marinides et al., 1994]. TSP1 has been suggested to be antiangiogenic in some systems and proangiogenic in others [Bornstein, 1995]. Its ability to interact with numerous proteins and cell surface receptors may be the basis of its different, in some cases opposite, activities in different settings. Our findings suggest a mechanism through which TSP1 could be proangiogenic in the sub-retinal space. Fluorescein angiography in patients with AMD show that some drusen are hyperfluorescent, indicating that they are accessible to serum and are therefore likely to contain TSP1 [Pauleikhoff et al., 1990, 1992]. Deposition of TSP1 and/or other ECM proteins in drusen or thickened Bruch's membrane inpatients with AMD could potentially modulate the secretion of angiogenic growth factors by RPE cells and could play a role in the development of choroidal neovascularization. Studies in animal models are needed to evaluate this hypothesis. These data suggest the potential utility of $\alpha v\beta 5$ in modulating VEGF secretion from RPE.

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