Effect of quiescence on integrin α5β1 expression in human retinal pigment epithelium

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Purpose: The retinal pigment epithelium (RPE) is a monolayer of polarized epithelial cells located between the photoreceptor outer segments of the neural retina and the choroidal blood supply [1]. RPE cells have many metabolic and biochemical functions essential for maintaining retinal integrity, such as phagocytosis of rod and cone outer segments, transport of metabolites to and from the visual cells and choroid, maintenance of the blood-ocular barrier and adhesion of the retina [1]. At their basal surface, the RPE basement membrane is separated from the basement membrane of the choroidal capillaries by the Bruch’s membrane. The Bruch’s membrane is composed of a number of extracellular matrix (ECM) macromolecules such as type I, III and IV collagen, laminin and fibronectin (FN), which are also synthesized by RPE cells in vitro [2]. Studies of cultured RPE cells have shown that they attach to and spread on individual matrix molecules such as collagen [3], laminin [3,4,5] and fibronectin [5], as well as on ECM produced by various cell lines [3-6].

The interactions between a cell and its ECM are, in part, mediated by a family of cell surface glycoproteins called integrins [7,8]. Signals transduced by integrins can influence cell adhesion, migration, differentiation and proliferation [7,8].

Integrins are heterodimers consisting of variable α and β subunits. The different combinations of these subunits determine the receptor specificity for the different ECM molecules [7,8]. Although integrins often bind to more than one ligand, some show selectivity. It is the case for the α5β1 integrin which binds specifically to FN [7-9]. The α5β1 integrin has been shown to mediate adhesion to FN for a number of cell types, including leukemic cells [10], mammary carcinoma cells [11], and RPE cells [12]. Cell spreading and proliferation are inhibited in α5-deficient NRK fibroblasts [13], whereas mammary carcinoma cell proliferation is inhibited by antibodies to α5β1 [11]. Furthermore, α5β1 has been shown to mediate migration of aortic endothelial cells [14], fibroblasts [15] and RPE cells [16] on FN, a further evidence that α5β1 also promotes cell proliferation and migration.

The FN integrin α5β1 also appears to play pathogenic roles in a variety of proliferative diseases. Indeed, changes in α5β1 expression have been correlated with malignancy of uveal melanoma [17]. Robbins et al. [18] showed the presence of the α5 subunit (amongst others) on pigmented cells (presumably of RPE origin) from proliferative vitreoretinopathy (PVR) membranes and concluded that its presence was abnormal when compared to the lack of α5 staining on normal retina [19].

Integrins have been postulated to play a major role in PVR [7,18], a pathology in which normally quiescent and differentiated RPE detach from the underlying Bruch’s mem-
brane, migrate and proliferate [20-22]. When RPE cells are cultured, they dedifferentiate, migrate and proliferate very much the same as those in PVR. Studying integrin expression of cultured RPE cells, and comparing integrin expression on proliferating and quiescent cells, could thus help understand the involvement of integrins in PVR.

Given the importance of the α5β1 integrin in cell adhesion, proliferation and migration, we sought to investigate the α5β1 protein expression and gene regulation in proliferating and quiescent human RPE cells. We determined if the integrin α5 subunit was present at the RPE cell surface in situ and in vitro and examined the α5 mRNA transcribed in human RPE cells in vitro at different cell confluences. We also assessed whether the changes in the amounts of α5 mRNA in vitro found with increasing cell confluence were reflected by differences in the transcriptional activity directed by the α5 gene promoter.

Our results demonstrate that the integrin α5 subunit is present at the RPE cell surface both in vitro and in situ, and that its expression is modulated with cell quiescence. Proliferating (sub-confluent and confluent) RPE cells have similar amounts of α5 protein and α5 mRNA transcripts, and both protein and mRNA expression are downregulated when RPE cells become quiescent. A small increase in α5 promoter activity is however observed between sub-confluent and confluent RPE cells, although promoter activity also decreases when cells become quiescent, providing further evidences that cellular quiescence dictates the level to which the α5 integrin is expressed in RPE cells.

**METHODS**

**Cell cultures:** Cultures of primary RPE cells were established from eyes of 14 human donors (age range: 6 days old to 48 years old, mean=22 years old) obtained from the National Eyerbank Inc. (Québec, Qc, Canada) within 24 h of death. This research was conducted in accordance with the tenets of the Declaration of Helsinki. Dissection was done by a modification of a previously published method [23]. We did not use eyes from donors older than 50 years old because we usually harvested more cells from younger adult eyes that also attached and spread faster than cells from older adult eyes. Cells were cultured with Keratinocyte-SFM medium (Canadian Life Technologies, Burlington, Ontario, Canada) supplemented with 5% bovine calf serum (Hyclone, Logan, Utah, USA) and 200 mg/ml albumax, 45 mg/ml ascorbic acid, 1 mg/ml carnitine, 500 mg/ml glucose, 112 mg/ml fructose, 5 mg/ml glutathione, 6 mg/ml hypoxanthine, 67 mg/ml oxalic acid, 0.15 mg/ml retinol acetate, 5 mg/ml taurine, 0.025 mg/ml D-α-tocopherol, 50 mg/ml transferrin and 0.3 mg/ml uridine. Medium was changed every 2-3 days and cultures were maintained in an incubator at 37 °C in 5% CO2/95% air atmosphere. For all experiments, cultures were used at passage 2 and cells were plated at different densities as follows: non-confluent cells: 2.5x10⁶ cells/cm², 2 days in culture; confluent cells: 1.5x10⁶ cells/cm², 2 weeks in culture; post-confluent cells: 1.5x10⁵ cells/cm², 2 days in culture.

**DNA synthesis of RPE cells at different cell confluences:** DNA synthesis of RPE cells at different confluences was monitored by the addition of the thymidine analogue bromo-deoxyuridine (BrdU; Sigma, Oakville, Ontario, Canada) to the culture medium (final concentration 10 μM). Labeling was allowed for 24 h upon which time cells were washed and fixed with an ethanol fixative (70% ethanol, 50 mM glycine, pH 2.0) for 30 min at -20 °C. Incorporated BrdU was detected by immunofluorescence. Briefly, RPE cells were first incubated with a mouse anti-BrdU antibody (clone BRD.2; Medicorp, Montreal, Quebec, Canada) diluted 1:50 in PBS containing 3000 U/ml of Exonuclease III (Sigma) and 150 U/ml of restriction endonuclease SAU 3A I (Sigma) for 30 min at 37 °C, followed by incubation with a secondary antibody (anti-mouse IgG-TRITC conjugated; Sigma) for 30 min at 37 °C. After 3 other washes in PBS and 2 final washes in distilled water, the coverslips were mounted on a microscope slide and fixed with nailpolish. The fluorescence was observed with an epifluorescence microscope (Nikon Diaphot 300). For negative controls, the primary antibody was replaced by PBS-BSA1%. The results shown are representative of a minimum of 3 independent experiments conducted on separate RPE populations derived from different donors.

**Immunohistochemistry:** Immunohistochemistry was performed on 2 different human donor eyes (a 21 year old donor and a 81 year old donor) which were obtained, respectively, 15.5 h and 4 h post-mortem. They were immediately 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, eyes were embedded in OCT and frozen in liquid nitrogen. Sections of 10 μm were cut using a Leitz 1720 digital cryostat, collected on superfrost/plus Fisherbrand slides (Fisher scientific, Nepea, Ontario, Canada) and stored at -80 °C until use. Sections were incubated for 20 min at room temperature in 95% ethanol, and then treated with 0.25% potassium permanganate for 15 min at room temperature [24] to reduce autofluorescence arising from lipofuscin present in RPE [25]. Blocking buffer and antibodies were diluted in 0.3% BSA in PBS. Sections were blocked overnight at 4 °C in 5% goat serum (Vector Laboratories, Burlington, Ontario, Canada) and then incubated with rabbit anti-integrin α5 polyclonal antibody (Chemicon, Temecula, CA), or with normal rabbit serum (Sigma) for negative control, both at a concentration of 103 μg/ml. Sections were blocked again in 5% goat serum for 60 min at 37 °C, and incubated with secondary antibody labeled with Alexa Fluor 488 at a 1:100 dilution (Molecular Probes, Eugene, OR) for 90 min at 37 °C. Sections were mounted with Prolong antifade mounting medium (Molecular Probes). Images were obtained using a MRC 1024 confocal system (Bio-Rad) mounted on a Nikon Diaphot TMD inverted microscope equipped with 60x 1.4 N.A. immersion optics. Confocal 8 bit images were captured using Bio-Rad’s Lasersharp acquisition software running under OS2.

In order to distinguish residual autofluorescence from specific immunoreactivity, images were obtained as described in Kennedy et al. [26]. Briefly, sections were subjected to sequential excitation with the 488 nm line (green) and the 568 nm line (red) of the krypton/argon laser. Autofluorescent ma-
In situ hybridization and immunohistochemistry. Cryosections were hybridized with digoxigenin-UTP-labeled probes as described above, postfixed with 4% PFA for 10 min at room temperature. Cells (5x10⁶ cells) were then lysed in lysis buffer (50 mM Tris-HCl, pH 7.5 containing 1% Igepal CA-630, 120 mM NaCl, 1 mM CaCl₂.2H₂O, 1 mM MgCl₂, 0.7 µg/ml pepstatin, 0.5 µg/ml leupeptin, 1 mM pefabloc). Insoluble materials were removed by centrifugation at 16 000 g for 10 min at 4 °C. Protein concentration was measured with the BCA method (Pierce) to ensure that equal amounts of protein were loaded in each lane. Each ml of supernatant was pre-cleared by a 1 h incubation with 50 µl of unconjugated protein-A-Sepharose CL-4B beads (Amersham Pharmacia Biotech, Baie d’Urfé, Quebec, Canada). Approximately 50 µl of goat anti-mouse IgG coated sepharose beads (Zymed Laboratories, San Francisco, CA) was incubated for 3 h at room temperature with 10 µg primary antibody (mouse anti-human integrin α5, clone IIA1, Pharmingen), then rinsed with wash buffer. Then, 50 µl of 2X Laemmli buffer containing 5% 2-mercaptoethanol was added to the 50 µl of rinsed beads, boiled for 5 min at 85 °C, cooled, and 20 µl was loaded per lane (corresponding to 7.5x10⁶ immunoprecipitated cells) on a 6% polyacrylamide gel and transferred to nitrocellulose (Bio-Rad Laboratories, Mississauga, Ontario, Canada). The membrane was blocked for at least 1 h with 3% BSA, incubated for 1 h with streptavidin-HRP (Pierce) and processed for autoradiography. Band density was evaluated using the QuantityOne 1-D image analysis software (Bio-Rad Laboratories).

Semi-quantitative Reverse Transcription-Polymerase Chain Reaction: RNA from RPE cells at different confluences was isolated using the Tri-Reagent (Sigma) according to the manufacturer’s instruction. Reverse transcription (RT) was performed using 5µg total RNA and 0.2µg of random hexamer primers (Fermentas; Burlington, Ontario, Canada) following the manufacturer’s protocol for synthesis of first strand cDNA. Polymerase chain reaction (PCR) was performed using the QuantumRNA 18S Internal standards protocol for semi-quantitative RT-PCR (Ambion, Austin, Texas) according to the manufacturer’s instructions. Briefly, the linear range of the α5 PCR product was determined using RNA from sub-confluent, confluent and post-confluent RPE. PCR products were in the linear range between 23 and 33 cycles for sub-confluent and confluent cells, and between 25 and 31 for post-confluent cells. The total number of cycles used for semi-quantitative RT-PCR was 28. The QuantumRNA 18S Internal standard (Ambion) used comes with 18S primers and 18S competimers that competes one another to decrease 18S PCR product in order to co-amplify and detect both 18S and gene specific PCR products. The optimal ratio of 18S primers: competimers was determined and the ratio used was 2:8 (18S primers:competimers). Bands were analyzed with the GelDoc2000 gel documentation system (Bio-Rad Laboratories) and the QuantityOne 1-D image analysis software (Bio-Rad Laboratories). Semi-quantitative RT-PCR was done twice in duplicate on 4 different donors. The DNA sequence of the primers used for the amplification of the human α5 transcript were: forward primer: 5'-GGC AGC TAC GTC CCA CTG TGG-3', reverse primer: 5'-GGC ATC AGA GGT GCG TGG AGG CTT-3' (171-bp PCR product) and cycle parameters were 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The 18S primers (Ambion) gave a PCR product of 489 bp.

Transient transfections and CAT assays: Plasmids containing the chloramphenicol acetyltransferase (CAT) reporter gene from the plasmid pSKCAT fused to various 5' deletions of the α5 gene and stripped of their multicloning site (MCS; -954α5CATAMCS, -178α5CATAMCS, -132α5CATAMCS, -92α5CATAMCS, -41α5CATAMCS) have been previously described [27]. RPE cells at different confluences were transiently transfected using the calcium phosphate precipitation method as described by Graham and Van der Eb (1973) [28].
Each individual plate received 15 µg of the test plasmid and 5 µg of human growth hormone (hGH)-encoding plasmid pXGH5 [29] for 4 to 6 h before being washed with PBS and incubated an additional 48 h in fresh medium. Levels of CAT activity for the transfected cells were determined as described [30] and normalized to the amount of hGH secreted into the culture media and assayed using a kit for quantitative measurement of hGH (Immunocorp, Montréal, Québec, Canada). The value presented for each individual test plasmid transfected corresponds to the mean of at least three separate transfections done with RPE cells from three different donors, each in triplicate. To be considered significant, each individual value needed to be at least three times over the background level. Standard deviation is also provided for each transfected CAT plasmid. Statistical analyses were performed using a single factor ANOVA (Excel, Microsoft, Bellvue, WA). A p value of less than 0.001 was considered statistically significant.

RESULTS

The α5 integrin subunit is expressed on RPE cells in situ: Integrin α5 subunit expression in situ with normal RPE cells has been studied previously with contradictory results. Indeed, the lack of α5 subunit has been reported in situ on human [19] and chicken RPE cells [31]. On the other hand, Anderson et al. [32] demonstrated its presence on monkey RPE cells. To verify if the α5 integrin subunit is indeed expressed in vivo on RPE cells, a 21 year old human donor eye was analyzed by immunohistochemistry using a monoclonal antibody directed against the α5 subunit (Figure 1). As revealed on Figure 1A, expression of α5 was readily detected in the RPE monolayer, and staining was primarily concentrated along the basal surface of the cells. Staining for the integrin α5 subunit was also detected in the choroid. The labeling pattern for the 81 year old retina was the same, although much more lipofuscin autofluorescence could be observed (data not shown).

Proliferating and quiescent RPE cells in vitro: To assess whether cultured RPE cells grown at the cell densities selected for this study were in a quiescent or actively proliferating state, incorporation of BrdU was performed. RPE cell cultures that are left confluent for 2 days (confluent cells) incorporated BrdU into their nuclei indicating their high proliferative activity (Figure 2A). On the other hand, RPE cell cultures that remained confluent for 2 weeks (post-confluent cells) previous to BrdU labeling showed a markedly reduced BrdU staining indicating that most cells at this stage are out of the cell cycle and have reached cellular quiescence (Figure 2B). These results are consistent with the study of Kaida et al. [33] who also demonstrated that RPE cells were proliferating when cells were left for 2 to 3 days at confluence, and that proliferation of RPE cells was infrequent when cells were maintained at confluence for 8 weeks.

Decrease of the α5 protein at the RPE cell surface with cellular quiescence: The α5 integrin subunit has already been observed in vitro on RPE cells [12,16,32,34-39], and some of these studies reported that the staining pattern differed depending on the culture state reached by RPE cells [32,34,35,38]. Indeed, immunofluorescence studies showed that proliferating (undifferentiated) RPE cells had a heterogeneous α5 staining with fluorescence clusters at the cellular surface and fluorescence on the border of the cell membrane [32,38]. When

![Figure 2. Proliferation assay. Primary human RPE cells were seeded at 1.5x10^5 cells/cm^2 and remained in culture for either 2 days (confluent cells; A) or 2 weeks (post-confluent cells; B) before BrdU labeling (see Methods). As a negative control, the primary antibody was omitted (C). Scale bar represents 100 µm.](http://www.molvis.org/molvis/v9/a60)
RPE cells reached confluence or remained confluent for 1 to 2 weeks, the α5 staining yielded a nearly continuous linear band around the cells [32,34,35]. We also found, through immunofluorescence analysis, that confluence alters the α5 staining of RPE cells (data not shown) and wished to determine whether confluence also affected α5 protein levels at the RPE cell surface. Cell surface levels of the α5 subunit was thus examined in RPE cells by immunoprecipitation of biotinylated cell surface proteins (Figure 3). A protein with a molecular mass corresponding to that reported for the α5 integrin subunit (~140 kDa) [40,41] was observed upon immunoprecipitation with a monoclonal antibody raised against the α5 subunit. A smaller band at ~120 kDa that corresponds to the co-precipitated integrin β1 subunit [40,41] was also observed (Figure 3). Figure 3 also shows that non-confluent and confluent RPE cells express similar amounts of the biotinylated α5 subunit for the same number of cells at the cell surface. In contrast, post-confluent cells exhibited a marked decrease in cell surface expression of α5 when compared to proliferating cells.

Transcription of the α5 gene varies with increasing cell confluence: To investigate whether the difference in the expression of the α5 subunit protein at the RPE cell surface is the consequence of a reduced transcription of the α5 subunit gene, total RNA from cultured RPE cells was isolated and semi-quantitative RT-PCR was performed to evaluate the amount of α5 mRNA transcript present at different stages of cell confluence. When normalized to the amount of transcripts encoded by the ribosomal 18S gene, both non-confluent and confluent RPE cells expressed similar levels of α5 transcripts whereas post-confluent cells yielded a 41% decrease in α5 mRNA (Figure 4).

Given that the levels of integrin α5 subunit mRNA was influenced by cell confluence, we next asked whether this was due to differences in the transcriptional activity normally driven by the α5 gene promoter. The identification of the regions that are important for the regulation of the α5 promoter was examined by comparing the activity directed by different α5 promoter-bearing recombinant constructs following their transient transfections into cultured RPE cells grown at different cell confluences. The recombinant constructs selected for these assays contained the CAT reporter gene fused to different lengths from the human α5 gene promoter that extend to 5’ positions -954 bp (in plasmid -954α5CAT∆MCS), -178 bp (in plasmid -178α5CAT∆MCS), -132 bp (in plasmid -132α5CAT∆MCS), -92 bp (in plasmid -92α5CAT∆MCS) and -41 bp (in plasmid -41α5CAT∆MCS) relative to the α5 mRNA start site. As indicated in Figure 5, no significant CAT activity was obtained for plasmid -41α5CAT∆MCS under any of the cell culture conditions selected. Extending further the α5 pro-
motor up to position -92 resulted in a dramatic increase in CAT activity (232-fold, 255-fold and 51-fold increase for sub-confluent, confluent and post-confluent cells, respectively) that reached maximal level of activity at confluence and then slightly decreased when RPE cells reached post-confluence. These results clearly suggested the presence of one (or a few) strong positive regulatory element(s) on the α5 promoter segment is comprised between positions -41 to -92. Extending the α5 promoter from -92 to -132 and -178 resulted in a marked decrease in promoter activity (3-fold, 3-fold and 7-fold decrease between -92 and -178 for sub-confluent, confluent and post-confluent cells, respectively), suggesting that negative regulatory elements functional in RPE cells are located between these positions. Further extension up to position -954 resulted in no statistically significant alteration in the α5 promoter activity relative to the level directed by the -178α5CATAMCS plasmid (2.0-fold, 1.3-fold and 1.6-fold increase for sub-confluent, confluent and post-confluent cells, respectively).

Figure 5 also shows that all of the α5 promoter constructs used in this study directed maximal transcriptional activity at confluence, which then considerably decreased when RPE cells became quiescent (post-confluence), especially for the -132α5CATAMCS, -178α5CATAMCS and -954α5CATAMCS plasmids. The -92α5CATAMCS, -132α5CATAMCS, -178α5CATAMCS and -954α5CATAMCS plasmids yielded 1.3-fold, 1.4-fold, 1.5-fold and 1.0-fold increases, in CAT activity, respectively, between sub-confluent and confluent cells, although this increase was not found to be statistically signifi-
cant, except for the -132α5CATAMCS plasmid (p=0.0005). All but one construct (the -92α5CATAMCS) were found to have a statistically significant decrease between confluent and post-confluent cells. This reduction between confluent and post-confluent cells was 1.3-fold (p=0.02), 1.9-fold (p=0.0001), 3.3-fold (p=0.0003) and 2.6-fold (p=1.5x10^-8) for the -92α5CATAMCS, -132α5CATAMCS, -178α5CATAMCS and -954α5CATAMCS plasmids, respectively. We therefore conclude that α5 promoter sequences that determine basal promoter activity are located between α5 positions -41 to -92, and that cell quiescence does affect α5 promoter function in vitro.

**DISCUSSION**

PVR is a disease in which normally quiescent RPE cells start to dedifferentiate, detach from their basement membrane and resume growth [20-22]. They migrate, proliferate and secrete ECM molecules that form fibrocellular membranes on the retina or in the vitreous [20-22]. As the disease progresses, the membranes contract and cause tractional retinal detachment [20-22]. Migration, proliferation and dedifferentiation of RPE cells should involve membrane proteins like integrins since they have been shown to play a predominant role in such processes [7,8]. Moreover since FN was clearly shown to be involved in epiretinal membranes in PVR [42-44], it has been postulated that its membrane receptor, the integrin α5β1, could be involved in PVR [45]. We therefore used cultures of RPE cells as a model for dedifferentiated cells that are actively proliferating and studied the expression of the α5 integrin subunit at the protein, mRNA and gene levels.

In order to establish if the integrin α5 subunit is differentially regulated on recently dedifferentiated RPE cells that are actively proliferating, we first needed to establish whether this subunit is normally expressed in vivo. We found that the integrin α5 subunit is indeed expressed on human RPE cells in situ. This contrasts with the data of Brem et al. [19] who found no α5 in human RPE cells. The presence of α5 on RPE cells in vitro is less unsettled, and its presence has been well documented [12,16,32,34-39]. Indeed, indirect evidence that RPE cells do express the α5 integrin subunit has been provided in a recent study by Zhao et al. [39]. They demonstrated that phagocytosis of FN-coated beads by human RPE cells was markedly inhibited by exposure of such cells to antibodies directed against the α5 integrin subunit. Direct evidence that human RPE cells do indeed express the α5 integrin subunit was also provided by the same group through both FACS and RT-PCR analyses [16]. In that study, α5 integrin subunit expression was shown to be substantially increased through the activation of the MAPK signaling pathway upon exposure of RPE cells to TNF-α [16]. Although these studies provided evidence that the α5 integrin subunit is expressed in RPE cells, none examined whether cell confluence has any influence on this integrin subunit expression and whether such alterations also translate into altered α5 promoter function as well.

It has been shown that RPE cells in culture demonstrate different patterns of expression for the same protein depending on how much time cells are left at confluence [35]. For

**Figure 5.** Cell density dependence of the α5 promoter activity in RPE cells. Sub-confluent (beige box; n=24), confluent (black box; n=30) and post confluent (white box; n=9) cultures of human RPE cells were transiently transfected with recombinant plasmids bearing the CAT reporter gene fused to various promoter segments from the human α5 gene. Levels of CAT activity for the transfected cells were normalized to the amount of hGH secreted into the culture media. Values are presented with standard deviation. An asterisk at the right side of the boxes indicates a promoter strength statistically different between the sub-confluent and confluent cells, or between the confluent and post-confluent cells, as indicated by the brackets (ANOVA; p<0.001). See text for individual p values.
example, adhesion molecules of the integrin and CAM families showed a similar diffuse distribution of varying intensity among individual cells early in confluence, then each accumulated in a distinct pattern at the cell periphery when cell reached full confluence. The time required for each molecule to achieve its characteristic distribution differed, ranging from 1-2 weeks post-confluence for the integrin α5β1, 5 weeks post-confluence for ICAM and PECAM, and 2 months post-confluence for N-cadherin and NCAM [35]. Our immunofluorescence data (not shown) are consistent with these results. However, immunofluorescence does not provide quantitative information on the amount of protein expressed at the cell surface. We thus evaluated if this differing pattern of expression was reflected with a change in the amount of protein at the cell surface. In our experiments with cultured RPE cells, immunoprecipitation studies showed that proliferating cells express much more α5 at the cell surface than quiescent cells. Thus, unlike proliferating cells, quiescent cells may need different integrins at their cell surface and the presence of α5β1 may not be required in large amount. This is in accordance with other studies [41,46-48] which demonstrated that terminal differentiation of keratinocytes in culture also involves loss of α5β1 at the cell surface. Also, Sastry et al. [49] demonstrated that α5-transfected myoblasts remained in the proliferative phase and that differentiation was inhibited even in confluent cultures, suggesting that loss of integrin α5 expression may be required in order for differentiation to take place.

Our results provided evidence that cell quiescence also affected expression of integrin α5 subunit at the gene level. Both mRNA levels and promoter strength decreased between confluent and post-confluent cells. This indicates that promoter activity is at its highest when proliferating cells reach confluence and then there is a general down regulation of the α5 promoter when cells become quiescent. These results are consistent with those from other studies [48,50]. Indeed, the reduced transcription of integrin α5 subunit in keratinocytes has been shown to correlate with the expression of differentiation markers [48]. In addition, when human fibrosarcoma HT-1080 cells are released from quiescence, an increase in promoter activity, mRNA level as well as protein levels has been observed for the integrin α5 subunit [50]. In a more recent study conducted by our group, expression directed by the human α5 integrin subunit gene promoter was found to be strongly affected by cell density in primary cultures of rabbit corneal epithelial cells (RCECs) [51]. Indeed, the α5 promoter activity was found to be 9 to 13 times lower in confluent RCECs when compared to the level measured in non-confluent cells, indicating that the α5 promoter is regulated differently in different cell types. It must be pointed out that RCECs reach quiescence after only a few days at post-confluence.

When we look more closely at the regulation of the α5 promoter in RPE cells, we found that the sequence located between positions -41 to -92 functions as a powerful transcriptional activator. Previous studies demonstrated that the -62 to -132 α5 promoter region indeed functions as a positive regulatory region in the fibrosarcoma HT-1080 cell line [50]. The -41 to -92 α5 promoter segment was also shown to positively modulate α5 promoter activity in the promonocytic U937 cell line [52]. The α5 -41/-92 promoter sequence bears target sequences for the transcription factors AP-1 (between positions -45 and -51 bp) [53] and Sp1 (between positions -61 and -77 bp) [54]. Sp1 has recently been shown to bind a target site within this α5 promoter segment designated the fibronectin responsive element (FRE) and located between positions -56 to -82, which confers responsiveness of the α5 promoter toward FN [54]. Whether AP-1 or Sp1 are involved in the -41/-92 mediated up regulation of the α5 promoter activity in RPE cells remains to be elucidated.

The present study shows that when RPE cells are dedifferentiated and proliferate, promoter activity, mRNA transcripts and α5 protein levels are highly increased compared to quiescent cells. This finding is important in view of the possible involvement of this integrin subunit in PVR. Indeed, given that FN was shown to be expressed in epiretinal membranes in PVR [42-44], that the α5β1 integrin binds specifically to FN [7-9] and that α5β1 promotes cell proliferation and migration [14,16], it can be postulated that the α5 integrin subunit plays a major role in PVR in which RPE cells detach from the underlying Bruch’s membrane, migrate and proliferate [20-22]. This integrin subunit could thus represent a therapeutic target for PVR if the mechanism of its upregulation can be identified. In this regard, a recent study [36] demonstrated that human RPE cells grown in the presence of vitreous resulted in an elevation of expression of the α5 subunit. In PVR, RPE cells come in contact with vitreous through a tear in the neural retina [55]. Therefore, a detailed study on how the transcriptional activity directed by the α5 promoter is modulated following exposure of RPE cells to vitreous would most certainly contribute to the understanding of why this integrin subunit is upregulated during PVR.

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