Inhibition of Retinal Pigment Epithelial Cell Migration and Proliferation With Monoclonal Antibodies Against the β1 Integrin Subunit During Wound Healing in Organ Culture

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**Purpose.** To investigate the effect that antibodies against the β1 subunit of integrin, a cell-surface extracellular matrix receptor, would exert on chick embryo retinal pigment epithelial (RPE) cells maintained in organ culture after mechanical wounding of the epithelium.

**Methods.** RPE cells maintained in organ culture in the presence of antibodies against the β1 subunit of integrin were observed to quantify their spreading and migration. Antibodies against proliferating cell nuclear antigen (PCNA) were used to assess cell proliferation under the experimental conditions.

**Results.** In the presence of monoclonal antibodies against the β1 subunit of integrin, cell migration is inhibited whereas the initial cell spreading response still occurs. This implies that the RPE cells along the wound edge use different mechanisms in interacting with the substratum in spreading and in migration. Moreover, the RPE cells along the wound edge of cultures in which migration is inhibited do not express PCNA. Higher concentrations of the anti-integrin antibodies, however, are required to inhibit cell proliferation than to inhibit cell migration.

**Conclusions.** These results suggest that specific cell-substratum interactions may be involved in the initiation of a proliferative response to wound healing in this model system. Invest Ophthalmol Vis Sci 1993;34:2761-2768.
grains also appear to be involved in cell migration because some cell types, such as keratinocytes migrating on various substrata, show increased staining with antibodies directed against the β1 subunit relative to stationary cells.

To try to model the in situ interactions of RPE cells with their substratum—the basal lamina of Bruch’s membrane—we have developed and previously described an organ culture system in which wounds can be made and the response of cells to wounding analyzed. RPE cells migrating from the wound edge in this system were observed to form stress fibers and vinculin-positive focal contacts along their ventral surface at the ends of the stress fibers, similar to the focal contacts commonly observed along the ventral surface of various cell types in cell culture, including RPE cells. Furthermore, in wounds greater than approximately 125 μm in width, migrating RPE cells at the wound edge expressed the proliferating cell nuclear antigen (PCNA), indicating that these cells had been induced to enter the S phase of the cell cycle and to proliferate. Because this wound width corresponds approximately to slightly more than the diameter of two maximally spread RPE cells, we hypothesized that spreading alone was not sufficient to induce cell proliferation and that, instead, active migration of the RPE cells was required.

In contrast to spreading cells, migrating RPE cells exhibit stress fibers and vinculin-positive focal contacts. We wished, therefore, to investigate the possible relationship between contact formation and proliferation in our organ culture system. Specifically, we attempted to block the integrin-substratum interactions and migration of RPE cells by using monoclonal antibodies to the β1 subunit of integrin to determine if proliferation of RPE cells along the wound edge was also inhibited. The results obtained indicate that proliferation of RPE cells is inhibited when the cells are prevented from migrating but not from spreading by antibodies to the β1 subunit of integrin.

METHODS

These investigations conformed to the ARVO Resolution on the Use of Animals in Research.

Embryos

Fertilized chicken (Gallus domesticus) eggs were obtained from Glen Fenlon Farms (Toronto, Ontario) and incubated for 12 days in a humidified atmosphere at 37°C.

Culture Medium

To dissect and maintain the RPE in organ cultures, alpha minimal essential medium (Flow Labs, Mississauga, Ontario) containing 10% heat-inactivated fetal calf serum (Gibco, Burlington, Ontario) and 100 IU penicillin, .25 μg fungizone, and 100 μg streptomycin (Gibco) per ml were used.

Primary Antibodies

Mouse IgG monoclonal antibodies 2A10 against the β1 subunit of integrin and 3C10 against an RPE cell surface antigen; mouse IgG monoclonal antibodies W1B10 against the β1 subunit of integrin; and rabbit polyclonal antibodies against the cytoplasmic domain of the β1 integrin subunit were gifts. Mouse IgM monoclonal antibodies (clone 19A2) against PCNA were purchased from Coulter Immunology (Hialeah, FL).

Organ Culture

Organ cultures and tissue cultures were prepared as previously described. Briefly, eyes were aseptically removed from 12-day old chick embryos and placed in serum-free culture medium. Anterior segments and the vitreous were removed and discarded, and then the neural retina was gently teased from the eye cup, taking care to avoid damage to the RPE. After removing the optic nerve, the eye cup was divided in half. To support the organ cultures, strips of sterile polycarbonate, 0.45 μm pore size Nuclepore filters (Nuclepore, Pleasanton, CA) coated with Cell-Tak adhesive (Lot #89-0928, Collaborative Research Inc., Bedford, MA) were placed on top of sterile HA-type 0.45μm pore size Millipore filters (Millipore Ltd., Mississauga, Ontario) and submerged in the medium. Sheets of RPE attached to the choroid were gently removed from the sclera and placed choroid-side down on these filters. The filters with the attached tissue were then transferred to 24 well culture dishes (Falcon 3001, Becton Dickinson Canada Inc., Mississauga, Ontario) containing 500 μl of medium per well. Cultures were maintained in a humidified incubator in a 5% CO₂ atmosphere at 37°C.

Wounding of Organ Cultures

After 6 hours of incubation, some of the RPE cells were detached from Bruch’s membrane by repeated gentle stroking of the epithelial surface with a single human hair to create tapering linear wounds varying in width from approximately 25 to 1000 μm. Loose cells were removed from the wounded area by gentle flushing with the medium. After wounding, some of the cultures were transferred to fresh medium in new wells containing various concentrations of antibodies against β1 integrins. Control cultures were incubated in either medium devoid of antibodies, or in medium containing various concentrations of 3C10 antibodies. After 1, 24, and 48 hours of incubation, the cultures were photographed using a Wild M5A Stereomicroscope (Wild Leitz Canada, Willowdale, Ontario).
Image Processing

Photographs of healing wounds were electronically superimposed and enhanced using a high dynamic range TV camera (Dage-MTI 70 Chalnicon) (Dage-MTI Inc., Michigan City, IN) and digital image processing equipment (Image-Pro 100, Media Cybernetics on Imaging Technology Inc. VS-100-AT board) (Imaging Technology Inc., Woburn, MA) on a Compaq 386/25 computer (Compaq Computer Corp., Houston, TX). Linear measurements were made perpendicular to the wound edge at 100 μm intervals to determine the distance cells had moved during the previous 24 and 48 hours (Fig. 1).

Immunofluorescence

After 72 hours of culture, the tissues on the filters were fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) at 4°C for 1 minute and then placed in PBS at room temperature. The Millipore filter was then cut away and the remaining Nuclepore filter with overlying tissue was fixed in 100% methanol at -20°C for 10 minutes and then washed in PBS for 15 minutes. The tissue was then incubated with mouse monoclonal anti-PCNA IgM at a dilution of 1:100 in PBS for 1 hour at room temperature. After washing in PBS, it was further incubated for 40 minutes at room temperature with a 1:30 dilution of fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgA + IgG + IgM (Lot #KG62-5, Kirkegaard and Perry Inc., Gaithersburg, MD). After washing, the preparations were mounted on slides in Vinol (St. Lawrence Chem., Toronto, Ontario) containing 0.25% 1,4-diazabicyclo-(2,2,2)-octane (Polysciences, Warrington, PA) to reduce photobleaching. In controls, primary antibodies were replaced with PBS.

RPE cells cultured on glass coverslips were similarly prepared, although the initial fixation with formaldehyde was omitted. Preparations were incubated with W1B10 antibodies used at a dilution of 1:100 (16 μg/ml) and then, after washing in PBS, were incubated with a 1:30 dilution of FITC conjugated F(ab')2 fragment donkey anti-mouse IgG (H+L) (Lot #15143, Jackson Immunoresearch, West Grove, PA).

The preparations were examined with a Zeiss Photomicroscope III (Carl Zeiss Canada Ltd., Toronto, Ontario) equipped with epifluorescence optics and FITC-specific filters. Photographs were recorded on either TMAX 100, 400, or P3200 (Eastman Kodak, Rochester, NY) film and processed with TMAX developer (Kodak).

RESULTS

RPE Cell Focal Adhesions Contain β1 Integrins

Although previous investigations12-13 have shown that RPE cells contain β1 integrins, we wanted to confirm that the antibodies we obtained positively stained chick RPE cells in tissue culture. The results obtained demonstrate that the W1B10 antibodies stained focal contacts and the larger focal adhesions7 located in the peripheral regions of well-spread RPE cells (Fig. 2). The staining pattern was similar to that previously obtained with antibodies to integrins in other cell types14'15 in that the margins of the focal adhesions labeled more strongly than their central regions. Similar results were obtained with a variety of fixation and permeabilization techniques attempted (methanol, methanol/acetone, formaldehyde, paraformaldehyde, Triton X-100). Because the W1B10 antibodies are directed against an extracellular epitope on the β1 integrin subunit and because the plasma membrane is in close contact with the substratum in these regions, the observed staining pattern may result from limited access to or physical exclusion of the antibodies, or both, from sites of close contact with the substratum.15 A similar staining pattern was also noted, however, when using polyclonal antibodies against the 37 amino acid oligomers of the cytoplasmic carboxyl terminal tail of human β1 integrin subunit, thus arguing against this hypothesis (results not shown).

Inhibition of RPE Cell Migration by Antibodies to the β1 Subunit of Integrin

As previously reported,5,6 the RPE cells along the wound edge rapidly spread and migrated into the wounded regions over the denuded basal lamina. In the presence of antibodies against β1 integrin, however, the migration of RPE cells into the wound was markedly impaired. Quantitative measurements of the
rate of cell migration into the wound in cultures treated with 2A10 and W1B10 antibodies are shown in Figure 3. Newman-Keuls multiple comparisons procedures for one-way analysis of variance revealed significant differences ($P < 0.01$) in the rate of cell migration between antibody-treated (W1B10 and 2A10) and control cultures at all dilutions tested. Each antibody was tested using a minimum of six samples with an average of 36 measurements per sample.

The morphology of RPE cells distant from the wound edge was not obviously affected by the antibody treatment. This is consistent with previous results that demonstrated that spread RPE cells adhering to fibronectin-coated substrata were not affected by 2A10 antibodies. It is interesting, however, that RPE cells spread on laminin substrata detached when exposed to the 2A10 antibody under similar conditions.

In wounded cultures incubated in the presence of 3C10 antibodies, which have been shown to bind to the cell surface of chick RPE cells, the spreading and the rate of migration of RPE cells was not significantly inhibited when analyzed using the above statistical procedure (Fig. 3). The inhibitory effect of the 2A10 and W1B10 antibodies, therefore, was specific and was not caused by the mere presence of mouse IgG in the culture medium.

Comparison of the Effect of Antibodies to the $\beta_1$ Subunit of Integrin on RPE Cell Spreading and Migration

Although the wound closure was greatly inhibited by the antibodies to the $\beta_1$ subunit of integrin, it was never completely blocked. At the highest concentrations of antibodies tested, 64 $\mu$g/ml for W1B10 and 400 $\mu$g/ml for 2A10, each of the wound edges moved closer by 41.8 ± 14.3 $\mu$m and 47.2 ± 24.4 $\mu$m in the first 24 hours, respectively. These values are similar to previously determined values for the diameter of RPE cells maximally spread on Bruch’s membrane, which was estimated to be 44 ± 5 $\mu$m. This implies that the partial closure of the wound observed in the presence of high concentrations of anti-$\beta_1$ integrin antibodies may be caused by spreading that was not noticeably impaired. Furthermore, this also suggests that in RPE cells in contact with their normal substratum, the Bruch’s membrane, different mechanisms of adhesion, extracellular matrix receptors, or both, are involved in cell spreading and in cell migration.

Inhibition of RPE Cell Proliferation by Antibodies to the $\beta_1$ Subunit of Integrin

When wounded control (medium devoid of antibodies as well as medium containing 3C10 antibodies) organ cultures were examined by indirect immunofluorescence for the presence of PCNA 72 hours after wounding, RPE cells along the wound edge in the narrower completely healed regions (Fig. 4a) and in the wider unhealed wounds (Fig. 4b) were positively stained. These results are consistent with our previous results, which demonstrated that maximal expression of PCNA in RPE cells in organ culture occurred approximately 72 hours after wounding. A similar examination of wounded cultures maintained in the presence of 2A10 antibodies at a concentration of 100 $\mu$g/ml (Fig. 4c), demonstrated that a large proportion of RPE cells along the wound edge were still positively stained for PCNA. It is interesting to note that at this concentration of 2A10 antibodies, RPE cell migration was significantly inhibited (Fig. 3). Fewer PCNA-positive RPE cells, however, were noted along the wound edges of cultures maintained in a 200 $\mu$g/ml concentration of 2A10 antibodies (Fig. 4d). Furthermore, at 400 $\mu$g/ml concentrations of 2A10 antibodies (Fig. 4e) and at 64 $\mu$g/ml concentrations of W1B10 antibodies (Fig. 4f), PCNA positive RPE cells were virtually absent. Of the approximately 800 RPE cells examined along the wound edge of organ cultures maintained at these concentrations of antibodies, none were found to be positive with W1B10 antibodies and only three with 2A10 antibodies. These results indicate that the antibodies to the $\beta_1$ subunit of integrin inhibits both PCNA expression and cell migration in this system. The inhibition of PCNA expression, however, appears to require higher concentrations of anti-$\beta_1$ integrin antibodies than did the inhibition of migration. This may, however, only indicate that the statistical methods used to assess cell migration were more sensitive.
Inhibition of RPE Wound Healing in Organ Culture

**DISCUSSION**

In this paper, we examine the wound healing response of RPE cells in organ culture in the presence of two different antibodies directed against the β1 subunit of integrin. Quantitative data indicate that the response observed under these conditions may be almost totally attributed to cell spreading and that this process is not significantly altered by the antibodies. In contrast, both antibodies significantly attenuated wound closure, demonstrating that the migration of RPE cells on Bruch’s membrane is effectively inhibited.

These results are in contrast to results from previous studies showing that 2A10 antibodies inhibited both the spreading and the migration of RPE cells cultured on fibronectin and on laminin substrata. This difference may result from the presence of additional adhesive extracellular matrix molecules in the considerably more complex substratum, the Bruch’s membrane, to which the RPE cells adhere and on which they spread in organ culture. Alternatively, adherent RPE cells spreading into a wound may use different adhesive mechanisms than RPE cells in suspension, which spread after coming into contact with a suitable substratum.

The apparently selective effect of the antibodies inhibiting the migratory response while allowing cell spreading to occur also suggests that spreading and migration are distinct cellular processes, each using specific extracellular matrix receptors. Alternatively, the RPE cell spreading observed under these conditions may be viewed as an initial event in the migratory response. In the absence of β1 integrin mediated contacts, the cell-substratum adhesion developed by the RPE cells may be only strong enough to permit spreading but not the migration of RPE cells. This is consistent with previous observations that during the migration of the epithelial sheet, the three to four rows of RPE cells nearest to the wound edge develop stress fibers (not present during the initial spreading response) and “lean” away from the wound when observed in a section cut perpendicularly to its edge, suggesting that these cells generate the force for cell migration along their basal regions while being re-

![Graph showing the effects of W1B10 (a) and 2A10 (b) antibodies on RPE cell migration in response to wounding in organ culture.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933175/)
FIGURE 4. Micrographs of RPE cells in organ culture stained for PCNA 72 hours after wounding when cultured (a) in the absence of antibodies and (b) in the presence of 360 µg/ml of 5C10 antibodies, (c) 100 µg/ml, (d) 200 µg/ml, (e) 400 µg/ml of 2A10 antibodies, and (f) 64 µg/ml of W1B10 antibodies. In all micrographs, unwounded regions of RPE are indicated by an asterix and the wound edge in (e) and (f) by arrowheads. In (a) and (b), note labeled PCNA-positive cells in healed regions of both narrow (a) and wider (b) wounds. In (c), many RPE cells in a narrow healed region of the wound are labeled for PCNA. In (d), there are some PCNA positive cells along the wound edge. In (e), a rarely seen positive RPE cell is indicated by an arrow. The RPE cells in (f) show no evidence of labeling for PCNA. In (d, e, and f) the positive labeled cells in the underlying choroid are seen through the denuded regions of basal lamina. Bar = 20 µm.
strained by junctions at their apical ends. In contrast, RPE cells away from the wound edge lean toward the wound as if they were being pulled in this direction by the first few rows of cells via their apical zonulae adhaerentes junctions. The blocking of β1 integrins by the antibodies, therefore, may not allow the cells near the wound edge to adhere sufficiently to the substratum to overcome the restraining tension in the epithelial sheet for cell migration to occur.

We have also demonstrated in this study that the presence of antibodies against β1 integrin subunits, the expression of PCNA in RPE cells along the wound edge is greatly inhibited. This result is of great interest because the possible role of integrin receptors in regulating cellular functions, such as proliferation, is currently the subject of some controversy. Because integrins have been shown to possess tyrosine phosphorylation sites in their cytoplasmic domains, one possible hypothesis suggests that adherent cells could be directly regulated by specific integrin-ligand interactions. In human T cells, for example, fibronectin-stimulated proliferation is mediated by α4β1 and α5β1 integrin receptors.

Alternatively, cell-cell and cell-substratum interactions could affect cell shape, which in turn could regulate cell proliferation through as yet undefined mechanisms mediated by the cytoskeleton. In this model, the specific receptor involved in cell-substratum adhesion, cell-cell adhesion, or both, would not be as significant as the change in cell shape that resulted from the interaction. Recent experiments have tried to differentiate between these two mechanisms. In rabbit synovial fibroblasts, for example, changes in gene expression that are regulated by integrin receptors are induced by a mechanism that is distinct from signaling through changes in cell shape.

Furthermore, we have previously shown that the shape changes associated with spreading are insufficient to induce PCNA expression and DNA synthesis during wound healing in this system. The present results support those previously reported, and both studies suggest that cell migration is associated with PCNA expression. Based on these results, one may not rule out the possibility, however, that cell shape changes function in concert with specific receptor (integrin) ligand interactions in controlling the proliferative response. At present, therefore, the precise mechanisms by which β1 integrin antibodies inhibit cell proliferation in this system are not known.

Key Words

wound healing, organ culture, retinal pigment epithelium, PCNA, integrins, migration

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References

15. Burridge K, Fath K. Focal contacts: Transmembrane