Functional Inhibition of Retinal Pigment Epithelial Cell–Substrate Adhesion With a Monoclonal Antibody Against the \( \beta_1 \) Subunit of Integrin

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In the preceding report, experiments were described which identify the 2A10 antigen of retinal pigment epithelial (RPE) cells as a \( \beta_1 \) subunit of the cell-surface extracellular matrix receptor protein integrin. In this article, experiments are presented which use the 2A10 and CSAT antibodies, both directed against the \( \beta_1 \) subunit, to investigate the role of integrin in RPE and fibroblast (FB) cell–substrate adhesion. When added to cultures simultaneously with cells, either the 2A10 or CSAT antibodies inhibit both FB and RPE cell adhesion and spreading on laminin (LM). However, although the 2A10 antibody blocks adhesion and spreading of FB and RPE cells on fibronectin (FN), the CSAT antibody has no effect. The inhibition of the 2A10 antibody is specific for integrin-mediated adhesion; it does not affect FB or RPE cell adhesion and spreading on tissue-culture plastic. When RPE cells are first allowed to attach to and spread on FN and LM and then the 2A10 or CSAT antibody is added to the cultures, both cause detachment and rounding of RPE cells from LM, but neither has any effect on the cells already spread on FN. These results indicate that there are differences in the way FB and RPE cells interact with LM and FN. Furthermore, these results provide the first direct functional demonstration that RPE cell–substrate adhesion is mediated by integrin. Invest Ophthalmol Vis Sci 32:1763–1769, 1991

The integrity and polarity of the retinal pigment epithelium (RPE) is in part dependent on appropriate molecular signals provided by Bruch’s membrane, the extracelluar matrix on which the RPE rests. As such, insight into the molecular basis for the interaction of RPE cells with components of Bruch’s membrane is essential for a complete understanding of both normal RPE function and the aberrant behavior of RPE cells under pathologic conditions, such as proliferative vitreoretinopathy (PVR). Although soluble vitreous factors may affect the behavior of RPE cells in PVR, altered cell–substrate interactions may also be involved. In addition to maintaining the integrity of the mature RPE, the development and differentiation state of RPE cells may be influenced by extracellular matrix components. Specific cell–substrate interactions have been implicated in the control of RPE cell transdifferentiation whereby mature RPE cells undergo a phenotypic transformation into neural retina or lens cells.

The known molecular components of Bruch’s membrane include the extracellular matrix proteins fibronectin (FN), laminin (LM), type IV collagen, and heparan sulfate. Among the molecules which have been identified as receptors for extracellular matrix molecules in many cell types are the integrins. A family of cell-surface transmembrane heterodimeric proteins composed of distinct \( \alpha \) and \( \beta \) subunits. Integrin has been shown to be present in the RPE, and it is presumed to serve a similar cell–substrate adhesive function in cells of the RPE as in other tissues in which it has been studied. However, such a function in the RPE has not been demonstrated directly. We previously described the initial identification and characterization of the 2A10 antigen, a monoclonal antibody-defined cell-surface molecule found in chick RPE cells. In the preceding accompanying report, we described experiments which identified the 2A10 antigen of chick RPE cells as a \( \beta_1 \) subunit of integrin. In this article, we describe in vitro functional studies using the 2A10 monoclonal antibody which provide, for the first time, direct evidence indicating that integrin mediates the formation of cell–substrate adhesion between RPE cells and the extracellular matrix proteins LM and FN.
Materials and Methods

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

Immunologic Reagents

The production and characterization of monoclonal antibody 2A10, which recognizes the β1 subunit of avian integrin, has been described previously.\(^{13-15}\) For these studies, the 2A10 antibody obtained from hybridoma supernatant was concentrated with ammonium sulfate, dialyzed against Tris-buffered saline (TBS), containing 0.01 M Tris HCl, pH 7.5, and 0.15 M NaCl, and adjusted to 10 mg/ml protein. The CSAT monoclonal antibody, which also recognizes the β1 subunit of avian integrin, was the generous gift of Dr. Clayton Buck (Wistar Institute, Philadelphia, PA).

Cell Culture

Fibroblast (FB) cultures were prepared from the skin of 10-day-old chick embryos by dissection in HEPES-buffered saline with glucose containing 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.4, 0.15 M NaCl, 3 mM KCl, 1 mg/ml of glucose, and 10 μg/ml of phenol red (HBSSG) and digestion of minced tissue fragments in 0.025% w/v trypsin in HBSSG (HBSG) for 10 min at 37°C. The dissociated cells were removed from the posterior half. The RPE was peeled out of the posterior half of the eye and incubated for 30 min at 37°C. The RPE was then triturated to disperse cells, and further incubated for 30 min at 37°C. The eyes were then transferred into 0.1% trypsin in HBSSG/Ca for 40 min at 37°C. Whole eyes were then washed three times with F12 medium, and cultured in F12. All cultures were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO₂ and contained penicillin, streptomycin, and amphotericin B at 100 units/ml (Hazelton, Lenexa, KS). All experiments using RPE cells were done as short-term experiments with primary cultures of freshly prepared RPE cells without subculturing. As a consequence, the cultures contained individual RPE cells, small RPE cell clusters, and a small number of FBs and moribund cells. No attempt was made to purify the cultures. The cell culture fields were chosen at random for photography.

Cell Adhesion and Spreading Inhibition Experiments

Cultures of both chicken skin FB and RPE cells were tested for the effects of antibodies on cell-substrate adhesion and spreading. Adherent and nonadherent cells were distinguished by disturbing the culture medium and observing the movement of cells; no quantitation of adhesion per se was made. Spread FB and RPE cells were distinguished clearly from unspread cells by their spindle or epithelioid shapes, respectively, as opposed to rounded cells. Polystyrene tissue culture treated dishes (35 mm; Falcon, Lincoln Park, NJ) were first coated either with 1 μg/cm² FN (bovine plasma; Sigma) in TBS (pH 7.5) or with 1 μg/cm² LM (murine EHS tumor; Sigma) in TBS at room temperature for 1 hr. The coated dishes were washed twice with TBS/Ca before use. The prepared cells were then added to the dishes at 3–4 x 10⁴ cells/ml of F12 medium per dish. Cultures were maintained as described. For adhesion-inhibition studies, antibodies were added either at the beginning of the culture period or after the cells had been allowed to adhere and spread for different experiments as detailed subsequently. The 2A10 antibody was used at 100 μg/ml and the CSAT antibody was used at 40 μg/ml.

Results

The 2A10 and CSAT Antibodies Specifically and Differentially Inhibit FB Cell-Substrate Adhesion

Since cell-substrate adhesion has been studied most extensively using FB cells, these were first used to compare the effects of the new 2A10 antibody with the previously characterized CSAT antibody. The FB cells adhered and spread rapidly when cultured on petri dishes precoated with either FN (Fig. 1A) or LM (Fig. 1B). The cells adhered within 15 min and were well spread within 2 hr. When the 2A10 antibody was added to the cultures simultaneously with cells, attachment and spreading of FB cells was inhibited completely on both FN (Fig. 1C) and LM (Fig. 1D). Under these conditions, the cells remain rounded and unat-
Fig. 1. Inhibition of FB cell-substrate adhesion with the 2A10 and CSAT antibodies. FB cultures were prepared as described in Materials and Methods. The cells were cultured for 4 hr in petri dishes precoated with either FN (A, C, E) or LM (B, D, F). The cultures contained either no antibodies (A, B) 2A10 (C, D), or CSAT (E, F) added simultaneously with the cells at the initiation of the culture. 2A10 blocks FB adhesion to both FN and LM, whereas CSAT blocks only adhesion to LM. Magnification x20.

Fig. 2. Inhibition of FB cell-substrate adhesion by 2A10 is specific for FN- and LM-mediated adhesion. FB cultures were prepared as described in Materials and Methods. The cells were cultured for 2 (A-F) or 4 (G-L) hr on noncoated tissue culture dishes (A, D, G, J) or on coated petri dishes treated with either FN (B, E, H, K) or LM (C, F, I, L). Control cultures received no antibody (A-C, G-I) whereas the others received 2A10 antibody (D-F, J-L) at the initiation of the culture. The 2A10 antibody has no effect on cell adhesion and spreading on tissue-culture dishes but inhibits adhesion and spreading on FN- and LM-coated dishes. Magnification x15.

attached to either substrate. The effect of the 2A10 antibody differed from that of the CSAT antibody. When the CSAT antibody was added simultaneously with FB cells at the beginning of the culture period, adhesion and spreading on FN was unaffected (Fig. 1E); the cells were prevented from adhering and spreading on LM (Fig. 1F).

To demonstrate the specificity of the inhibitory effect of the 2A10 antibody, FB cells were cultured in tissue culture-treated dishes and in petri dishes precoated with either FN or LM. The cells adhered and spread on tissue culture-treated dishes due to electrostatic forces in the absence of additional substrate components and in an integrin-independent fashion; adhesion and spreading on petri dishes was dependent on the addition of FN or LM and occurred in an integrin-dependent fashion. Thus, the behavior of cells under these different conditions may be used to assess the specificity of the effect of the 2A10 antibody. When FB cells were cultured on tissue culture-treated dishes in the absence of added adhesion factors, adhesion and spreading occurred (Figs. 2A, 2G) although somewhat less efficiently than when cells were cultured on FN (Figs. 2B, 2H) or on LM (Figs. 2C, 2I). However, adhesion and spreading on the tissue culture treated dishes was not affected by the 2A10 antibody (Figs. 2D, 2J), which completely inhibited adhesion and spreading on FN (Figs. 2E, 2K) or LM (Figs. 2F, 2L) in parallel cultures. These results indicate that the 2A10 antibody did not have a generalized toxic effect on the cells, and the antibody did not interfere in a general way with adhesion and spreading of the cells. Rather, the results indicate that the 2A10 antibody specifically inhibits integrin-dependent cell ad-
hesion and spreading, presumably due to direct binding and interference with the function of integrin.

The 2A10 and CSAT Antibodies Specifically and Differentially Inhibit RPE Cell-Substrate Adhesion

To examine the effect of antiintegrin antibodies on RPE cell-substrate adhesion directly, experiments which parallel those described for FB cells were done with RPE cells. Although RPE cells do not attach to or spread on uncoated petri dishes (data not shown), they attach and spread well on petri dishes precoated with FN (Figs. 3A–D). The clusters of spread RPE cells appear to have an epithelial morphology, tightly adhering to one another at areas of intercellular contact, and extending fine processes at the peripheral free edges of the clusters (Figs. 3B, 3D). When the 2A10 antibodies were added at the beginning of the culture period, adhesion and spreading of RPE cells to FN was inhibited, although the cells remained in clusters (Figs. 3E–H). When the CSAT antibody was added at the beginning of the culture period, many attached and spread clusters of RPE cells on FN could still be observed (Figs. 3i–L).

When RPE cells were added to petri dishes precoated with LM, they also attached to and spread on the dishes (Figs. 4A–D). Again the RPE cell clusters were tightly adherent in regions of intercellular contact, with many fine extensions in the periphery of cell clusters (Figs. 4B, 4D). The adhesion and spreading of RPE cells on LM was inhibited by both the 2A10 (Figs. 4E–H) and CSAT (Figs. 4i–L) antibodies. Thus, the inhibitory effects of antiintegrin antibodies on RPE cell-substrate adhesion paralleled those on FB cell-substrate adhesion, with the 2A10 antibody blocking RPE cell adhesion and spreading on both FN and LM and the CSAT antibody only affecting RPE cell adhesion and spreading on LM.

The specificity of the effect of 2A10 antibodies on RPE cell adhesion was also tested by adding the antibody to RPE cells cultured on tissue culture-treated dishes. As in the case of FB cells, RPE cells also adhered and spread on these dishes (Figs. 5A–D). How-
Fig. 5. RPE cell-substrate adhesion on tissue culture-treated plastic is not inhibited by the 2A10 antibody. RPE cells were prepared and cultured directly on tissue culture-treated dishes in the absence (A-D) or presence (E-H) of 2A10 antibodies as described in Materials and Methods. The cultures were photographed at 4 (A, B, E, F) and 10 (C, D, G, H) hr of incubation at ×18 (A, E, C, G) and ×72 (B, F, D, H). In the low-magnification fields, the RPE cells which are indicated by arrowheads, are the same as shown for high magnification. Unlike RPE cells in petri dishes whose adherence is dependent on LM or FN, those adhering directly to tissue culture-treated plastic are not affected by the 2A10 antibody.

However, this adhesion was not affected by the 2A10 antibody (Figs. 5E–H). These results indicate that the 2A10 antibody was neither generally toxic to RPE cells nor interfered with the general process of adhesion and spreading. Rather, the results further indicate that the 2A10 antibody specifically inhibited RPE cell adhesion and spreading on FN and LM through perturbation of integrin.

The 2A10 and CSAT Antibodies Differentially Reverse the Adhesion of Already Spread RPE Cells on FN and LM

The specificity of the 2A10 and CSAT antibody effects on RPE cell-substrate adhesion was tested further by adding the antibodies to cultures of RPE cells which had already been allowed to become established on FN and LM substrates. After 4 hr of culture, RPE cells were found to be attached and well spread on petri dishes which had been precoated with either FN (Figs. 6A–B, 6E–F) or LM (Figs. 6I–J, 6M–N). When either 2A10 (Figs. 6C–D, 6K–L) or CSAT (Figs. 6G–H, 6O–P) antibodies were added to these established cultures, neither antibody affected the morphology of RPE cells spread on FN (Figs. 6C–D, 6G–H); both antibodies caused the detachment and rounding of RPE cells previously attached and spread on LM (Figs. 6K–L, 6O–P). The detachment of cells from LM could be seen as early as 1 hr after addition of antibody (data not shown). These results are a further indication that neither the 2A10 nor CSAT antibodies are generally toxic since they had no effect on...
RPE cells prespread on FN even after 6 hr of exposure; their effect on cells attached to LM was rapid. Furthermore, the results indicate that events subsequent to the initial adhesion and spreading of RPE cells differed with respect to FN and LM, i.e., the 2A10 antibody can cause the rounding of already spread and attached RPE cells from LM but not from FN even though initial adhesion to both was blocked by the antibody.

### Discussion

The distribution and presumed functional interaction of integrin, FN, and LM are generally coincident in various tissues. In the chick, the role of integrin in mediating cell-substrate adhesions between RPE cells and the basal lamina on which it sits has been inferred from immunocytochemical localization studies which demonstrated the presence of integrin in the RPE. Both FN and LM have also been demonstrated to be present in the chick RPE basal lamina. Adhesion of RPE cells to FN is inhibited competitively by peptides containing the tripeptide sequence RGD (arginine, glycine, aspartic acid), found in FN and recognized by integrin. Although a cell-substrate adhesive role for integrin in the RPE was suspected, our experiments provide the first direct evidence that integrin serves as a ligand linking RPE cells to matrix proteins such as FN and LM.

The FB cells have often served as the paradigm for studies of cell-substrate adhesion and were among the first cell types whose adhesion was demonstrated to be inhibited by antiintegrin antibodies. Other cell types whose adhesion is also affected by antiintegrin antibodies are myoblasts, neural crest cells, and neurons. However, the adhesive properties of different cell types on different substrates are not equally susceptible to the adhesion-perturbing effects of these antibodies. We used skin FBs for our comparison with RPE cells and found that they both respond similarly in our experiments. The biochemical and immunocytochemical analyses reported in the accompanying article suggest that FB and RPE integrins are similar in their electrophoretic mobility and immunoreactivity with the 2A10 antibody. The functional similarities between RPE and FB cells we found are consistent with the biochemical findings, i.e., cells adhering by similar molecules would be expected to demonstrate similar behaviors in vitro when the factors being manipulated are limiting. In vivo and, to some extent, in vitro, FB cells have a mesenchymal morphology, and RPE cells have an epithelial morphology. Thus other factors which remain to be elucidated, in addition to the integrin-mediated adhesions to LM or FN demonstrated here, may be expected to contribute to the overall morphology of RPE cells. Such factors may include cell-cell adhesion mediated by N-cadherin, which we previously demonstrated to be present in the RPE.

As shown, the 2A10 and CSAT antibodies, although both directed against β1 subunits of integrin, are not functionally identical in their adhesion-blocking capacity. At the concentrations used in our study, the CSAT antibody is more effective at inhibiting cell adhesion to LM than to FN, and the 2A10 antibody is an effective inhibitor against both substrate molecules. Although heterodimers, all avian integrins identified to date share a common β1 subunit; the interaction of integrins with different molecules depends in part on the nature of the α subunits. Thus the β1 subunit (recognized by the 2A10 and CSAT antibodies) may be combined with one or more α subunits, and the resulting distinct heterodimers in turn may interact with different molecules. It is possible that the 2A10 and CSAT antibodies, while directed against the same molecule, recognize and bind to distinct epitopes. If these epitopes are involved differentially in recognition of FN and LM, and/or these sites are unequally exposed and accessible to the 2A10 and CSAT antibodies in different heterodimers, this could account for the substrate-dependent differences observed with these two antibodies.

A related explanation may also account for the observation that inhibition of initial adhesion and detachment of already spread cells are not caused equally by the 2A10 and CSAT antibodies. Both antibodies were found to block initial adhesion of RPE cells to, and also cause detachment of RPE cells from, LM. The CSAT antibody was neither effective in blocking initial adhesion to, nor in causing detachment from, FN. However, although the 2A10 antibody was found to block initial adhesion of RPE cells to FN completely, it had little, if any, effect on these cells once they were attached and spread on FN. The different effects on early and late stages of cell-substrate adhesion to FN, as opposed to LM, may be due to either a steric hindrance of accessibility to already formed adhesion sites or to some specific biomechanical difference between FN- and LM-mediated adhesions. For example, it is possible that RPE cells spread on a FN substrate adhere closer and/or tighter than those on LM, preventing the antibodies from diffusing under the cells. Alternatively, since detachment of already spread cells implies that the antibodies are capable of competing with FN or LM for sites on integrin, it is possible that the affinity of RPE cell integrins is greater for FN than for LM, rendering the integrin–FN bonds less reversible. A third possibility is that subsequent to the formation of integrin–FN bonding, additional biochemical steps occur to render...
the RPE cell–substrate attachment less labile and that these steps do not occur after the formation of integrin–LM bonds.

In conclusion, our results demonstrate that the formation of cell–substrate adhesions by RPE cells to FN and LM is mediated by integrin. Furthermore, the effects of perturbations to these adhesions are dependent on the nature of the substrate to which the RPE cells are attached. These observations are interesting because the behavior of RPE cells under pathologic conditions, such as PVR, could be influenced, in part, by the nature of, and perturbations to, the cell–substrate adhesion molecules to which the RPE cells are exposed. Future experiments may determine the nature of the in vivo molecules which serve as substrates for RPE cells both in their normal environment, as in Bruch’s membrane, and under pathologic conditions, as in the vitreous humor.

Key words: retinal pigment epithelium, RPE, monoclonal antibody, cell–substrate adhesion, integrin

References