A Peptide From Fibronectin Cell-Binding Domain Inhibits Attachment of Epithelial Cells

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Fibronectin is thought to contribute to the adhesion of corneal epithelium during epithelial repair, presumably by mediating epithelial attachment to stroma during epithelial migration. To understand the mechanism of the interaction of fibronectin with corneal epithelial cells, the effects of intact plasma fibronectin and of the synthetic peptide GRGDS from the cell binding domain of fibronectin on the attachment of rabbit corneal epithelial cells have been examined. When dissociated epithelial cells were plated on fibronectin-coated culture dishes, the number of cells that attached increased in proportion to the concentration of fibronectin used for coating. Attachment of the cells was inhibited in a dose-dependent manner by the addition of GRGDS to the medium. When the structurally similar control peptide GRGES was added to the medium, no inhibition of epithelial attachment was observed. Our results demonstrate that corneal epithelial cells use an adhesive recognition system for fibronectin related to that characterized in fibroblastic cells. GRGDS thus presumably competes for receptors for fibronectin on epithelial cells of the cornea, resulting in the observed inhibition of attachment. These results identify a mechanism for corneal epithelial cell adhesion, compatible with that identified in other fibronectin-dependent systems. Invest Ophthalmol Vis Sci 29:1820–1825, 1988

Successful corneal epithelial resurfacing after injury requires attachment and migration over a temporary matrix of fibrin and fibronectin.1-3 After severe injury to the cornea, persistent epithelial defects can develop which are correlated with subsequent stromal ulceration.3 Although the pathobiology of persistent defect formation is not well understood, an unregulated plasminogen activator (PA)/plasmin system is thought to degrade a temporary fibrin/fibronectin substratum on the corneal surface to result in diminished adhesion, an epithelial defect and subsequent stromal ulceration.3

The ability of topically applied autologous plasma fibronectin to promote healing of persistent human epithelial defects4-6 and of rabbit plasma fibronectin to stimulate rabbit corneal epithelial migration in vivo7 and in vitro8 has been reported previously. The fibronectin molecule consists of disulfide-linked subunits containing a variety of binding domains with different specificities.9,10 One of these domains, termed the cell binding domain, binds to the cell surface receptor (integrin) for fibronectin. The tetrapeptide sequence, RGDS11,12 or the pentapeptide, GRGDS (glycine-arginine-glycine-aspartic acid-serine)13,14 from the cell binding domain has been found to be essential for fibronectin-mediated cell attachment. In some cell systems, including fibroblast adhesion to fibronectin, the addition of GRGDS inhibits the attachment of cells.11,14 Other domains of fibronectin are thought to bind to different components of the extracellular matrix (ECM) such that structural continuity via fibronectin from the cell surface to the ECM is obtained (including binding to components of basement membrane or to fibrin after injury).9,10,15

To examine the mechanism of the interaction of fibronectin with corneal epithelial cells, we investigated the effects of GRGDS on fibronectin-mediated attachment of rabbit corneal epithelial cells in vitro. The structurally similar pentapeptide GRGES (glycine-arginine-glycine-glutamic acid-serine), which lacks competitive inhibitory activity in other systems,13,14 was used as a control peptide for GRGDS.

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Materials and Methods

Albino rabbits (2.5 to 3 kg) were purchased from Shimizu Animals (Kyoto, Japan). TC-199 culture medium, trypsin (0.25%) and EDTA solution (0.53 M) were obtained from the Research Foundation for Microbiological Diseases of Osaka University (Suita, Osaka, Japan). Dispase (300,000 units/g) was from Sanko Junyaku (Tokyo, Japan). Hepes buffer (2 M) was from Flow Laboratories (McLean, VA). Fetal calf serum (FCS) was from Commonwealth Serum Labs (Melbourne, Australia). Multiwell tissue culture plates (96 wells) were from Corning Glass Works (Corning, NY). Tissue culture chamber/slides (#4808) were from Miles Scientific (Naperville, IL). Gelatin-coupled Sepharose 4B was from Pharmacia Fine Chemicals (Uppsala, Sweden). Bovine serum albumin (BSA, fraction V, 5 times crystallized) was from Armour Pharmaceutical Co. (Kankakee, IL). The synthetic peptides GRGDS and GRGES were synthesized to our specifications by Peninsula Laboratories (Belmont, CA). The semipurified peptides were further purified to homogeneity by reverse-phase HPLC on a 2.1 × 25 cm C18 column (Dynamax, Rainin Instrument Co., Woburn, MA). This study adhered to the ARVO Resolution on the Use of Animals in Research.

Cell Culture

Rabbits were euthanized by intravenous injection of an overdose of sodium pentobarbital (60 mg/kg body weight). Whole corneas were excised, and the endothelium and Descemet’s membrane were removed mechanically by jeweler’s forceps under a dissecting microscope. Epithelium on subjacent stroma was incubated with Dispase (2 mg/ml in TC-199 medium) at 37°C for 45 min. After incubation, epithelial sheets were removed from corneal stroma by jeweler’s forceps. Sheets were then cut into small pieces by razor blade and treated further by trypsin (0.125%) and EDTA (0.26 M) at 37°C for 15 min. A single cell suspension was obtained by repeated aspiration through a 21 gauge needle. Resultant suspensions were washed by centrifugation (1200 g) in TC-199 medium containing 15% FCS. Then epithelial cells were cultured in TC-199 medium containing 15% FCS in a humidified incubator (37°C, 5% CO₂ in air). After 4 days, the cells were harvested by washing with phosphate buffered saline and incubating with 0.125% trypsin and 0.26 M EDTA. The single cell suspension obtained was further washed three times with unsupplemented TC-199 medium by centrifugation, and cell number was adjusted to 2 × 10⁶ cells/ml after counting in a hemocytometer.

Coating of Plates

Rabbit plasma fibronectin was purified by gelatin-coupled Sepharose-4B according to the method of Engvall and Ruoslahti. Fibronectin was dialyzed against phosphate buffered saline. Fibronectin concentrations were determined by the method of Lowry.
Fig. 2. Effects of fibronectin matrix on the attachment of cultured rabbit corneal epithelial cells. For detailed experimental conditions, see legend for Figure 1. Each point shows the average of triplicate assays. Bars indicate SEM. (*: P < 0.005).

et al, with bovine serum albumin as a standard. The purity of fibronectin was assessed by SDS gel electrophoresis and a single band was observed under the reduction condition. Fibronectin was added to TC-199 containing 20 mM hepes buffer at the indicated concentrations. The surfaces of multi-well culture plates were coated by fibronectin (20 μg/ml, 75 μl/well) at room temperature for 1 hr and then washed extensively in TC-199 medium. Heat-inactivated BSA (80°C for 3 min, 10 mg/ml in TC-199) was used to coat areas which had not been coated by fibronectin (75 μl/ml at room temperature for 30 min) so as to prevent nonspecific (fibronectin-independent) adhesion of the epithelial cells in the attachment assay. The coated plates were washed three times by unsupplemented TC-199 medium prior to the assay.

Attachment Assay

A single cell suspension of cultured corneal epithelial cells (5 × 10⁴ cells in 0.1 ml) and an equal volume of various concentrations of GRGDS or GRGES in hepes-buffered TC-199 were plated into each well and incubated at 37°C for 3 hr. After the incubation, the cells were fixed with 10% formalin for 2 hr at room temperature. Fixed cells were stained by 1% crystal violet in 95% ethanol for 20 min, washed with water and air-dried. After the cells had been stained with crystal violet, attached cells were counted under an inverted light microscope. Each point in the figures was expressed as mean ± SEM (cells/mm²) in triplicate assays. Statistical analysis was carried out by the student t-test.

Results

The effects of fibronectin on the attachment of cultured corneal epithelial cells are shown in representative microscopic views of the epithelial cells incubated on an uncoated plastic tissue culture plate as compared with plates coated with various concentrations of fibronectin (Fig. 1). When the culture dish was not coated with fibronectin, almost no epithelial cells attached during the 3 hr assay. With increased concentrations of fibronectin coating, the number of...
attached cells increased, up to 5 µg/ml fibronectin (Fig. 2). Thereafter, the number of cells attached reached a plateau and remained relatively constant up to the 20 µg/ml examined. These results demonstrate that attachment of corneal epithelial cells depends on the concentration of fibronectin being used for coating. Thus, attachment of corneal epithelial cells is mediated by fibronectin.

The effects of GRGDS on fibronectin-mediated attachment of corneal epithelial cells were examined by incubation of cells, plated on plastic culture dishes that had been precoated by fibronectin at a concentration of 10 µg/ml, in the presence of GRGDS. When no GRGDS was added to the culture medium, the cells attached on the fibronectin matrix (Fig. 3). Quantitative analysis showed that when GRGDS was added simultaneously with the cell suspension, the numbers of epithelial cells attached decreased in a dose-dependent fashion as the concentration of GRGDS was increased (Fig. 4). Above a GRGDS concentration of 6 µg/ml, cells became progressively rounder, indicating decreased capacity of epithelial cells to attach in the presence of the peptide (Fig. 3). These results demonstrate that the addition of GRGDS inhibits the fibronectin-mediated attachment of corneal epithelial cells.

In order to examine the specificity of the inhibitory effects of GRGDS on fibronectin-mediated attachment of epithelial cells, we compared the effect of adding the structurally similar pentapeptide GRGES to the culture medium. This peptide contains the same amino acids as GRGDS, except that a glutamic acid in GRGES replaces aspartic acid. As shown in Figure 5, no prominent morphological changes were noted with this control peptide: the cells attached on the fibronectin matrix regardless of the concentrations of GRGES added. No inhibitory effects of GRGES on the attachment of corneal epithelial cells were observed by quantitative parameters (Fig. 6). GRGES also caused an initial increase in cell attachment compared to cell attachment in wells without peptide. Thus GRGES, unlike GRGDS, does not inhibit the attachment of corneal epithelial cells on the fibronectin matrix.

![Fig. 5. Effects of the control peptide GRGES on the attachment of rabbit corneal epithelial cells on a fibronectin matrix. Plastic tissue culture wells were coated by rabbit plasma fibronectin at a concentration of 10 µg/ml, and then epithelial cells were plated with various concentrations of GRGES. Note the absence of significant differences in morphology of the epithelial cells with or without GRGES. (A) 10 µg/ml fibronectin only, (B) 3.13 µg/ml GRGES, (C) 25 µg/ml GRGES, (D) 200 µg/ml GRGES. Bar shows 0.1 mm.](image_url)
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of many types of cells. Most of the cells reported to use fibronectin for attachment are mesenchymal cells; epithelial cells are generally thought to use laminin, except during wound healing. As we report in this paper, individual ectodermal corneal epithelial cells can also respond to fibronectin, so that cell attachment, a requisite event in epithelial resurfacing of injured cornea, is enhanced greatly by the presence of fibronectin.

Fibronectin has many important functions such as its interaction with cell surface receptors to form an adhesive link between the cell surface and the underlying substratum. As reported in this paper, fibronectin readily mediates the attachment of even isolated corneal epithelial cells, thus suggesting a direct mechanism for the attachment of corneal epithelium that could ultimately result in complete epithelial resurfacing.

A variety of recent studies have implicated the GRGDS sequence (and particularly its central three RGD residues) in the adhesion of a variety of cell types to fibronectin; most cells tested have been of mesenchymal/fibroblastic origin. Synthetic peptides containing RGDS directly inhibit the interaction of fibronectin with its receptor on fibroblastic cells by simple competitive kinetics, but GRGES does not. The results obtained in this study show similar peptide inhibition of corneal epithelial cell attachment, even though the cells are of epithelial (ectodermal) origin. The fact that the cell binding domain of fibronectin is required for corneal epithelial cell attachment, as in other cell systems, is indicated by the ability of the peptide GRGDS, derived from the cell binding domain of fibronectin, but not the structurally similar peptide GRGES, to inhibit cell attachment.

Inhibition of fibronectin function by biologically active peptide sequences of the molecule has been documented for fibroblasts. Injection of peptides related to GRGDS can, moreover, block important in vivo biological events which are thought to involve adhesion and migration, including gastrulation and experimental metastasis. With regard to possible significance of the present results for the cornea, Berman has reported that persistent epithelial defects after alkali burns of rabbit corneas are correlated with accelerated degradation of subepithelial fibrin/fibronectin and with elevated levels of active plasminogen activator (PA). Therefore, abnormally elevated plasmin levels could degrade fibronectin to uncouple cell- and ECM-matrix binding domains in such a way that fibronectin, once degraded, could not function as an adhesive macromolecule. The possible additional relationship of the results of the present studies to the pathophysiologic role of an unregulated PA/plasmin system in epithelial defect formation and stromal ulceration has been reviewed elsewhere.

Key words: cornea, epithelial cells, attachment, cell culture, fibronectin, cell binding domain, GRGDS

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References