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Effect of Serum, Fibronectin, and Laminin on Adhesion of Rabbit Intestinal Epithelial Cells in Culture

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Rabbit intestinal epithelial cells, obtained after a limited hyaluronidase digestion, were incubated in medium with or without calf serum, on bacteriological plastic dishes. The dishes, either plain or coated with an air-dried type I collagen film, were pretreated with medium alone or with medium containing purified laminin or purified fibronectin. Cells did not attach in significant numbers to untreated bacteriological plastic, even in the presence of serum. Cells did attach to collagen-coated dishes, and were judged viable on the basis of their incorporation of radiolabeled leucine into cell protein. Cell adhesion to the collagen substrate increased in proportion to the concentration of serum in the medium, with maximal attachment at 5% serum or greater. Pretreatment of plain or collagen-coated dishes with increasing amounts of fibronectin enhanced cell adhesion in a concentration-dependent manner. Either serum, or fibronectin-free serum in the medium enhanced cell attachment to substrates pretreated with either fibronectin or laminin. Thus, intestinal epithelial cells appear to possess surface receptors for both laminin and fibronectin. The evidence further suggests that calf serum may contain factors, other than fibronectin, capable of enhancing intestinal epithelial cell attachment to collagen substrates.

Key words: fibronectin, intestinal epithelial cell adhesion, laminin

Intestinal epithelial cells proliferate in pouch-like crypts, migrate as a sheet along a basement membrane, and slough off at the tip of the villus into the intestinal lumen. By this steady-state process, the entire intestinal epithelium is replaced every 3 to 5 days. This tissue, therefore, provides a useful model for the study of cell adhesion and migration. These experiments are part of the effort to establish an in vitro system to study the mechanism of epithelial cell attachment as the initial event in the overall phenomenon of cell motility. (For a recent review of cell attachment see Kleinman et al [1].)

Fibroblast adhesion to collagen, in vitro, has been well characterized, and a glycoprotein, fibronectin, has been identified as an attachment factor [2–4].

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Fibronectin is found both in plasma and on cell surfaces. Although the fibronectins from the two sources differ somewhat in their physical and biological properties, both enhance the attachment and spreading of cells onto collagen equally well [5]. Other adhesion factors exist for epithelial cells, since epithelial cells do not require fibronectin for adhesion to collagen [6–10]. Unlike fibroblasts, some epithelial cells have been found to attach to type IV (basement membrane) collagen in preference to other types of collagen [7, 8]. Laminin, a glycoprotein component of the basement membrane found in the lamina lucida, greatly enhances the adhesion of epithelial cells to type IV collagen [7]. Whereas laminin is apparently the sole adhesion factor for some epithelial cells [7], others can use either laminin or fibronectin [6–10]. Both fibronectin [12] and laminin [13] have been identified in association with the intestinal epithelial basement membrane. Therefore, we have examined the role of these two adhesion factors, fibronectin and laminin, on intestinal epithelial cell adhesion to type I collagen.

MATERIALS AND METHODS

Animals and Materials

Type I collagen was obtained from Collagen Corporation, Palo Alto, California. [^{3}H]-L-Leucine (52 Ci/mmol) was purchased from Amersham/Searle. All other chemicals were purchased from Sigma Chemical Company.

New Zealand white male rabbits (1.2–1.5 kg), obtained from the NIH Small Animal Section, were fasted overnight and killed by CO_2 asphyxiation.

Attachment Proteins

Rat plasma fibronectin was prepared following the procedure of Engvall et al [16] as modified by Murray et al [8]. Cell surface fibronectin was prepared according to the procedure of Yamada et al [17]. Laminin was prepared according to the procedure of Timpl et al [18].

Isolation of Intestinal Epithelial Cells

Intestinal epithelial cells were isolated by the procedure of Towler et al [15] with some modifications. Briefly, a 40- to 50-cm segment of the proximal jejunum was excised and washed with 300 ml of cold phosphate-buffered saline (PBS). The ends of the segment were tied to infusion sets (Abbot Hospitals, Chicago, Illinois), the intestine was filled with 30 ml of isolation medium [96 mM NaCl, 8 mM KH_2PO_4, 5.6 mM Na_2HPO_4, 50 mM trisodium citrate, pH 7.2, containing 1.5 mg/ml hyaluronidase (type II) and 2.5 mg/ml bovine serum albumin (BSA)] and incubated in PBS at 37°C with gentle shaking for 15 min. The lumen was rinsed once with medium (a 50:50 mixture of Dulbecco's modified Eagle's medium (MEM) with 25 mM N-2-hydroxy-ethylpiperazine-N′-ethanesulfonic acid (HEPES) and NCTC 135, supplemented with 4 mM L-glutamine, 100 μg/ml gentamicin, 100 U/ml mycostatin, to which was added 5 mg/ml BSA) and the cells were released by gentle patting of the medium filled gut supported by a water-filled bag. The collection procedure was repeated three or four times, and the released cells were pooled, washed, resuspended in medium (50:50 mixture without BSA), and filtered in succession through 200- and 25-μm mesh Nytex filters (Tetko, Elmsford, New York).

Attachment Assays

Six-well cluster plates of Linbro bacteriological plastic (Flow Laboratories, Rockville, Maryland) were used throughout. The 35-mm wells were coated with a film of type I collagen by placing 1 ml of a 10 μg/ml aqueous collagen solution into them, and allowing it to dry overnight in a laminar flow hood. Plain or collagen-coated wells were pretreated for 1 hr at room temperature with 1 ml of medium, with or without varying concentrations of purified fibronectin or purified laminin. Pretreatment solutions were removed from the wells, the wells were rinsed with medium, and 2 ml of medium with or without calf serum were placed into each well. Finally, filtered cells, 10^5 in 1 ml of medium, were added to the wells and incubated at 37°C in a humidified, 5% CO_2 atmosphere for the times indicated.

Following incubation, unattached cells were removed from the wells with PBS; attached cells were detached by trypsinization (0.1% trypsin in PBS) for 10 min at room temperature and counted with a Coulter Counter (Coulter Electronics, Hialeah, Florida).

Each figure is representative of observations from at least three animals; individual points in the figures are means of at least two assay wells, which did not differ by more than 10%.

RESULTS

Intestinal epithelial cells were capable of attaching to collagen-coated bacteriological plastic dishes in a time-dependent manner (Fig. 1). The presence of 5% calf serum in the incubation medium appreciably enhanced cell adhesion to the collagen substrate. Cells did not attach to untreated bacteriological plastic dishes either in the presence of calf serum or after pretreatment of the plastic dishes with 1 ml of 200 μg/ml BSA or 5% calf serum (data not shown).

The incorporation of [^{3}H]-L-leucine into protein of attached cells was measured to determine cell viability (Fig. 2). The rate of incorporation into attaching cells was biphasic, with an apparent lag period lasting approximately 1 hr, followed by a sixfold increase in the rate of incorporation over the second hour of incubation. The presence of serum had little or no effect on these rates. The addition of cycloheximide to the incubation medium reduced the rate of incorporation at 2 hr by more than 90% (Fig. 2), but had no effect on cell attachment (data not shown). Unattached cells also incorporated [^{3}H]-L-leucine, but at a rate three to five times lower than that observed in attached cells (data not shown). Thus, the attached cells are viable as measured by their ability to synthesize protein.

At concentrations above 0.1%, cell attachment increased with increasing concentrations of calf serum (Fig. 3). Similar levels of attachment are obtained with sera from the fetal calf, horse, chicken, and rabbit (data not shown).

Fibronectins are glycoproteins found both on cell surfaces and in plasma. This class of proteins has been shown to be involved in cell–cell cohesion as well as cell–substrate adhesion [5]. Quaroni et al [12] have implicated fibronectin in the attachment of rat intestinal epithelial cells to the basement membrane. Purified rat plasma fibronectin enhanced epithelial cell attachment to plain bacteriological plastic dishes (Fig. 4). The combination of fibronectin and collagen was a

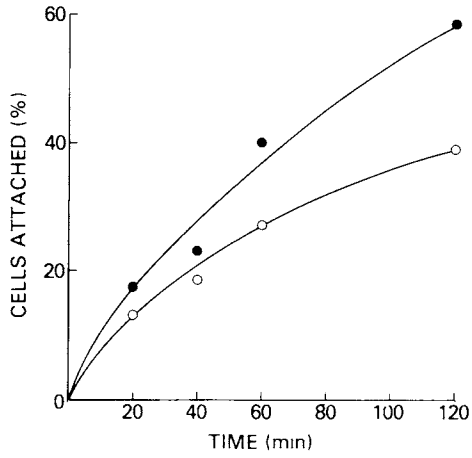

Fig. 1. Time course of attachment of rabbit intestinal epithelial cells to collagen-coated dishes. Cells (10^5) were plated on 35-mm bacteriological dishes coated with 10 μg of type I collagen in medium with (●) or without (○) 5% calf serum and incubated at 37°C. At the indicated times, unattached cells were washed off and the attached cells were removed by trypsinization and counted electronically.

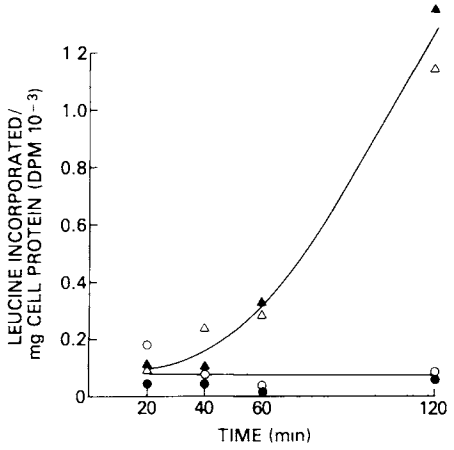

Fig. 2. L-Leucine incorporation into cell protein. Epithelial cells (1.6×10^5 cells/ml) were pre-incubated with [^{3}H]-L-leucine (1.5 μCi/ml, 52 Ci/mmol) for 5 min prior to plating on collagen-coated dishes. After incubation at 37°C for the indicated times, attached cells were harvested and washed with PBS and the cell pellet was collected by centrifugation. Cell protein was precipitated with 5% trichloroacetic acid (TCA), dissolved in 1 N NaOH, and neutralized, and an aliquot was counted in a Beckman LS-255 liquid scintillation counter. (△, ▲) Without cycloheximide; (○, ●) with 67 μg of cycloheximide/ml; with serum (▲, ●); without serum (△ ○).

more effective substrate than fibronectin alone. At all concentrations of fibronectin, the addition of 5% calf serum further stimulated cell adhesion to either type I collagen film or plastic substrates. Thus, whereas serum alone is ineffective in promoting intestinal epithelial cell attachment to plastic dishes, purified fibro-

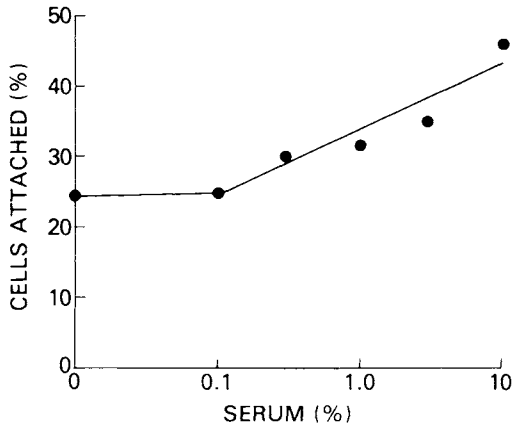

Fig. 3. The effect of calf serum on the attachment of epithelial cells. Cells were plated on collagen-coated dishes in the presence of the indicated serum concentrations and incubated for 1 hr at 37°C.

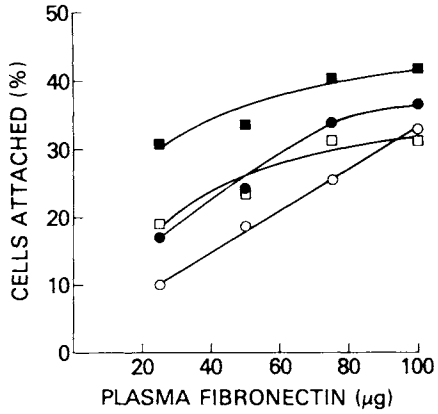

Fig. 4. Effect of plasma fibronectin on cell attachment. Bacteriological dishes either plain (○, ●) or coated with type I collagen (□, ■), were pretreated with the indicated amounts of rat plasma fibronectin in 1 ml of medium for 1 hr at room temperature. Dishes were rinsed with medium and 10^5 cells were plated in the presence (●, ■) or absence (○, □) of 5% calf serum and incubated 1 hr at 37°C.

nectin can promote cell adhesion to bacteriological plastic substrates. Cell surface fibronectin was also effective in stimulating cell attachment to collagen but at a concentration 5 to 10 times lower than plasma fibronectin (Fig. 5). The addition of either serum or fibronectin-free serum could further promote the fibronectin-mediated adhesion, suggesting that yet another adhesion (co)factor exists in serum for epithelial cells.

Laminin has also been found to serve as an attachment protein for epithelial cells [7]. Consistent with the findings of Terranova et al [7], we did not observe an increase in cell attachment to type I collagen with increasing concentrations of laminin in the absence of serum. In the presence of a constant amount

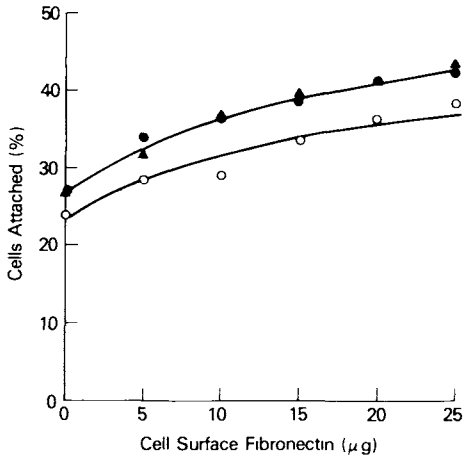

Fig. 5. Effect of cell surface fibronectin on the attachment of epithelial cells. Collagen-coated dishes were preincubated with the indicated amounts of cell surface fibronectin in 1 ml of medium for 1 hr at room temperature. Dishes were rinsed with medium and 10^5 cells were plated in normal serum (5%) (●), fibronectin-free serum (5%) (▲), or in the absence of serum (○), and were incubated for 1 hr at 37°C.

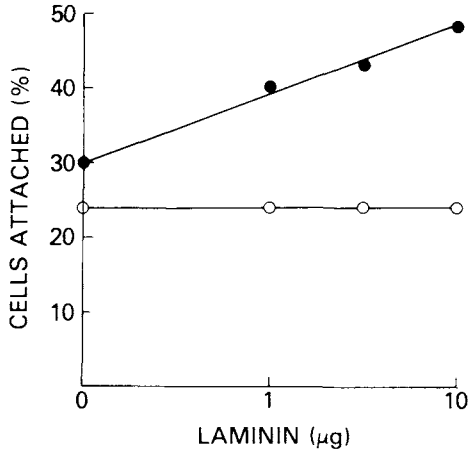

Fig. 6. Effect of laminin on the attachment of epithelial cells. Collagen-coated dishes were pre-incubated with the indicated amounts of laminin in 1 ml of medium for 1 hr at room temp. Dishes were rinsed with medium and 10^5 cells were plated in the presence (●) or absence (○) of 5% calf serum and were incubated for 1 hr at 37°C.

of serum, however, laminin could increase cell attachment to type I collagen in a concentration-dependent manner (Fig. 6). Thus, laminin is also an adhesion factor for epithelial cells, but only in the presence of serum.

DISCUSSION

We have characterized the in vitro attachment properties of intestinal epithelial cells to a variety of substrates. When bacteriological plastic dishes were

used, less than 1% of the epithelial cells attached, with or without serum present in the medium. In contrast, 20% of human skin fibroblasts attached to this plastic in the absence of serum and 50% attached in the presence of serum (J. Butler and P. Burrill, unpublished observations). The intestinal epithelial cells showed a significant level of attachment to bacteriological plastic dishes that had been coated with collagen. This attachment was enhanced by the addition of serum (Figs. 1 and 2) and by pretreatment with either cell surface or plasma fibronectin (Figs. 4 and 5). This observation is consistent with the findings of Quaroni et al [12] who detected fibronectin associated with the intestinal basement membrane by immunofluorescent microscopy. We found that both fibronectin-free and normal serum were similarly effective in increasing cell adhesion to collagen, to collagen plus fibronectin (Fig. 5) or to collagen plus laminin (Fig. 6). Two explanations exist for the additive effect of serum on fibronectin or laminin-mediated cell adhesion. First, these results suggest that there is an additional component of serum, other than fibronectin, which promotes epithelial cell attachment. Stenn [18] has reported a serum factor other than fibronectin that can promote epidermal cell adhesion and spreading. Barnes et al [19] have also reported a similar activity in serum for breast epithelial cells. It is likely that such a factor also exists for intestinal epithelial cells based on our findings. Second, this serum enhancement effect is not substrate specific and may simply be due to the presence of protease inhibitors in the serum. These inhibitors could protect both cell surface receptors and attachment proteins from proteolytic activity released by the intestinal cells.

Terranova et al [7] demonstrated that a transformed epithelial cell line, Pam 212, preferentially attached to basement membrane collagen (type IV) and that laminin, also a constituent of basement membranes, increased cell attachment to this collagen about ninefold. Their observations suggest either that laminin binds to type IV collagen better than to other collagens, or that the conformation of laminin, when bound to type IV collagen, is critical for cell attachment. Foidart [13] also found laminin associated with the intestinal basement membrane.

In our system, we found laminin to be at least as effective as either plasma or cell surface fibronectin in enhancing intestinal adhesion to type I collagen, but only in the presence of serum. Thus, the intestinal epithelial cell apparently has surface receptors for fibronectin and laminin. Hepatocytes [9, 10] and endothelial cells [7, 11] have also been shown to respond to both these adhesion factors. In addition, the capacity of both normal and fibronectin-free serum to stimulate the attachment of epithelial cells to these substrates suggests the existence of still another adhesion factor for these cells. Multiple mechanisms for attachment may be a requirement for these cells to survive and migrate to the villus tip. Also, since these cells have three different surfaces (luminal, basement membrane, and cell side), multiple adhesion mechanisms are presumably a prerequisite for normal intestinal architecture.

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