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Intestinal epithelial cells preferentially attach to a biomatrix derived from human intestinal mucosa

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SUMMARY Primary intestinal epithelial cells have a very short lifespan *in vitro* when cultured free of mucosal elements. Support of the basal plasma membrane by a more natural substrate may thus enhance the initiation of primary cell cultures. A cell free biomatrix consisting of native interstitial collagens, basement membrane fragments and microfibrils was extracted from the lamina propria of human intestinal mucosa. Immunofluorescence revealed the presence of collagens type III, IV, and VI and procollagens type I and III as well as fibronectin, laminin and undulin. Primary crypt cells of suckling mice displayed a significantly increased affinity to pepsin and collagenase solubilised intestinal biomatrix when compared with plastic and fibronectin. Colonies of primary crypt cells survived for up to four days and longer on pepsin solubilised biomatrix but only for 48 hours on fibronectin. The intestinal biomatrix preparation has proved to be a useful substrate for the initiation and prolongation of primary intestinal cell cultures.

Macromolecules of the extracellular matrix surrounding the parenchymal cells *in vivo* have been shown to enhance the initiation and maintenance of cell cultures *in vitro*. Their ability to promote cell attachment, cell proliferation or cellular differentiation depends on the cellular phenotype.[1,2,3] In general epithelial cells which *in situ* adhere to a basement membrane prefer contact with the basement membrane constituents laminin, collagen type IV and heparansulphate proteoglycan while adult fibroblastic cells display greater affinity to fibronectin and the interstitial collagens. Compositions of these factors seem to have an even greater potential in stabilising the primary cellular phenotype.[4,5] Consequently, preparations of the whole extracellular matrix of a certain organ should comprise a maximum of attachment factors for the parenchymal cells of the same organ *in vitro*. A liver derived so called biomatrix has recently been shown to qualify as a substrate for adult rat hepatocytes.[6,7]

Primary cell cultures of adult intestinal epithelial cells are especially difficult to maintain. Homogenous epitheloid cell lines have been established from intestinal mucosa of different species retaining some characteristics of intestinal cells, but the maintenance of a population of differentiated absortive epithelia in a monolayer technique has not yet been achieved.[8-10] We have therefore extracted a cell free biomatrix of human intestinal mucosa to promote the attachment and viability of primary intestinal epithelial cell cultures.

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Methods

PREPARATION OF BIOMATRIX

Biomatrix was prepared from human small intestinal mucosa within two hours post mortem from two patients 29 and 45 years of age. They had no history or evidence of gastrointestinal disorders, systemic infections or chemotherapy and had died of intracerebral haemorrhage and myocardial infarction respectively. The scheme of preparation is outlined in the Table.

ANALYSIS OF BIOMATRIX

The carbohydrate content was measured by the orcinol reaction and the amount of sialic acid by the thiobarbiturate reaction.[11,12] Soluble biomatrix extracts were prepared from the native, fibrillar material by digestion with pepsin (Sigma, Munich, FRG) in 0·5 M acetic acid or bact. collagenase (CISPA, Worthington, Freehold, NJ, USA) in 0·2 M NH_4

Table 1. *Preparation of biomatrix*

Work in cool room at 4° C, centrifuge at 4° C! Evert 2 m jejunum and rinse with tap water briefly
↓
Scrape the mucosa carefully off leaving submucosa
↓
Wash scraped material in 500 ml aqua bidest
↓
Homogenise 10 g/10 min in aqua bidest on ice
↓
Extract in aqua bidest for 30 min
↓
Centrifuge twice, (always 15000 rpm 20 min)
↓
Extract residue in 2 × volume 1 M NaCl 1 hour
↓
Centrifuge 3 × residue with 5 × volume 1 M NaCl
↓
Centrifuge 2 × residue with 5 × volume aqua bidest
↓
Degrade adherent nuclear debris with 1 mg DNAse and 5 mg RNAse/3 gr residue in culture medium 2 h at 36° C
↓
Centrifuge 2 × with 5 × volume 1 M NaCl
↓
Extract with ether/butanol (2:1) for 45 min at 21° C
↓
Extract with 25% ether for 10 min at 21° C
↓
Centrifuge 4 × residue with 5 × volume aqua bidest
↓
Store ready biomatrix frozen at −80° C

HCO_3, 10 mM $CaCl_2$ at 1:100 w/w at 15° C over 24 h, followed by centrifugation and dialysis of the supernatants against the same solutions. An acid soluble extract was prepared by dissolving crude biomatrix in 0·5 M acetic acid at room temperature over night. Protein content was estimated according to Lowry.[13]

The presence of defined extracellular matrix components within the fibrillar biomatrix was studied by immunofluorescence using monospecific antibodies against collagen type III, procollagen type I and III, collagen type IV and VI, fibronectin, laminin and undulin as described before[14–18] on cryostat sections of frozen biomatrix embedded in pig liver. For controls, rabbit and goat non-immune IgG, rabbit antibodies to intestinal alkaline phosphatase and to human IgG, albumin and factor VIII (Dakopatts, Hamburg, West Germany) were used.

Ultrastructural analysis of biomatrix components by transverse electron microscopy was done as described before using a 1% tannin/1% glutaraldehyde fixation procedure.[19]

ATTACHMENT ASSAYS

Crypt cell suspensions were prepared as follows: 10–15 day old C57 Black mice were anaesthetised with ether and the abdominal cavity exposed. Ten

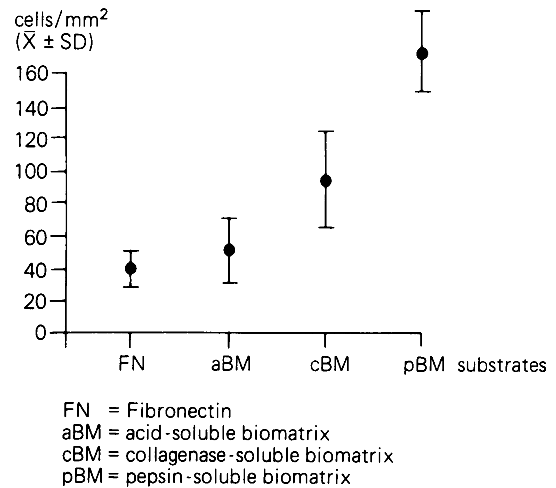

Fig. 1. *Attachment assay of primary crypt cells to solubilised biomatrix preparations (for experimental detail see* Methods). *Values represent the mean number of attached cells/mm² after incubation for 3 hours on five coated coverslips per substrate and extensive washing. 10 mm² were counted per substrate.*

centimetres of the jejunum were excised, immediately everted onto a plastic rod and incubated for 15 minutes in 30 mM EDTA, 1% glucose in phosphate buffered saline at 15° C equilibrated with 95% O_2/5% CO_2. The epithelium of the villi is then spun off by immersion in ice cold Hanks solution vibrating the plastic rod at approximately 300 rpm and discarded. Crypt cells are sequentially detached in the same way, collected immediately by centrifugation and stabilised by the addition of calcium chloride to 10 mM. Sequential isolation of villus epithelial and crypt epithelial cells had been monitored by measuring brush border enzyme activity (alk phosphatase, α-glucosidase and lactase) *versus* 3H-thymidine uptake in previous experiments. Crypt cells were then suspended in Dulbecco's modified Eagles medium plus 5% fetal calf serum, 4 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 50 mU/ml insulin (Biochrom, West Berlin and Hoechst, Frankfurt, FRG), quickly pipetted to disperse them into single cells (5–18 × 10^5 cells/ml) and incubated with the different substrates at 36° C, 8% CO_2 in air for three hours, after which the unattached cells were washed off with phosphate buffered saline (three washes) and the remaining cells counted under an inverted microscope per mm². Crypt cell suspensions which had not been dispersed were seeded onto the substrates and the survival of colonies comprising more than eight cells was followed microscopically and by trypan blue exclusion test after six days *in vitro*.

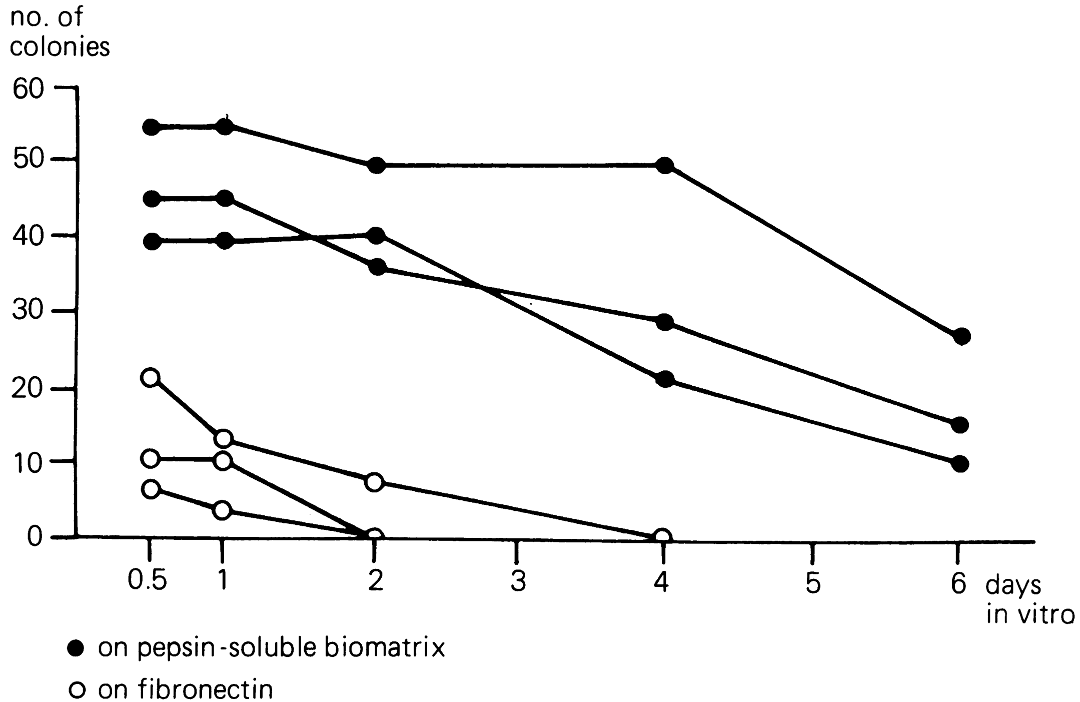

Fig. 2. *Survival of primary crypt cell colonies on pepsin-soluble biomatrix and on fibronectin. Identical aliquots of a suspension of primary crypt cells were seeded onto the two substrates. The values represent the number of viable colonies during the course of one typical experiment over six days. Colonies with more than eight cells were counted and the mean cell number per colony was 22 cells.*

Cover slips had been coated with 2 $\mu g/mm^2$ of soluble biomatrix digests or fibronectin air dried under an UV-light source and extensively washed with culture medium without serum before use.

Attachment assays were undertaken five times for each substrate and 12 animals were killed to yield the intestinal cells (experiment of Fig. 1). For the assay of survival of crypt cell colonies on biomatrix, crypt cell suspensions of two animals were pooled and the assay repeated nine times. The yield of vital crypt cell colonies varied considerably from assay to assay but the relationship of survival rates on the two substrates used was always similar (see Fig. 2).

Results

Biomatrix prepared from human small intestine is composed of native collagen fibrils, microfibrils, basement membrane fragments and amorphous ground substance as visualised by transverse electron microscopy (Fig. 3). It is free of cellular debris ultrastructurally. This was achieved by carefully keeping the preparation temperature under 4° C (Table). We also considered it essential to minimise the time lag between the post mortem examination and the beginning of the preparation. The carbohydrate content of crude biomatrix was estimated to be 15% disaccharides and 8% sialic acid confirming the presence of glycoproteins.

By immunofluorescence the collagens type III, IV and VI and the procollagens type I and III were detected ubiquitously within the biomatrix as well as fibronectin, laminin and undulin. Staining with antibodies to laminin and collagen type IV resulted in a more delicate, network like pattern leaving some areas unstained while all the other antibodies to matrix molecules stained the fibrillar, crude biomatrix almost homogenously.[20] Antibodies to non-immune IgG as well as to albumin, intestinal alkaline phosphatase and factor VIII did not stain the biomatrix, again signalling that no cellular debris or plasma had been retained although it is essentially a non-enzymatic and non-denaturing preparation.

Primary crypt cells of adult mice are difficult to isolate in a viable state and show very little tendency to adhere at all to plain plastic surfaces or to a number of single or combined matrix molecules according to our own experiences. Results are better

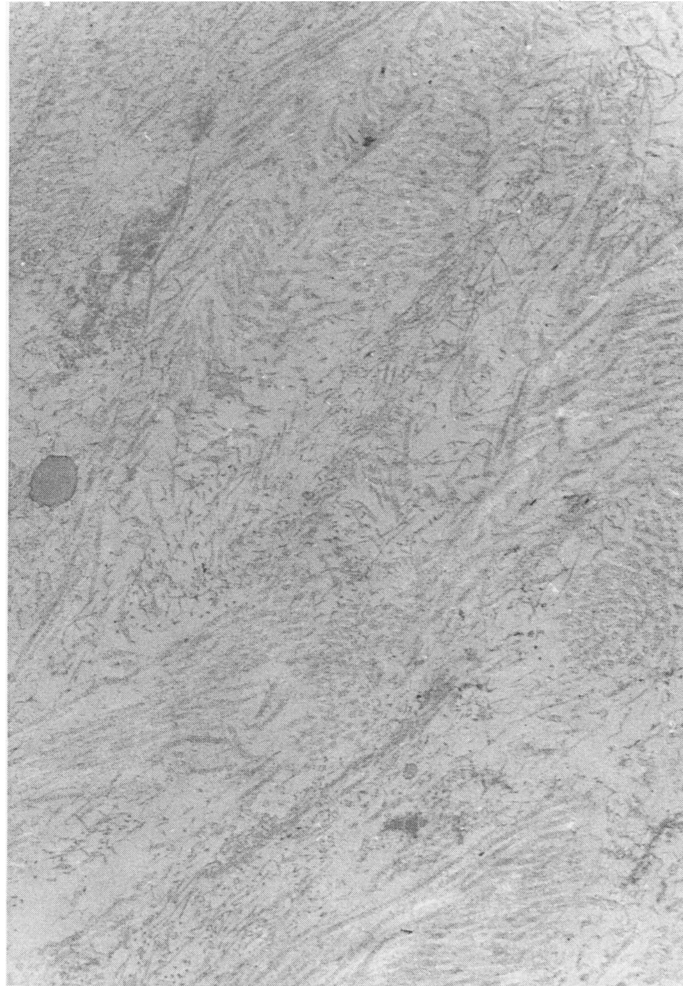

Fig. 3. *Ultrastructural aspect of freshly prepared intestinal biomatrix. Cross striated collagen fibres, microfibrils and amorphous ground substance are dispersed in the biomatrix. Transverse electron microscopy,* × *35 000.*

with the respective cells from suckling mice corresponding to previous reports.[8] These cells displayed markedly increased affinity to pepsin solubilised biomatrix compared with collagenase digested biomatrix, the acidic extract and fibronectin. In all assays there was an at least four fold increase in the number of attached cells on coverslips coated with the pepsin digest and the number of attached cells on the collagenase extract was usually double that on fibronectin (Fig. 1).

Morphologically, these cells spread out onto the substrates to a limited degree compared with cells of intestinal epithelial cell lines[21] and did not survive more than 48 hours when plated as single cells. If the crypt cell suspension, however, was not thoroughly dispersed by pipetting, crypt cell aggregates of 8–30 cells were preserved. These spread out well and maintained their well defined appearance in phase contrast optics for 24 hours on fibronectin but up to four days and longer when plated onto the pepsin digest of intestinal biomatrix (Fig. 2). Very few mitoses were observed in the colonies. Survival of intestinal crypt cells therefore apparently depended both on cell to cell contact as well as on the culture substrate.

Discussion

The cell free, fibrillar residue extracted from human intestinal mucosa termed intestinal biomatrix was

shown to be a valuable substrate for the initiation and maintenance of freshly isolated intestinal crypt cells. Crude intestinal biomatrix contains native collagens and non-collagenous glycoproteins of the extracellular matrix. These were identified by immunofluorescence and were identical to the components of the extracellular matrix of mouse and human intestinal mucosa as described before and comprised procollagen type I and III, collagen type III, IV and VI, laminin, fibronectin and undulin.[14,22,23]

Primary crypt cells attached to and also survived on the crude, fibrillar matrix residue. As the crude biomatrix is difficult to handle in culture experiments because of its fibrillar nature and as it is reasonable to assume that attachment factors may persist after treatment with acid, collagenase or pepsin,[7,24] we tested the respective biomatrix extracts as culture substrates.

Pepsin solubilised biomatrix proved to be the most efficient substrate. Cell attachment is most likely to be the result of the interaction of specific receptors of the epithelial cell plasma membrane with components of the extracellular matrix, notably the basement membrane. One of these putative receptors, the laminin receptor, has recently been identified on intestinal epithelia as defined by a monoclonal antibody.[25] Laminin is a major glycoprotein of basement membranes and consists of several domains, some of which are protease resistant and have been shown to promote cell attachment.[24,26,27] Protease resistant domains of collagen type IV and heparan sulphate proteoglycan retaining affinity for cell surfaces have also been described.[28] The pepsin solubilised material of human intestinal biomatrix is therefore likely to be enriched in factors promoting the attachment of intestinal epithelial cells and providing the necessary support for cellular polarisation and survival. Evidence for the presence of laminin and collagen type IV peptides in the biomatrix extract has been derived from measuring the contents of the pepsin fragment laminin P1 and the 7-S domain of collagen type IV by radioimmunoassay. Current investigations are completing these observations. These ubiquitous basement membrane proteins, however, may not be the most important factors involved in the adhesion of intestinal epithelial cells *in vitro* and *in vivo*. Acidic glycoproteins from biomatrix preparations of different organs have been shown to be tissue-specific: hepatocytes attach preferentially to hepatic acidic glycoproteins in comparison with identically prepared fractions from spleen, lung and heart.[7] The physiology of the mobile intestinal epithelium strongly suggests different mechanisms of cell adhesion compared with those exerted by sessile hepatocytes or pancreatic acinar cells. Thus, a promising route has been started to facilitate intestinal primary cell cultures but more analytical investigations are needed to identify all the matrix factors involved in cell-matrix interactions of the intestinal epithelium. We expect important insights into the regulation of cell proliferation in the intestinal mucosa from these studies.

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