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Human fetal enterocytes *in vitro*: Modulation of the phenotype by extracellular matrix

(Nonmalignant/keratin/villin/Matrigel/insulin-like growth factor binding proteins)

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ABSTRACT **The differentiation of small intestinal epithelial cells may require stimulation by microenvironmental factors *in vivo*. In this study, the effects of mesenchymal and luminal elements in nonmalignant epithelial cells isolated from the human fetus were studied *in vitro*. Enterocytes from the human fetus were cultured and microenvironmental factors were added in stages, each stage more closely approximating the microenvironment *in vivo*. Four stages were examined: epithelial cells derived on plastic from intestinal culture and grown as a cell clone, the same cells grown on connective tissue support, primary epithelial explants grown on fibroblasts with a laminin base, and primary epithelial explants grown on fibroblasts and laminin with n-butyrate added to the incubation medium. The epithelial cell clone dedifferentiated when grown on plastic; however, the cells expressed cytokeratins and villin as evidence of their epithelial cell origin. Human connective tissue matrix from Engelbreth–Holm–Swarm sarcoma cells (Matrigel) modulated their phenotype: alkaline phosphatase activity increased, microvilli developed on their apical surface, and the profile of insulin-like growth factor binding proteins resembled that secreted by differentiated enterocytes. Epithelial cells taken directly from the human fetus as primary cultures and grown as explants on fibroblasts and laminin expressed greater specific enzyme activities in brush border membrane fractions than the cell clone. These activities were enhanced by the luminal molecule sodium butyrate. Thus the sequential addition of connective tissue and luminal molecules to nonmalignant epithelial cells *in vitro* induces a spectrum of changes in the epithelial cell phenotype toward full differentiation.**

Epithelial function is defined by the phenotype and spatial arrangement of its constituent cells. The balance between internal mechanisms controlling differentiation and factors external to the enterocyte has long been the subject of debate. These external mechanisms are ill defined, but in the fetus they include: cell–cell interactions (1), the composition of basement membranes (2), and nutrients/growth factors in amniotic fluid (3). External influences are further elaborated after birth when luminal factors such as bacterial colonization and diet may affect differentiation. The basement membrane and the connective tissue from which it is derived may act in concert with luminal factors to influence extrauterine differentiation.

A study of the external influences on human intestinal epithelial cell maturation requires the capacity to culture human enterocytes. However, such cells have proven difficult to grow *in vitro*. To date, the only human epithelial cells that have remained viable in culture for indefinite periods have been derived from colonic carcinomas (4). Moreover, it is not clear to what extent their malignant transformation has allowed them to escape a possible dependence on external microenvironmental influences. Studies on nonmalignant cell lines isolated from rat (5) suggest that connective tissue is important in stimulating enterocyte differentiation (6–8) but may not be the only critical factor in that process. The epithelium is now considered to be only one of four elements of an integrated functional unit that also includes extracellular matrix molecules, mesenchyme-derived cells and luminal factors (2). The aim of this work was, therefore, to test the hypothesis that nonmalignant cells from the human small intestinal epithelium require the constant application of exogenous signals to stimulate differentiation. We first isolated human fetal enterocytes and maintained their growth on plastic. We then examined the effect of connective tissue proteins on the differentiation of these cells. We further expanded our model to include an examination of fibroblasts and luminal elements on primary explants of human small intestinal epithelium. Our results constitute *in vitro* a model of the external influences on enterocytes that stimulate their differentiation. We are now in a position to identify and further define the relative importance of these mechanisms involved in the molecular pathways of differentiation. Such mechanisms may not only be important in the differentiation of cells from crypt to villus, but may also be involved in the changes observed in the ontogeny of the epithelium from fetus to adult.

METHODS

Culture of Human Nonmalignant Epithelial Cell Lines. Epithelial cells were obtained from whole small intestine of fetuses aged from 20 to 22 weeks gestation. Approval was obtained from the Human Studies Committee of the Brigham and Women's Hospital (Boston). After resection, fetal intestine was washed with Hanks' balanced salt solution (GIBCO) at 4°C. The intestine (which contained both villi and crypts) was cut into pieces 3 mm × 7 mm and incubated for 20 h in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum (FCS) (HyClone) in 5% CO_2. The fragments were transferred to fresh dishes containing DMEM, 10% FCS, insulin (4 μg/ml) (Sigma), and collagenase I (40 μg/ml) (Sigma) for 1 week at 37°C. Two days later clones were occasionally observed growing from the intestinal segments in the dish. These cells were transferred to new dishes. Dishes containing islands of fibroblasts were discarded. However, many islands were seen containing only epithelial-like cells. Cells in these islands were isolated by cylinders greased with

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Abbreviation: IGFBP, insulin-like growth factor binding protein.

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a sterile seal. Eight cell clones, each from a different fetus, were developed by using this technique.

Primary Cultures of Epithelial Cell Sheets. Epithelial cell sheets were grown from human intestine by methods adapted from Kedinger *et al.* (6). Briefly, sterile fetal intestine 3–4 cm in length was placed on an agar support (75% F-10; 25% of 1% agar). Intestine was incubated in CMRL media (GIBCO) with 0.1% collagenase for 1 h. The endodermal cells were separated from the serosa of longitudinally incised intestine by using a circular glass coverslip. Epithelial cell sheets were then transferred to a fibroblast support (on laminin) by using a glass Pasteur pipette. [Fibroblasts were previously grown from human fetal skin. They were seeded at 5×10^4 cells/cm^2 onto laminin (Collaborative Research) and grown to confluence.] The epithelial cell/fibroblast co-cultures were grown in coculture media [44% F-12, 44% DMEM, and 10% fetal bovine serum (FBS)].

Morphology and Immunocytochemistry. Cells were stained with anti-keratin antibody (AE-3) (Chemical Credential, ICN) diluted 1:50 with (1:200) fluorescein-conjugated goat anti-mouse IgG (Jackson ImmunoResearch). Their staining properties were compared with fibroblasts and Caco-2 cells. To study the distribution of f-actin filaments, cells were stained with (1:50) phalloidin conjugated with rhodamine (Molecular Probes).

Villin expression was examined in cells by immunofluorescence by using a rabbit polyclonal anti-villin antibody. The presence of villin in cells derived on plastic was detected by enhanced chemiluminescence (ECL) Western blot analysis (Amersham) according to manufacturer's recommendations.

Cell Proliferation and Monolayer Electrical Resistance. Proliferation of derived cells was determined by plating cells at 1×10^4. The media were changed daily, and cell counts were performed in triplicate at 24-h intervals. Cell numbers were determined by hemocytometer. Cell viability was estimated by trypan blue exclusion. To determine whether cell monolayers formed tight junctions after confluence, cells were also plated onto well inserts (Falcon), and transmembrane resistance was measured daily.

In experiments designed to examine the effect of basement matrix on cell polarity and differentiation, isolated human enterocytes were plated at a higher density (1×10^6 cells/cm^2) onto 6-well plastic plates coated with Engelbreth–Holm–Swarm sarcoma basement membrane (Matrigel; Collaborative Research). Cells were recovered from the gel surface by the addition of dispase (100 g/liter, 500 units/g; Collaborative Research). One hundred percent Matrigel reduced the longevity of the seeded cells to 1–2 days. To increase longevity, experiments were also performed on 50% Matrigel from a lower initial plate density (6×10^4).

Brush Border Enzyme Activities. Brush borders of cells were obtained by the method of Kessler *et al.* (10). Culture plates were placed on ice and cells were rinsed twice with ice-cold Hanks' balanced salt solution and harvested by gentle scraping in 0.5 mol/liter Tris·HCl buffer (pH 7.0) (Sigma). Cells were transferred to Tris·mannitol buffer containing phenylmethylsulfonyl fluoride. The cell suspension was sonicated twice for 15 sec at half maximum power (Kontes microultrasonic cell disrupter). Cells were then centrifuged in 18 mM $CaCl_2$ at $950 \times g$ for 10 min at 4°C. The supernatants were centrifuged at $105{,}000 \times g$ for 1 h to obtain a membrane fraction. The resulting brush border membrane fractions were used to measure specific enzyme activity. Alkaline phosphatase (EC 3.1.3.1) was determined according to Garen and Levinthal (11) with *p*-nitrophenyl phosphate as a substrate. Sucrase (EC 3.2.1.48) activity was also measured spectrophotometrically (12) Protein was measured using the BCA protein assay kit (Pierce) according to manufacturer's recommendations. In experiments examining the effects of sodium butyrate on enzyme activity, endodermal cells from different intestinal preparations were divided into two, each being placed on a bed of laminin and fibroblasts. After 24 h, one of each pair was incubated in 2.5 mM butyrate, the other acting as a control without butyrate.

Insulin-Like Growth Factor Binding Proteins (IGFBPs). The profile of IGFBPs secreted by cultured intestinal epithelial cells changes with differentiation (13, 14). This profile was therefore used as an independent measurement of the phenotypic expression of intestinal epithelial cells. IGFBPs in the incubation media from cells were analyzed by Western ligand blotting (15). Sixty microliters of each sample was applied to 10% SDS/polyacrylamide gel. After transfer of proteins to nitrocellulose membranes, the membranes were probed with [^{125}I]IGF-I (DuPont/NEN). The relative positions of IGFBP-2, -3, and -4 on ligand blotting have been verified previously by immunoblotting (14).

RESULTS

Connective Tissue Elements Induce a More Differentiated Morphology in Enterocytes. The morphology of enterocytes was examined in the isolated cell clone on plastic and on Matrigel and in primary explants on fibroblast culture. Establishment of viable human epithelial cells was greatly facilitated by the previous collective experience in establishing rat epithelial cell lines (5, 16). Eight attempts to obtain viable cells in long-term culture were successful. The data described in this report refer to one particular cell clone, designated H-4** (the fourth epithelial cell line to survive initial passage). Examination of the chromosomes of H-4 cells demonstrated a normal 46 chromosome XY karyotype.

The morphology of H-4 cells (Fig. 1*a*), on plastic or glass, was of a homogeneous population of epithelial-like cells with large, oval nuclei growing as tight colonies of polygonal, closely opposed cells. Growth to confluence (Fig. 1*b*) resulted in a sheet of cells that covered the surface on which they grew. H-4 cells became more compact on confluence, but unlike Caco-2 or TE84 carcinoma cells, they never developed "domes" after reaching confluence. Lack of dome formation indicated that it was unlikely that these monolayers were completely sealed by tight junctions on plastic. This was confirmed by a lack of increase in transepithelial resistance after confluence. H-4 cells were stained with ZO1 antibodies to further characterize the production of tight junctions. Consistent with the monolayers' inability to develop a high electrical resistance, ZO1 was barely detectable along the margins of the cells, but was present diffusely in the cytoplasm.

Growth on Matrigel modulated the phenotype of the intestinal epithelial cells. They became markedly more pleiomorphic (Fig. 1*c*). Some cells remained similar to those seen on plastic; however, after 2–3 days most cells develop pseudopodial extensions. After 1 week, a proportion of cells coalesced into spherical structures. They were less tethered to the connective tissue support, reminiscent of Madin–Darby canine kidney epithelial cells grown on similar support (17, 18).

Cells in the primary explants maintained the spatial arrangements derived from their origins in the fetal epithelium from which they were taken (Fig. 1*d*). Sheets of cells from the villus tip remained distributed as a semicircle, and those from the side of the villus were disposed in straight sheets. Cells remained in these configurations for 1–2 weeks, but as time passed they began to intermingle with the fibroblasts (Fig. 1*d*).

Extracellular Matrix Induces Polarized Structures in Enterocytes. The phenotype of H-4 cells was also assessed by

**H-4 cells will be deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD,20852, subject to approval by the Massachusetts General Hospital Office of Technology Affairs. Investigators wishing to use this dedifferentiated clone are requested to obtain cells from this source.

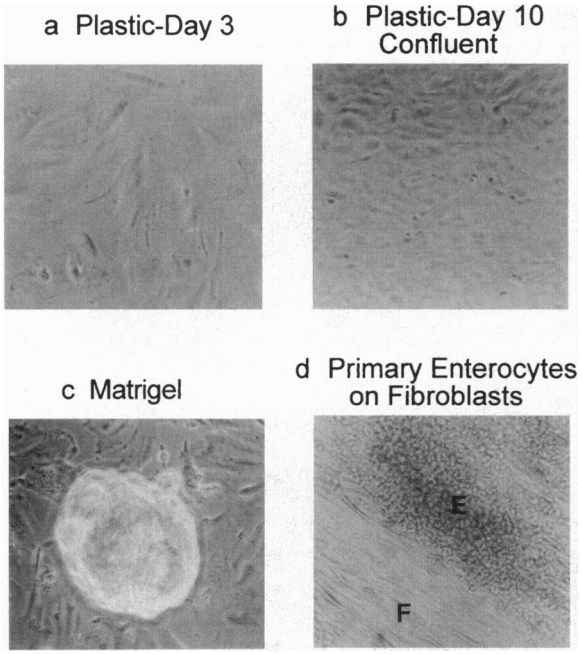

FIG. 1. Morphology of cultured human fetal enterocytes. (*a*) H-4 cells cloned from small intestinal segments cells on plastic at 3 days after plating and (*b*) grown to confluence. (*c*) H-4 cells grown on Matrigel showing appearance of a spherical pattern of cells. (*d*) Primary epithelial cell explants (E) amongst fibroblasts (F) on a laminin support. Endodermal cells were removed as sheets from intestine (see *Methods*) and plated onto fibroblasts without an intervening isolation step on plastic. (×4.)

examining filamentous (f-) actin with rhodamine phalloidin. f-Actin was organized mainly into stress fiber bundles on the basal region in H-4 cells grown on plastic or glass, but was not bundled into microvilli (Fig. 2*a*). However, culture of H-4 cells on the basement membrane support led to the development of apical microvilli (Fig. 2 *b* and *c*). In addition, the phenotype was also modulated at the basolateral pole, because H-4 cells that had developed microvilli demonstrated an associated loss of stress fibers at this pole. These experiments demonstrate that in nonmalignant enterocytes, the acquisition of a polarized cytoskeletal architecture depends on contact with external matrix structures at the basolateral membrane.

Expression of Cytokeratins and Villin in Enterocytes. Staining with anti-keratin antibody demonstrated strands of cytokeratin in H-4 cells (Fig. 3). Similar staining was found in the human colonic carcinoma cell line (Caco-2). However, no staining was seen in human fibroblasts.

Although cytokeratins distinguish cells of epithelial origin from fibroblasts, keratins are not specific to intestinal epithelial cells. A more specific cytoskeletal protein is villin (19–21), a component of the cytoskeleton of enterocytes and in other epithelial cells that contain brush borders. Clear aggregates of villin were not identified in H-4 cells on glass using immunohistochemistry (data not shown), but in cells grown on Matrigel, villin was detectable and colocalized to those positions where actin bundles were seen with phalloidin (Fig. 4 *a* and *b*), recapitulating events *in vivo* where actin is bundled at the brush border by villin. Villin synthesis may therefore be a step mediating the effects of connective tissue on brush border development. Villin was, however, detected by immunoblotting in H-4 cells grown on glass (Fig. 4*c*). Therefore, villin may be synthesized in small amounts in cells without connective tissue support but not positioned among the actin bundles in sufficient amounts to produce microvilli. In primary enterocytes plated onto fibroblasts, villin is very clearly identified in

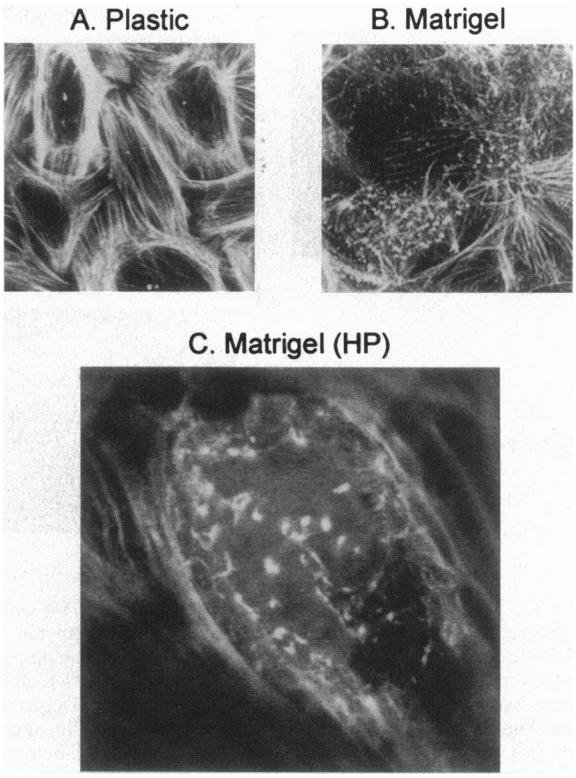

FIG. 2. Confocal microscopy of H-4 epithelial cells 5 days after plating. (×40.) After fixation cells were stained with phalloidin to examine the distribution of f-actin bundles. (*a*) Cells grown directly on glass are polygonal and flat with no polarized features. (*b*) H-4 enterocytes simultaneously plated onto Matrigel demonstrate the appearance of microvilli in a high proportion of cells. (×40.) The depth of focus includes both the microvilli at the apical aspect and the stress fibers at the basolateral aspect. The disposition of stress fibers is lessened in those cells that have microvilli. (*c*) High power (×100) of microvilli at the apex of an H-4 epithelial cell. Depth of focus is shortened at this magnification and distinguishes between the microvilli and the stress fibers of the adjacent cells which lie below the plain of focus, demonstrating their different positions in the vertical plane of the cell monolayer.

bundles near the apex of cells (Fig. 4*d*), consistent with the high brush border enzymes activities in this model (see below).

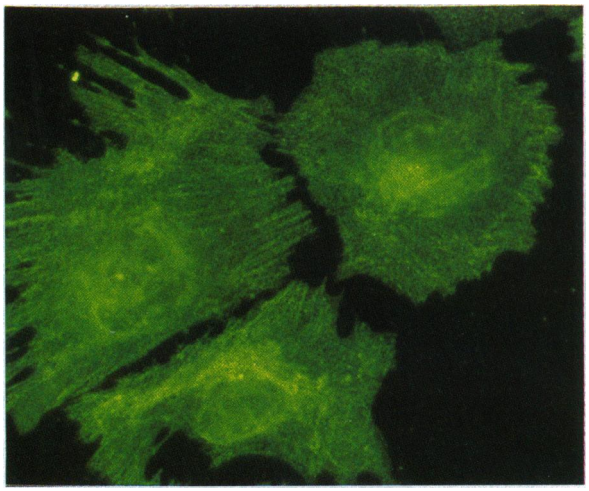

FIG. 3. H-4 enterocytes (day 3 after plating) stained with anti-cytokeratin antibody (AE-3) and examined by fluorescence microscopy to demonstrate their epithelial cell origin. (×40.) Caco-2 cells were also positive when stained with the antibody, but fibroblasts were negative.

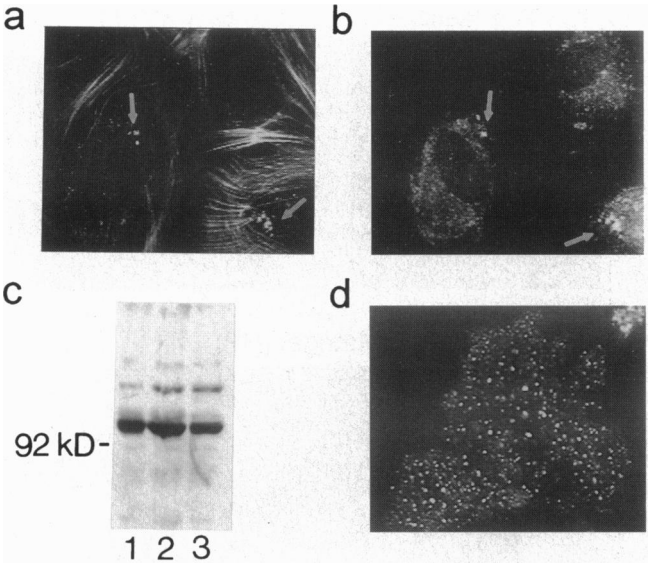

FIG. 4. Expression of villin in enterocytes. Villin in H-4 cells on Matrigel colocalized with actin bundles. (*a*) Actin detected with rhodamine-phalloidin. (×40.) (*b*) Villin demonstrated in the same cells by immunofluorescence using fluorescein. (*c*) Villin detected by Western blotting. The protein was extracted from H-4 cells grown on plastic. The Western blots were incubated with anti-pig villin antibody which, in turn, was detected by horseradish peroxidase-labeled antibodies using the ECL light sensitive Western blotting detection system. Lanes 1–3, growth of H-4 cells on plastic for 1–3 weeks, respectively. (*d*) Villin disposition in primary cell explants grown on fibroblasts. (×100.) Villin was stained with a anti-pig villin and stained with a fluorescein second antibody.

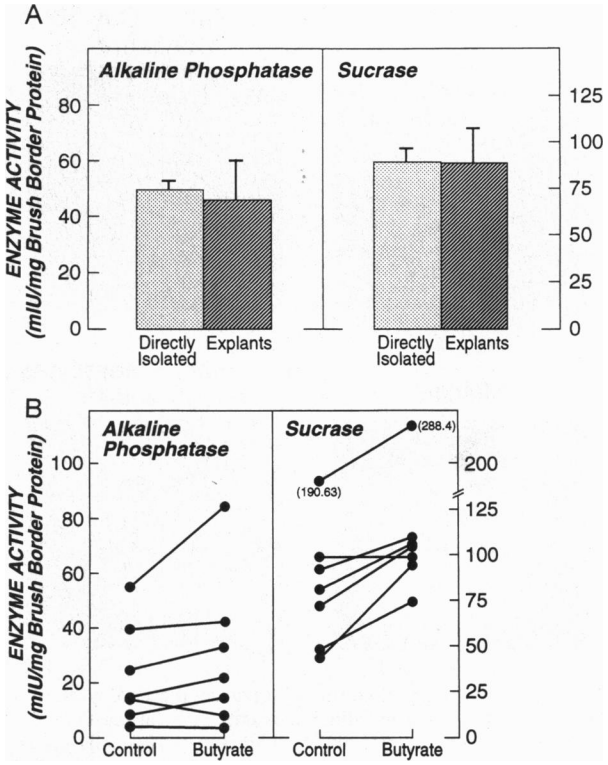

FIG. 5. (*a*) Expression of alkaline phosphatase and sucrase on primary cell explants after 48-h growth on fibroblasts. Enzyme activities in brush borders removed from cultured cells were comparable to those obtained from freshly isolated tissue. (*b*) Sodium butyrate (2.5 mM) for 24 h significantly increased sucrase activity ($t = 3.38$, $P < 0.05$). Explants were divided into butyrate and control samples.

Brush Border Enzyme Activity Is Enhanced by Enterocyte Microenvironment. Brush border enzymes are an integral part of the microvillus membrane and are quantitatively associated with enterocyte differentiation. To examine whether extracellular matrix altered the activity of these enzymes, H-4 cells were grown on Matrigel over 17 h. Alkaline phosphatase activity was enhanced from 0.95 ± 0.032 to 1.65 ± 0.016 mIU/mg brush border protein by this maneuvre ($P < 0.005$), but there was no significant sucrase activity detected. The specific activity of brush border enzymes of H-4 cells was compared with primary cell explants grown directly on fibroblasts. After 2 days, cells were removed and the brush borders isolated. Alkaline phosphatase activity in these explants was an order of magnitude greater than in H-4 cells grown on Matrigel (Fig. 5*a*). Furthermore, although connective tissue matrix did not induce significant sucrase activity in H-4 cells, sucrase activity was readily detected in primary sheets grown on fibroblasts. Sucrase activities were enhanced by the further addition of sodium butyrate to the medium 24 h before collection (Fig. 5*b*). Thus the differentiation lost during isolation of epithelial cells on plastic was not fully replaced by growing them again on connective tissue. Cells grown on fibroblast and connective tissue support maintained their enzyme activities. Indeed, the enzyme activities of primary cells grown for 48 h on fibroblasts were similar to those seen in brush borders isolated directly from fetal intestine (Fig. 5*a*).

The effect of connective tissue matrix on H-4 proliferation was studied by counting cell densities over time. H-4 cells reached confluence by 7 days on plastic. There was a reduction in cell numbers 24 h after plating, a phenomenon characteristic of nonmalignant cell lines, but not normally seen in malignant cells. Cells grown on connective tissue matrix did not increase in density, cell numbers always being well below those at plating. Thus there is a reciprocal relationship between the ability of H-4 cells to proliferate and their ability to differentiate. This reduction of proliferation is, therefore, an example of the recognized reduction of cell turnover that occurs in association with differentiation (22).

Connective Tissue Proteins Alter the Profile of IGFBPs. The profile of IGFBPs secreted by Caco-2 cells changes with differentiation, with an increase in IGFBP-2 and -3 and a fall in IGFBP-4 (14). Therefore, examination of the conditioned media for IGFBPs gives an independent assessment of whether factors modulate the phenotype of the epithelial cell. We

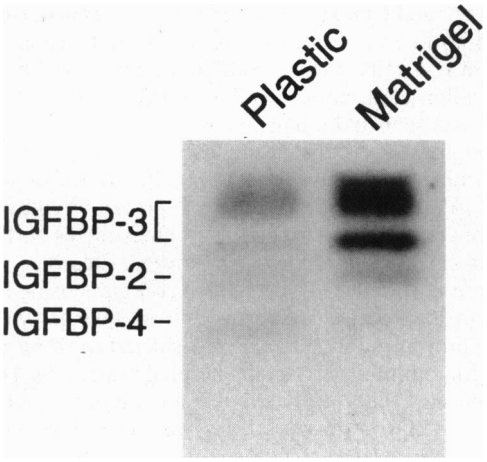

FIG. 6. Increase in IGFBP-2 and -3 secreted by H-4 cells relative to IGFBP-4 on plastic compared with on 50% Matrigel. Protein transfer (Western) blots were made of conditioned media collected during differentiation. Serum-free conditioned media incubated for 48 h were collected 7 days after plating. Samples were analyzed by Western ligand blotting with [^{125}I]IGF-I.

therefore examined the effect of connective tissue on IGFBP secretion by H-4 cells. Matrigel induced an increase in IGFBP-2 and -3 as identified by ligand blotting relative to IGFBP-4 (Fig. 6). Thus the pattern of IGFBP secretion changed toward that seen with enterocyte differentiation and is further evidence that the differentiation of enterocytes is enhanced by contact with an extracellular matrix. These experiments were not repeated with fibroblast support because the fibroblasts themselves secrete IGFBPs that cannot easily be dissected from the epithelial derived binding proteins in the supernatant (23).

DISCUSSION

In this study, we have developed techniques to examine epithelial cells derived from normal small intestine of the human fetus and have studied the effect of microenvironmental factors on their differentiation. These studies in a human nonmalignant epithelial cell line simulate the known stages of differentiation *in vivo* and show that external factors modulate the phenotype of intestinal epithelial cells.

The small intestinal epithelium resides in a functional unit in contact with the extracellular matrix and cells of mesodermal origin (24). The enterocyte, as it develops *in vivo* from the stem cell of the crypt, is in contact with such mesenchymal elements. An examination of the function of the epithelium may therefore be affected by this contact. To date, the study of the effect of extracellular factors on differentiation has been hampered by the lack of preparations of nonmalignant enterocytes. The best-characterized human enterocyte cultures presently available are of neoplastic origin. Neoplastic cells, by definition, have deranged cellular pathways that control differentiation, and, although they have proven to be excellent models for studying epithelium formation, information derived about mechanisms that affect differentiation has always been open to question.

This work shows clearly that isolation of enterocytes from their mesenchymal environment results in loss of differentiation. Both polarization and activity of brush border enzymes were lost in H-4 cells during the isolation of human fetal enterocytes. Culturing the cells in a mesenchymal environment resulted in only a partial return of polarization and a modest increase in alkaline phosphatase activity. It was not possible to completely mimic, *in vitro*, the characteristics of the mesenchyme of the basement membrane and the cell components of the lamina propria. Therefore we cannot say whether loss of differentiation would be fully reversed given the right mesenchymal environment, or whether some of the dedifferentiation is irreversible. Primary epithelial cell sheets grown directly from the human fetus had a greater activity of brush border enzymes when grown on fibroblasts than the H-4 cell line. The preserved extracellular components of the intestinal epithelial structure were more suitable for enterocyte differentiation than Matrigel. It seems probable that, for full differentiation to occur, both an uninterrupted series of events within the cell from crypt to villus (25) and an appropriate mesenchymal environment are needed. In addition, cells require other mechanisms that monitor differentiation and provide corrective adjustments when the differentiation process develops inappropriately. Studies of membrane analysis from crypt to villus by both Kedinger and coworkers (26) and Weiser *et al.* (27) support this view. The cells of the connective tissue through their interaction with the epithelium via connective tissue matrix are in a position to mediate influences on epithelial cells. Thus, corticosteroids, for example, may affect epithelial cell differentiation, both directly (28) or indirectly (29). Cytokines, also, may have different effects on the epithelium in contact with fibroblasts than in enterocytes alone (30).

In nonintestinal epithelial cell lines, microenvironmental factors including extracellular matrix have a central role in establishing a greater cellular and organizational complexity. Engelbreth–Holm–Swarm matrix and collagen gels both enhance the cellular differentiation of mouse mammary tumor cells (31, 32). Furthermore, amniotic basement membrane induces colonization of rat acinar tumor cells (33). The malignant colonic cell line Caco-2 does not require external basement membrane for its differentiation which occurs spontaneously in culture. It is likely that one of the steps that occurs in malignant transformation is a release from dependence on extracellular factors for differentiation. One mechanism by which this independence may be achieved is through the production of basement membrane molecules by the transformed epithelial cells (34). If part of the transformation process allows epithelial cells to differentiate independently, then it is possible that immortalizing cells with viral oncogenes will result in differentiation of cell lines that have been established and grown on plastic. This strategy is currently under investigation in our laboratory.

Data presented in this report supports the notion that both extracellular matrix and mesenchymal cells are required to maintain differentiation of the intestinal epithelium. An additional contributor is the role of luminal factors in achieving differentiation. Several factors are present in the intestinal lumen that have marked effects on enterocyte differentiation (35). These include (*i*) factors present in breast milk including epidermal growth factor (36, 37); (*ii*) factors produced by endogenous bacteria—for example, butyrate production (38, 39); and (*iii*) factors secreted into the gastrointestinal tract from other organs such as salivary glands, the gall bladder, and pancreas (40). The relative importance of these factors will depend on the age of the individual from fetus to adult.

This report has not specifically examined the role of cell–cell interactions on enterocyte differentiation. Cell–cell and matrix–cell contact have been disrupted *in vivo* in the intestinal epithelium of transgenic mice that express a mutant N-cadherin. This resulted in the development of intestinal inflammation (41). However, no loss of markers of enterocyte differentiation was reported in these animals. Nevertheless, cell–cell interactions are compromised in the H-4 cell clone, and the question as to the importance of this deficiency on cell differentiation remains unanswered.

Epithelial differentiation can alter not only the expression of structural proteins and brush border enzymes, but may also alter the production of certain secreted proteins. An example is the production of IGFBPs that alter epithelial cell growth (42, 43). Recent work from our laboratory has shown that Caco-2 cells produce at least three IGFBPs (14). We have now shown that H-4 cells secrete all the IGFBPs that are secreted by Caco-2 cell lines (Fig. 6). In Caco-2 cells the spectrum of IGFBPs was related to their degree of differentiation (14), and the present study shows that when connective tissue matrix is added, H-4 cells similarly vary their IGFBP secretion, in association with other changes in differentiation.

In addition to the proteins secreted by the enterocyte that are linked to differentiation, the epithelium also secretes cytokines that now define it as a part of the mucosal immune system. For example, human epithelial cells of carcinoma cell origin secrete interleukin 8 (IL-8) after a variety of stimulations (44); however, IL-8 production is increased in some malignant cell lines when compared with their nonneoplastic organ of origin (45). Therefore we cannot be certain, at present, that the production of Caco-2 cells is not merely a feature of their malignant state. Preliminary data has now shown that a combination of lipopolysaccharide and butyrate (both produced by luminal bacteria) stimulates the secretion of IL-8 by H-4 cells (9), showing definitively that IL-8 production occurs in nonmalignant enterocytes.

In conclusion, this study demonstrates that the addition of sequential extracellular factors are necessary for human nonmalignant enterocyte differentiation *in vitro*. Similar factors are also likely to be important *in vivo*. Epithelial cells derived

from normal human fetal small intestinal epithelium can grow in culture. However, isolating them on plastic alone results in dedifferentiation and loss of polarization. These attributes can be partially reversed by growth on mesenchymal elements and luminal factors. The question arises whether transformation can alter the dependence of intestinal epithelial cells on extracellular factors. To answer this we will immortalize epithelial cells with a temperature sensitive simian virus 40 T antigen. This will allow us to examine the degree to which malignant transformation allows the epithelium to escape from a dependence on extracellular elements in regard to its differentiation.

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