

Restitution at the Cellular Level: Regulation of the Migrating Phenotype

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Intestinal epithelial cells migrating across a mucosal defect are generally described as dedifferentiated, a term that suggests a loss of regulatory biology. Since cell biology may be more readily studied in established cell lines than *in vivo*, a model is developed using the human Caco-2 intestinal epithelial cell migrating across matrix proteins. This resembles *in vivo* models of mucosal healing in its sheet migration and loss of the brush border enzymes, which are conventional markers for intestinal epithelial differentiation. Immunohistochemical studies of migrating Caco-2 cells suggest, however, that the rearrangements of cytoskeletal, cell-cell and cell-matrix proteins during migration are not random but seem adapted to the migratory state. Indeed, Caco-2 migration may be substantially regulated by a variety of physiologic and pharmacologic stimuli and differentiation, measured by the specific activity of the intestinal epithelial brush border enzymes alkaline phosphatase and dipeptidyl dipeptidase, may be independently pharmacologically programmed during the stimulation or inhibition of cell motility.

INTRODUCTION

Intestinal epithelial wound healing occurs primarily by restitution, a process by which epithelial cells at the edge of a mucosal wound lose their conventionally differentiated characteristics including a columnar morphology and the expression of brush border enzymes such as alkaline phosphatase and dipeptidyl dipeptidase [1-5]. The migrating cells assume a squamous morphology and migrate across the defect as a sheet until it is resurfaced. Proliferation is a secondary phenomenon, which does not occur until 24 hr after the initial mucosal injury and which serves primarily to replace cells lost in a large mucosal injury.

Although migrating intestinal epithelial cells are conventionally described as "dedifferentiated," it may be more appropriate to define these cells as re-differentiating toward a migratory phenotype. The difference is more than semantic, since the description of such cells as dedifferentiated implies that motility is an unregulated or unregulatable process. If the cells are re-differentiating, however, then one could invoke standard paradigms of the regulation of differentiation to raise the possibility that the migratory phenotype is regulated physiologically and may be further modulated pharmacologically to program characteristics desirable for rapid wound healing and thereafter for resumption of a physiologically normal mucosa. The latter concept is not trivial. Tarnawski and colleagues [6, 7] have recently emphasized the importance of the quality of ulcer healing as well as its

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^bAbbreviations: BCECF, 2', 7'-bis (carboxyethyl)-5(and 6) carboxy fluorescein.

quantity, suggesting that abnormal neomucosa may exhibit abnormal function and increased recurrence rates.

The issue is difficult to study *in vivo* since mucosal biology *in vivo* represents a heterogeneous population of cells influenced by a complicated array of factors including systemic nutrition, post-operative or post-injury phenomena, growth factors, luminal nutrients, vascularity, matrix synthesis and inflammation. We have therefore chosen to address these questions in a preliminary fashion in an isolated cell line in culture, using a subclone of the human Caco-2 intestinal epithelial cell line as a model and a "fence" migration assay to quantitate cell migration after pharmacologic stimulation. We have also measured the specific activity of two common differentiation markers: alkaline phosphatase and dipeptidyl dipeptidase, in response to similar treatment with exogenous agents. We summarize here our recent investigations, suggesting that Caco-2 cells undergoing sheet migration exhibit a loss of brush border enzyme activity similar to that observed in healing mucosa in a rat model; that intracellular pH is modulated and the organization of a cytoskeletal protein (actin) and a cell-cell adhesion molecule (ZO-1) are specifically regulated during cell motility, and that both cell motility and brush border enzyme activity may be independently regulated in these cells.

MATERIALS AND METHODS

Rat model

Sixty Sprague Dawley rats weighing 150-200 g were fasted overnight and then subjected to laparotomy using sterile technique. A 0.5 cm incision was made lengthwise along the antimesenteric border of the mid-jejunum. This original defect was allowed to remain open, but was then patched using a seromuscular patch created by tacking the antimesenteric serosa of a distal loop of mid-jejunum across the defect using four 6-0 proline sutures. Control animals underwent sham laparotomy and tacking together of two loops of jejunum without jejunal enterotomy. Animals were maintained without oral intake for 12 hours and then permitted to eat *ad libitum*. Animals were then observed for one to 60 days prior to sacrifice and fixation of the surgically manipulated segment of bowel. There were no septic deaths in either group of rats and the serosal patch was found to be grossly intact at sacrifice in all animals subjected to enterotomy and patching.

Cells

The BBE clonal subline of Caco-2 cells [8] was used for all studies in cell culture. These cells were maintained at 37°C in five percent CO₂ in Dulbecco's minimal essential medium with 10 percent fetal calf serum, 10 mg/ml transferrin (Boehringer Mannheim, Indianapolis, IN), 2 mM glutamine, 1 mM pyruvate, 10 mM HEPES (N-2 Hydroxyethyl-piperazine-N'-2-ethanesulfonic acid), 100 units/ml penicillin-G, and 0.1 mg/ml streptomycin. The experiments represented in each figure were performed on the same day using the same passage of cells.

Alkaline phosphatase staining

For studies of alkaline phosphatase in tissue sections, the segment of intestine treated surgically was carefully excised intact after euthanasia and was fixed in 10 percent neutral buffered formalin. The tissue was embedded in paraffin and sectioned at 4 µm for light microscopy. Routine sections were stained with hematoxylin and eosin, and selected levels were stained for detection of alkaline phosphatase using aminoethylcarbazole chromagen, counterstained with hematoxylin, and mounted in Crystalmount. For studies of alkaline phosphatase in Caco-2 cells, the cells were similarly fixed in 10 percent neutral buffered formalin and then stained with naphthol AS-TR phosphate and Fast Red salt TR (Sigma), counterstained with hematoxylin, and mounted in Crystalmount.

Immunohistochemical staining

Immunohistochemical staining was performed in Caco-2 cell monolayers fixed using periodate-lysine-paraformaldehyde fixative and permeabilized with 0.2 percent Triton X-100. ZO-1 staining used an antibody which was a generous gift of Dr. James M. Anderson of the Yale University Department of Medicine [9] while rhodamine-conjugated phalloidin was visualized to image actin organization.

Intracellular pH measurement:

Confluent and migrating Caco-2 cells cultured in standard culture medium (extracellular pH 7.6) were trypsinized and triturated to a single cell suspension, which was then placed in a glass chamber mounted over a cover slip previously coated with Cell Tak (Collaborative Biomedical, Bedford, MA). The cells were allowed to settle and adhere to the cover slip and then were washed with oxygenated Modified (calcium, magnesium and bicarbonate free) Hanks Balanced Salt Solution containing 137 mM NaCl, 5.6 mM glucose, 5.6 mM KCl, 0.44 mM KH_2PO_4 and 0.33 mM Na_2HPO_4 . Cells were then loaded with 1 μM of the pH-sensitive dye 2',7'-bis(carboxyethyl-5-(and 6)-carboxyfluorescein-acetoxy methyl ester (BCECF^b-AM, Molecular Probes, Eugene, OR) at room temperature for 20 min [10] and intermittently washed with oxygenated Modified Hanks Balanced Salt Solution to remove extracellular dye and toxic by-products of BCECF-AM cleavage [11]. Chambers containing the cells were then mounted on a Diaphot 100 inverted microscope (Nikon, Tokyo, Japan) interfaced with a digital imaging system [12], which consisted of a Hamamatsu C2400 intensified CCD camera (Hamamatsu Photonics, Japan), an Image-LC image capture board (Matrox, Quebec, Canada) and a 486-based IBM compatible computer. Cells were viewed through a 40x oil immersion objective (Nikon CF-Fluor) and alternately excited at 490 nm and 440 nm using a 75 watt xenon fluorescent light source through a Lambda-10 optical filter wheel (Sutter Instrument Co., Novato, CA), and emissions were monitored at 520 nm. Fluorescence images were captured and analyzed using MetaFluor ratiometric fluorescent imaging software (Universal Imaging), which automatically calculated the 490/440 nm fluorescence ratio used for calculating intracellular pH. Intracellular pH was calibrated at the end of each experiment by measuring the fluorescence ratios of cell-free Modified Hanks Balanced Salt Solution titrated to five different pH values and loaded with a free acid form of BCECF.

Motility

Epithelial sheet migration was quantitated using the "fence" migration assay previously described [13, 14]. Briefly, cells are plated to confluence within the central well of a stainless steel fence which is then removed, permitting outward radial migration. After six days, each monolayer is fixed and stained with hematoxylin. The fixed monolayers are scanned into an IBM-compatible computer using a Microtek IIXE scanner, and the monolayer areas are measured using image analysis software (Sigma Scan/Image, Jandel Scientific, Anaheim, CA). The migrated area is then determined by subtracting the area of the original central well of the fence from the total monolayer area.

Enzyme assays

Cells were plated on bacteriologic plastic dishes (Falcon, Oxnard, CA) coated with type I collagen and allowed to grow to confluence. Cells were then treated with a medium supplemented with experimental agents for 24 hr and lysed on ice in a solution containing 0.5 percent Triton X-100 and 0.35 M NaCl for 1 hr. After separation of insoluble lipid components by centrifugation, the supernatants were assayed for protein (BCA assay, Pierce, Rockford IL), and all samples were diluted to equal protein content. Assays of enzyme activity were then performed as previously described, incubating protein-

matched samples of cell lysates with synthetic substrates and then quantitating the digestion products thus produced spectrophotometrically [15]. Standard dilution curves were performed simultaneously with each assay. Conditions for all digestive enzyme assays were initially calibrated by assay of serial dilutions of known standards, and all experimental assays were then performed within the range of linear response (data not shown). Results were standardized during each experiment against known standards and expressed as enzyme activity in international units per microgram of protein.

RESULTS

Studies of the regenerating neomucosa in the rat jejunum after enterotomy demonstrated a profound loss of histochemical staining for alkaline phosphatase in early mucosal healing which persisted even at 25 days, when the neomucosa appeared virtually normal histologically (Figure 1). A similar phenomenon was observed in migrating Caco-2 monolayers in which histochemical staining for alkaline phosphatase remained intact in the confluent cells within the monolayer but was completely ablated in the migrating cells at the edge of the monolayer (Figure 2a).

Quantitation of brush border enzyme activity during Caco-2 migration revealed substantial decreases in the specific activity of alkaline phosphatase and dipeptidyl dipeptidase in cells seeded into circumscribing fences and then allowed to migrate radially as compared with confluent (non-migrating) cells that had been seeded at the same time and density from the same parent flask of cells (Figure 2b). In particular, lysates of the migrating cell population expressed 85.9 ± 1.5 percent of the alkaline phosphatase specific activity of non-migrating cells and 55.1 ± 1.9 percent of the dipeptidyl dipeptidase specific activity of non-migrating cells. ($n = 9$, $p < .001$ for each)

The organization of actin and ZO-1 appeared sharply different in migrating cells. Although actin cables were not clearly organized in a discernible pattern in non-migrating cells, actin staining in cells at the migrating edge of the monolayer demonstrated a paucity of actin staining in the lamellipodium itself and a transverse cable of actin oriented at right angles to the direction of migration (Figure 3a). ZO-1 staining in non-migrating cells uniformly outlined the circumference of the cell in cross section. However, ZO-1 immunoreactive staining in migrating cells outlined only areas of cell-cell contact. ZO-1 staining was absent from the migrating edge of these cells (Figure 3b).

In addition to these changes in cytoskeletal and adhesion protein organization, the intracellular pH was also different between static and migrating cells. In non-migrating (confluent) cells, the intracellular pH was 7.28 ± 0.09 while the intracellular pH of migrating cells seeded simultaneously was 7.68 ± 0.1 ($n = 50$, $p < .001$).

Cell motility and brush border enzyme activity were inversely regulated according to the matrix protein substrate upon which the cells were cultured. Table 1 summarizes previously published data in this regard [14, 16] ($n > 6$, data normalized to type I collagen values, $p < .01$ for all comparisons) Motility was most rapid and alkaline phosphatase and dipeptidyl dipeptidase activity least on interstitial type I collagen, while migration was slower and brush border enzyme specific activity higher on the basement proteins laminin and type IV collagen. We next considered whether cell motility and brush border enzyme activity could independently be regulated by candidate pharmacologic agents directed at membrane receptors, metabolic pathways or intracellular signal transduction. Table 2 summarizes some previously published data in combination with new data that addresses this issue. ($n > 10$, data normalized to control values, $p < .01$ for all comparisons except NS as shown) Indeed, this proved the case. Both epidermal growth factor and pentagastrin tended to stimulate both cell motility and brush border enzyme specific activity (although the effect of pentagastrin on alkaline phosphatase did not achieve statistical significance). However, the gut neuropeptide PYY selectively stimulated alkaline phosphatase specific

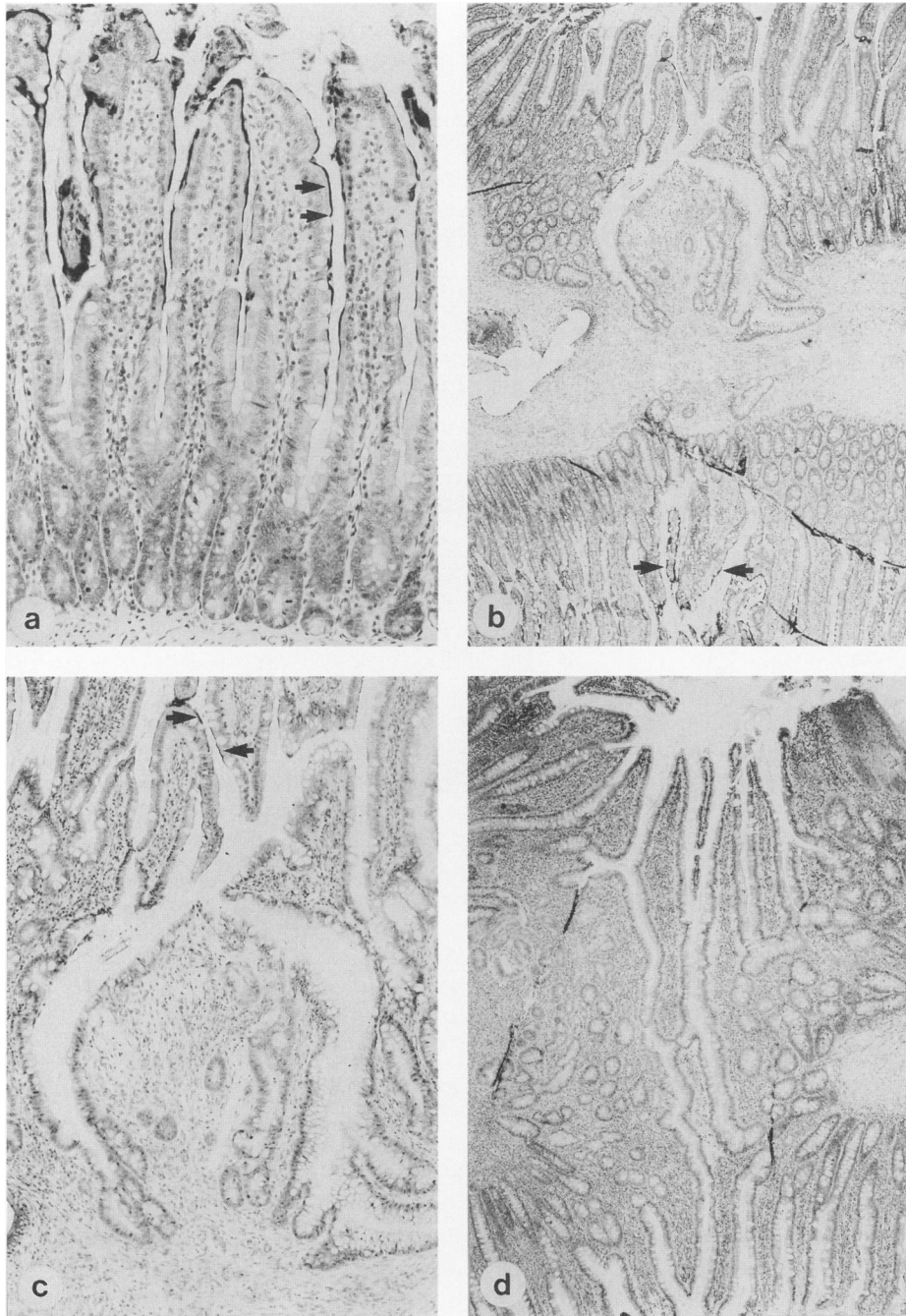


Figure 1. Alkaline phosphatase expression in rat jejunal mucosa (aminoethylcarbazole chromagen counterstained with hematoxylin). A: Normal jejunum. Alkaline phosphatase is expressed in the brush border of the villous epithelial cells (black line) but not in crypt epithelium (165x). B: Day seven. No alkaline phosphatase is detectable in the regenerating neomucosa, but the normal mucosa at the bottom of the field shows positivity on the surface of the villi (41x). C: Day seven. Higher power view of the upper part of the field illustrated in B. Scant alkaline phosphatase positivity is evident in the brush border of a few villi in the upper center of this field, but it is absent from the area of regeneration (82x). D: Histologically healed neomucosa: Alkaline phosphatase, present in the normal mucosa of this preparation as illustrated in A, is absent from the neomucosa. (50x).

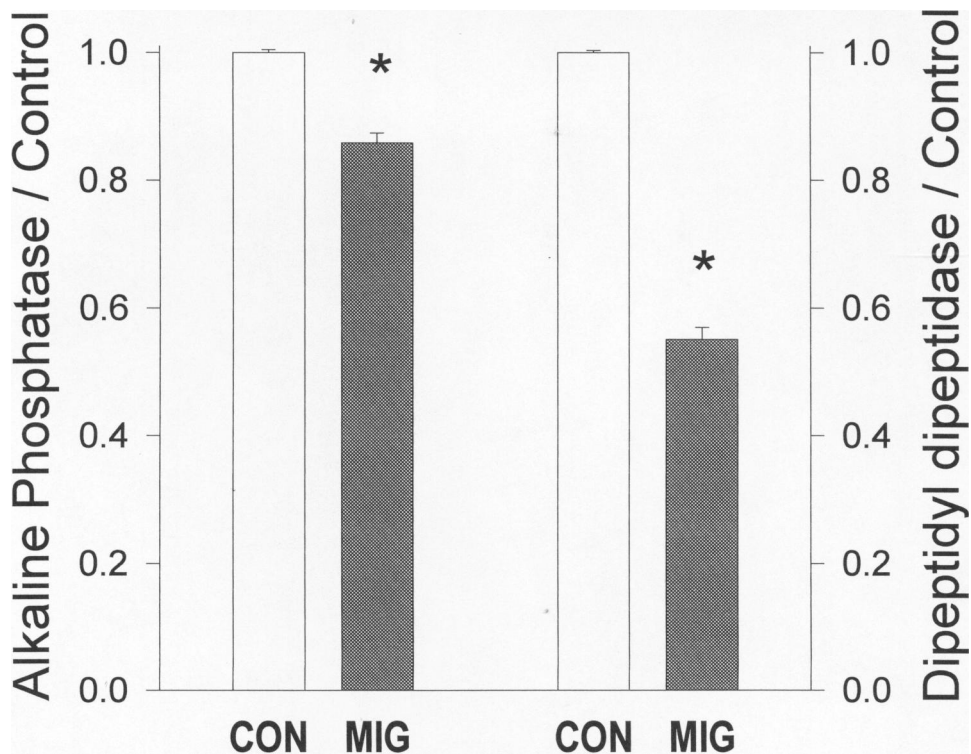
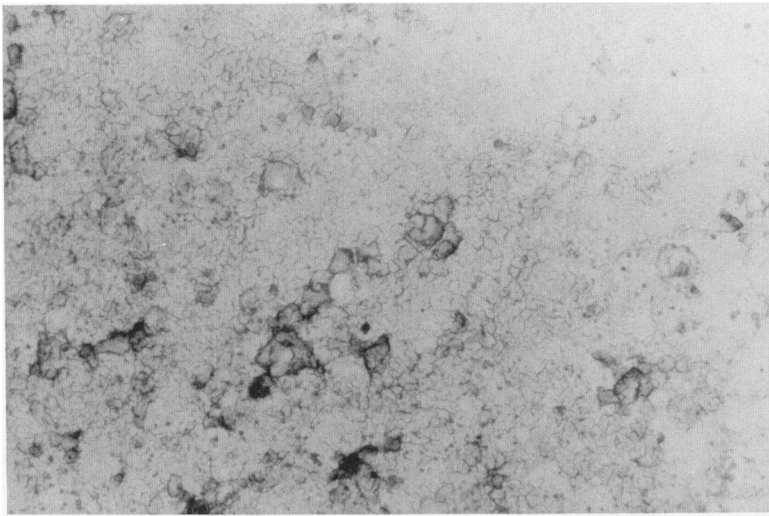


Figure 2a (top). Migrating Caco-2 cells stained histochemically for alkaline phosphatase (naphthol AS-TR phosphate and Fast Red salt TR, counterstained with hematoxylin). Histochemical staining for alkaline phosphatase is evident within the confluent monolayer in the center of the figure, but is decreased in the migrating front at the periphery of the monolayer.

Figure 2b (bottom). Differences in specific activity of alkaline phosphatase (first two bars) and dipeptidyl dipeptidase (second two bars) in static confluent Caco-2 cells (CON, open bars) and migrating Caco-2 cells of the same passage seeded simultaneously (MIG, shaded bars) six days after seeding. Results are expressed as percent of confluent control cells and represent pooled data from three similar experiments (n = 9, asterisk = p < .001).

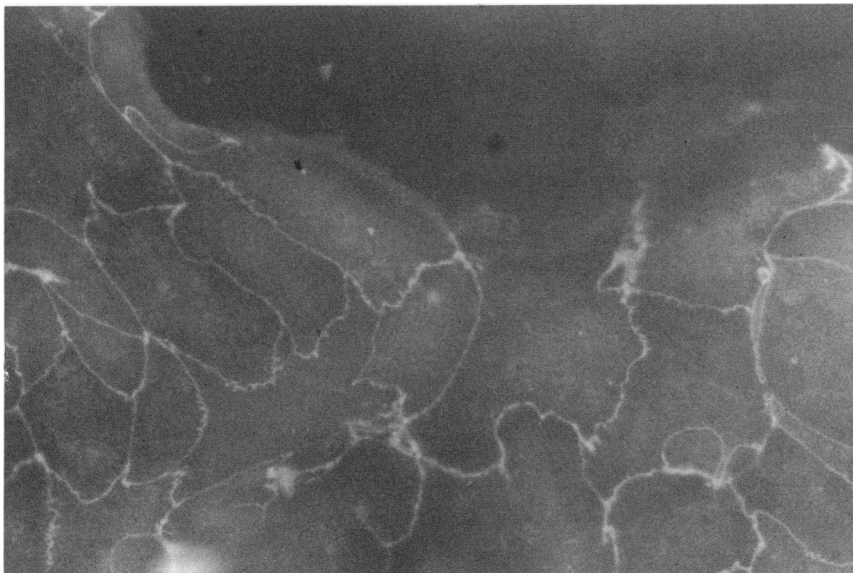


Figure 3a (top). Migrating Caco-2 cells stained for actin using rhodamine-conjugated phalloidin. Actin organization differs substantially between cells within the cell monolayer and migrating cells at the edge of the monolayer in which a paucity of actin staining is observed within the lamellipodial edge and a thick transverse actin cable is observed behind the lamellipodial edge perpendicular to the direction of migration.

Figure 3b (bottom). Migrating Caco-2 cells immunohistochemically stained for ZO-1. ZO-1 organization differs substantially between cells within the cell monolayer and migrating cells at the edge of the monolayer in ZO-1 staining is lost at the lamellipodial edge.

Table 1. Effects of extracellular matrix substrates on Caco-2 motility and brush border enzymes.

Matrix protein	Motility	Alkaline phosphatase	Dipeptidyl dipeptidase
Type I collagen	100±1.2%	100±1.2%	100±2.9%
Type IV collagen	90±2.3%	121±1.2%	162±4.0%
Laminin	68±1.6%	140±4.2%	179±1.2%

Table 2. Effects of soluble agents on Caco-2 motility and brush border enzymes. References refer to work previously published by our laboratory demonstrating these findings.

Agent	Concentration	Motility	Alkaline phosphatase	Dipeptidyl dipeptidase
EGF	30 ng/ml	210±20% [14]	229±2%	125±1%
PYY	10 ⁻⁷ M	98.8±3.1% (NS) [20]	138±2% [20]	92±6% [20]
Pentagastrin	10 ⁻⁵ M	107±1%	105±3% (NS) [20]	131±1% [20]
Butyrate	10 mM	17±1% [19]	131±1% [19]	140±5% [19]
Glutamine	10 mM	100±6% (NS)	65±2% [15]	47±2% [15]
Asparagine	10 mM	118±4%	88±5%	151±6%
TPA	0.7 ug/ml	2±2% [17]	92±1% [17]	555±26% [17]
NaOV	1.5 uM	137±5%	189±16%	47±4%
Sucralfate	33.9 mM	87±1% [33]	258±28%	115±1%

activity without altering either cell motility or dipeptidyl dipeptidase activity. Treatment with the short-chain fatty acid butyrate actually inhibited motility and promoted specific activity of the two differentiation markers studied, but glutamine supplementation inhibited brush border enzyme activity without altering motility, and the closely related amino acid asparagine actually stimulated motility and dipeptidyl dipeptidase specific activity while inhibiting alkaline phosphatase activity in these studies. We have previously demonstrated that prolonged treatment with the phorbol ester TPA (0.7 ug/ml for 24 hr) down-regulates intracellular protein kinase C activity in Caco-2 cells [17]. Treatment with 0.7 ug/ml TPA for 24 hr also stimulates dipeptidyl dipeptidase activity while inhibiting alkaline phosphatase activity and motility. Conversely, treatment with the tyrosine phosphatase inhibitor sodium orthovanadate actually stimulates motility and alkaline phosphatase activity while inhibiting dipeptidyl dipeptidase activity.

DISCUSSION

Although the extrapolation from a cultured cell to *in vivo* phenomena must always be cautious [18], the use of cultured cell lines offers substantial advantages for the study of mammalian biology. A large and homogenous cell population can readily be obtained without confounding influences relating to the neuroendocrine milieu or other similar variables. Although originally derived from a colonic adenocarcinoma, the Caco-2 cell is a commonly used model for enterocytic biology, while the subclone utilized in these studies is characterized by the morphological and cytoskeletal features of a mature enterocytic brush border [8] as well as the ability to regulate brush border enzyme expression [15, 17, 19-22] and epithelial sheet migration [14, 17, 23, 24] in response to a wide variety of extracellular and intracellular stimuli. In addition, we report here that these cells exhibit decreased brush border enzyme activity during motility just as mucosal enterocytes *in vivo* exhibit decreased brush border enzyme activity during the regeneration of a neomucosa

after injury. The quantitative data in Figure 2b are likely to substantially underestimate the degree to which brush border enzyme specific activity is lost during Caco-2 migration since these were derived from lysates of cell monolayers in which the inner cells were confluent and the outer cells were migrating. The degree to which topographical variation in loss of brush border enzyme activity occurs in these monolayers is suggested by the gradient of alkaline phosphatase activity represented in Figure 2a.

Caco-2 cells, therefore, would appear to represent a reasonable model in which to study the independent regulation of intestinal epithelial phenotype and cell motility. Migrating cells are frequently described as dedifferentiated. However, actin staining demonstrates not disorganization but a specific and ordered reorganization in migrating Caco-2 cells. Similarly, immunoreactive staining for the cell-cell adhesion molecule ZO-1, which associates with cadherin and alpha-catenin in tight junctions [9, 25-27], occurs in migrating cells along areas of cell-cell contact but not along the lamellipodial edge. One could expect a dedifferentiated cell to lose ZO-1 staining completely, but not to develop an ordered pattern of staining. Thus, these staining patterns suggest that these cells are not dedifferentiated but dedifferentiated towards a specialized phenotype adapted to cell motility and mucosal healing. The relative intracellular alkalization associated with Caco-2 cell migration may also reflect the adaptation to cell motility. We have previously described a similarly intricate pattern of reorganization of the $\alpha 2$ integrin subunit, which regulates epidermal growth factor-stimulated Caco-2 cell motility over a laminin substrate [14]. The regulation of rates of cell motility by a wide array of extracellular and intracellular agents further suggests that epithelial sheet migration is not random movement by dedifferentiated cells but an ordered and regulatable process.

However, these data suggest that classical dedifferentiation is not obligatory in migrating cells, since peptides such as epidermal growth factor and pentagastrin upregulate brush border enzyme activity as well as cell motility. Indeed, the present data would suggest not only that motility and brush border enzyme activity are independently regulated, but also that the activity of individual brush border enzymes may be selectively modulated by gut peptides and luminal nutrients. The mechanism of the sucralfate effect awaits further investigation. It seems possible that the effect of sucralfate on motility might be steric since sucralfate binds avidly to type I collagen [28-30]. The effect of sucralfate on cell differentiation may reflect the ability of this compound to bind to growth factors in the environment and present them to the cells [31, 32].

The mechanism of all of these effects awaits elucidation. In particular, it remains unclear to what extent these phenomena occur at the pre-translational or post-translational level. However, it is intriguing in this regard to note that protein kinase C downregulation via the phorbol ester TPA downregulates motility and alkaline phosphatase while tyrosine phosphatase inhibition by sodium orthovanadate has the opposite effects on all three parameters. Such observations raise the possibility that pathways mediated by both tyrosine and serine phosphorylation may converge to regulate intestinal epithelial motility and differentiation.

Much work remains in order to begin to understand the regulation of intestinal epithelial motility and differentiation. However, the data summarized here suggests that the migrating intestinal epithelial cell alters its phenotype to exhibit specific characteristics including decreased brush border enzyme activity and altered organization of cytoskeletal and cell adhesion molecules. Cell motility may be exogenously modulated by pharmacologic agents directed at membrane receptors or intracellular signaling pathways as well as by luminal nutrients. In particular, pharmacologically promising manipulations in the future may include treatment with analogs of epidermal growth factor or pentagastrin, asparagine supplementation, or interventions targeted at tyrosine phosphorylation or protein kinase C mediated signaling. Furthermore, these results suggest that although migrat-

ing intestinal epithelial cells exhibit decreases in the brush border enzymes required for nutrient absorption during and after the regeneration of a neomucosa, these other aspects of the migrating phenotype may be modulated independently of the rate of cell motility by appropriately targeted pharmacologic manipulation.

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