Differentiated intestinal epithelial cells exhibit increased migration through polyamines and myosin II

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Rao, Jaladanki N., Ji Li, Li Li, Barbara L. Bass, and Jian-Ying Wang. Differentiated intestinal epithelial cells exhibit increased migration through polyamines and myosin II. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G1149–G1158, 1999.—Early mucosal restitution is a rapid process by which differentiated intestinal epithelial cells migrate to reseal superficial wounds. However, most of the in vitro studies for restitution employ undifferentiated intestinal crypt cells as a model. The transcription factor, Cdx2, plays an important role in the regulation of intestinal epithelial differentiation. Forced expression of the Cdx2 gene in undifferentiated intestinal crypt cells induces the development of a differentiated phenotype. The current study was designed to determine changes in differentiated intestinal epithelial cell migration after wounding in the stable Cdx2-transfected IEC-6 cells and then to examine involvement of polyamines and nonmuscle myosin II in the process of cell motility. Cdx2-transfected IEC-6 cells were associated with a highly differentiated phenotype and exhibited increased cell migration after wounding. Migration of Cdx2-transfected IEC-6 cells were approximately four times that of nontransfected IEC-6 cells. Migration after wounding was associated with significant increases in polyamine synthesis. Depletion of cellular polyamines by 5 mM α-difluoromethylornithine (DFMO), a specific inhibitor of polyamine biosynthesis, inhibited cell migration without affecting the differentiated phenotype. DFMO also decreased levels of nonmuscle myosin II mRNA and protein and resulted in reorganization of myosin II, along with a marked reduction in stress fibers. Exogenous spermidine given together with DFMO not only returned nonmuscle myosin II levels and cellular distribution toward normal but also restored cell migration to control levels. These results indicate that 1) Cdx2-transfected IEC-6 cells exhibit increased cell migration after wounding and 2) cellular polyamines are absolutely required for stimulation of cell migration in association with their ability to modulate the structural organization of nonmuscle myosin II.

Cdx2 gene; restitution; ornithine decarboxylase; cytoskeleton; mucosal injury; differentiation

RESTITUTION OF SUPERFICIAL wounds in the gastrointestinal mucosa occurs by sloughing off the damaged epithelial cells and migration of remaining viable cells from areas adjacent to, or just beneath, the injured surface to cover the wounded area in vivo (26, 28). This early mucosal reepithelialization does not require cell proliferation and is the function of differentiated intestinal epithelial cells from the surface of the mucosa, rather than from the proliferative zone of undifferentiated stem cells within the crypts. However, most of the studies that used an in vitro system that mimicked the early cell division-independent stage of epithelial restitution employed undifferentiated intestinal crypt cells such as the IEC-6 line as a model (6, 7, 18, 20, 21). The IEC-6 cells are derived from neonatal rat small intestine and have characteristics of crypt-type epithelial cells, which do not exhibit differentiated morphology or specific gene expression (24). The ability and the regulation of normal differentiated intestinal epithelial cells to migrate after wounding in an in vitro system are still unclear, although its importance in understanding the mechanisms of mucosal restitution under physiological conditions is obvious.

The intestine-specific homeobox gene, Cdx2, encodes a transcription factor that is involved in the regulation of intestinal epithelial cell differentiation (31, 34). During intestinal development in the mouse embryo, an important transition is that Cdx1 and Cdx2 genes are expressed when the visceral endoderm transforms into a simple columnar epithelium with nascent villi (10, 15). In the adult animal, Cdx genes continue to be expressed at high levels in differentiated epithelial cells but not in undifferentiated crypt cells of the small intestine and colon (17, 27). When Cdx2 is forced to express in undifferentiated IEC-6 cells, it significantly induces the development of a differentiated phenotype (32). The stable Cdx2-transfected IEC-6 cell line expressing high levels of Cdx2 has multiple morphological characteristics of villus-type enterocytes and expresses sucrase-isomaltase, a specific molecular marker of small intestinal epithelial cell differentiation (31, 32).

The current study was designed to address several questions regarding the natural process of epithelial restitution in cells with highly differentiated phenotypes by using the cultured stable Cdx2-transfected IEC-6 cells. First, we wanted to determine the changes in the rate of Cdx2-transfected IEC-6 cell migration after wounding. Second, we were particularly interested in the involvement of polyamine synthesis in the mechanisms responsible for Cdx2-transfected IEC-6 cell migration, since our previous studies (36, 37) and those of others (16, 21, 38) have demonstrated that cellular polyamines are absolutely required for mucosal restitution in vivo and for stimulation of nontransfected IEC-6 cell migration in vitro. Third, we wished to determine whether observed changes in polyamines affected the concentration and distribution of nonmuscle myosin II.

MATERIALS AND METHODS

Materials. Disposable culture ware was purchased from Corning Glass Works (Corning, NY). Tissue culture medium...
and dialyzed fetal bovine serum (dFBS) were obtained from GIBCO BRL (Gaithersburg, MD), and biochemicals were from Sigma (St. Louis, MO). L-[14C]ornithine (specific activity of 51.6 mCi/mmol), [α-32P]dCTP and [γ-32P]ATP were purchased from NEN (Boston, MA). The primary antibody, an affinity-purified rabbit polyclonal antibody against nonmuscle myosin II, was purchased from Biomedical Technologies (Stoughton, MA). Anti-rabbit IgG-FITC isomer conjugate was purchased from Sigma. Myosin II oligo probe was a gift from Merrell Dow Research Institute of Marion Merrel Dow (Cincinnati, OH).

Cell culture and general experimental protocol. The IEC-6 cell line was purchased from ATCC at passage 13. The cell line was derived from normal rat intestine and was developed and characterized by Quaroni et al. (24). IEC-6 cells originated from intestinal crypt cells as judged by morphological and immunologic criteria. They are nontumorigenic and retain the undifferentiated character of epithelial stem cells. Cells were grown in DMEM supplemented with 5% heat-inactivated FBS, 10 µg/ml insulin, and 50 µg/ml gentamicin sulfate and incubated at 37°C in a humidified atmosphere of 90% air-10% CO2. Stock cells were subcultured once a week at 1:20; medium was changed three times weekly. The cells were restarted from frozen stock every five to six passages. Tests for mycoplasma were routinely negative.

The stable Cdx2-transfected IEC-6 cell lines were developed and characterized by Suh and Traber (32) and were kind gifts from Dr. Peter G. Traber (University of Pennsylvania, Philadelphia, PA). The expression vector, the LacSwitch system (Stratagene, La Jolla, CA), was used for directing the conditional expression of Cdx2, and isopropyl-β-D-thiogalactopyranoside (IPTG) served as the inducer for the gene expression. IEC-6 cells were transfected with pO discouraged technique, and when resistant to selection medium containing 0.6 mg G418/ml and 0.3 mg hygromycin B/ml were isolated and screened for Cdx2 expression by Northern blot, RNase protection assays, and electrophoretic mobility shift assay. Stock cells were grown in DMEM used in parental nontransfected IEC-6 cells. Before experiments, cells were grown in DMEM containing 4 mM IPTG for 16 days to induce cell differentiation.

The general protocol of the experiments and the methods used were similar to those described previously (38). In brief, IEC-6 and Cdx2-transfected cells were plated at 6.25 x 10^4 cells/cm^2 in DMEM plus 5% dFBS, 10 µg/ml insulin, 50 µg/ml gentamicin sulfate, and 4 mM IPTG. The cells were incubated in a humidified atmosphere at 37°C in 90% air-10% CO2. Stock cells were subcultured once a week at 1:20; medium was changed three times weekly. The cells were restarted from frozen stock every five to six passages. Tests for mycoplasma were routinely negative.

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In the first series of studies, we examined changes in migration rates of Cdx2-transfected IEC-6 cells after wounding and then compared the differences between Cdx2-transfected cells and nontransfected cells. Cells were grown in standard DMEM for 4 days after initial plating, and cell migration was assayed at various times after removal of part of the cell layer.

In the second series of studies, we examined the effect of polyamine synthesis on cell migration in Cdx2-transfected IEC-6 cells. Cells were grown in the presence or absence of 5 mM DFMO, a specific inhibitor of ornithine decarboxylase (ODC, the first rate-limiting enzyme for polyamine synthesis), for 4 days. The ODC mRNA and enzyme activity, cellular polyamine levels, and cell migration were assayed from cell layers that were intact or from layers at various times after wounding. Dishes were placed on ice, the cell layers were washed three times with ice-cold Dulbecco's PBS, and then different solutions were added according to the assays to be conducted. Electron microscopic analysis was also used to determine the effect of polyamine depletion by DFMO on cellular structures of Cdx2-transfected IEC-6 cells.

In the third series of studies, we examined whether depletion of cellular polyamines by DFMO could affect content and distribution of nonmuscle myosin II in Cdx2-transfected IEC-6 cells. After cells were exposed to DFMO or DFMO plus polyamine spermidine for 4 days, the mRNA and protein levels and cellular distribution of nonmuscle myosin II were assayed in normal and migrating cells.

Measurement of cell migration. The migration assays were carried out as described in our earlier publications with a few modifications (37). Cells were plated at 6.25 x 10^4/cm^2 in DMEM-dFBS with or without DFMO and polyamines on 35-mm dishes thinly coated with Matrigel according to the manufacturer's instructions. The cells were fed on day 2, and migration was tested on day 4. To initiate migration, the cell layer was scratched with a single-edge razor blade to a ∼27 mm in length. The scratch began at the diameter of the dish and extended over an area 7–10 mm wide. After the scratch, the cell layer was immediately photographed. Care was taken to include some identifying mark on the dish to serve as a future reference point. The dishes were then returned to the incubator, and cell migration was allowed to occur over the denuded area for different time periods. At the end of the desired time, the dishes were removed and rephotographed in the same area as before, and the migrating cells were counted by means of an eyepiece reticle. The migrating cells in six contiguous 0.1-mm squares were counted at x 10 magnification beginning at the scratch line and extending as far out as the cells had migrated. All experiments were carried out in triplicate, and the results were reported as the number of migrating cells per millimeter of scratch. An inverted phase-contrast microscope with attached Polaroid camera was used for the cell counts and photographs.

ODC assays. ODC activity was determined by a radiometric assay in which the amount of 14CO2 liberated from 1-[L-14C]ornithine was estimated as previously described (36). Briefly, after cells were sonicated and centrifuged at 12,000 g at 4°C for 15 min, the ODC activity of an aliquot of the supernatant was determined during incubation in stopped vials in the presence of 7.6 nmol of [14C]ornithine (specific activity of 51.6 mCi/nmol) for 15 min at 37°C. The 14CO2 liberated by the decarboxylation of ornithine was trapped on a piece of filter paper impregnated with 20 µl of 2 N NaOH, which was suspended in a center well above the reaction mixture. The 14CO2 trapped in a filter paper was measured by liquid scintillation spectroscopy at a counting efficiency of 95%. Aliquots of the 12,000 g supernatant were assayed for total protein using the method described by Bradford (3). Enzymatic activity was expressed as picomoles of CO2 per milligram of protein per hour.

HPLC analysis of cellular polyamines. The cellular polyamine content was determined as previously described (36). Briefly, after the cells were washed three times with ice-cold Dulbecco's PBS, 0.5 M perchloric acid was added, and the cells were frozen at −80°C until ready for extraction, dansylation, and HPLC. The standard curve encompassed 0.31–10 µM. Values that fell >25% below the curve were considered not detectable. Protein was determined by the Bradford method (3). The results are expressed as nanomoles of polyamines per milligram of protein.
solution containing 10% dextran sulfate and 32P-labeled DNA. Hybridization was carried out in the same centrifuged through a 5.7 M CsCl cushion at 150,000 g RPM for 16 h. The cells were washed with Dulbecco's PBS and lysed in 4 M guanidinium isothiocyanate. This was then further processed by Chirgwin et al. (5). Briefly, the cells were washed with Dulbecco's PBS and then lysed in 4 M guanidinium isothiocyanate. The lysates were applied to a 1 ml column of Q-Sepharose Fast Flow for 0.1 M sodium cacodylate (pH 7.4). Cells were postfixed in 2.5% glutaraldehyde-3.2% paraformaldehyde buffered with 0.1 M sodium cacodylate (pH 7.4). The resulting RNA pellet was dissolved in Tris-HCl (pH 7.5), which contained 1 mM EDTA, 5% sodium laurylsarcosine, and 5% phenol (added just before use). The addition of 0.1 vol of 3 M sodium acetate and 2.5 vol of ethanol precipitated the purified RNA from the aqueous phase in the presence of 10% dextran sulfate and 32P-labeled DNA.

RNA isolation and Northern blot analysis. Total RNA was extracted with guanidinium isothiocyanate solution and purified by CsCl density gradient ultracentrifugation as described by Chirgwin et al. (5). Briefly, the cells were washed with Dulbecco's PBS and then lysed in 4 M guanidinium isothiocyanate. The lysates were brought to 2.4 M CsCl concentration and centrifuged through a 5.7 M CsCl cushion at 150,000 g at 20°C for 24 h. The resulting RNA pellet was dissolved in Tris-HCl (pH 7.5), which contained 1 mM EDTA, 5% sodium laurylsarcosine, and 5% phenol (added just before use). The addition of 0.1 vol of 3 M sodium acetate and 2.5 vol of ethanol precipitated the purified RNA from the aqueous phase in sequence. In most cases, 30 µg of total cellular RNA were denatured and fractionated electrophoretically and then transferred to nitrocellulose filters. Blots were prehybridized with 5× Denhardt’s solution-5× standard salmon sperm DNA. Hybridization was carried out in the same solution containing 10% dextran sulfate and 32P-labeled DNA probes. After the final wash, the filters were autoradiographed with intensifying screens at −70°C. The probe for myosin II 40-mer was 5′-end labeled by T4 polynucleotide kinase in the presence of [γ-32P]ATP, and the DNA probes for ODC and GAPDH were labeled with [α-32P]dCTP by using a standard nick translation procedure. All procedures were performed according to the supplier’s instructions.

Western immunoblot analysis of nonmuscle myosin II. Cell samples, dissolved in SDS sample buffer, were sonicated and centrifuged at 2,000 rpm for 15 min. The supernatant was subjected to electrophoresis on 7.5% acrylamide gels according to Laemmli (14). Briefly, the filters were incubated in 5% nonfat dry milk in 10× PBS-Tween 20. Immunologic evaluation was then performed in 1% BSA-PBS-Tween 20 buffer containing 2 µg/ml affinity-purified rabbit polyclonal antibody against nonmuscle myosin II protein. The filters were subsequently washed with 1× PBS-Tween 20 and incubated with anti-rabbit IgG antibody conjugated to peroxidase by protein cross-linking with 0.2% glutaraldehyde. After extensive washing with 1× PBS-Tween 20, the immunocomplexes on the filters were reacted for 1 min with chemiluminescence reagent (NEL-100, DuPont NEN). Finally, the filters were placed in a plastic sheet protector and exposed to autoradiography film for 30 or 60 s.

Nonmuscle myosin II staining. Cells were plated at 6.25 × 10^4/cm² in chambered slides thinly coated with Matrigel according to the manufacturer’s instructions and incubated with medium containing DMEM plus 5% dFBS, 10 µg/ml insulin, and 50 µg/ml gentamicin sulfate. DFMO at a dose of 5 mM with or without 5 µM spermidine was added as treatment. On day 4 after initial plating, approximately one-third of the cell layer was removed diagonally across the chamber slide with a razor blade. The medium was changed to remove floating or damaged cells, and the cells were returned to the incubator for 6 h, during which time they began to migrate over the denuded area. The immunofluorescence procedure was carried out according to the method of Vliekind and Swierenga (35) with minor changes. Briefly, the cells were washed with Dulbecco’s PBS and then with Dulbecco’s PBS without Ca²⁺ and Mg²⁺ and fixed for 15 min at room temperature in 4% paraformaldehyde diluted with Dulbecco’s PBS. The cells were postfixed for 5 min with ice-cold methanol. The cells were rehydrated in Dulbecco’s PBS (without Ca²⁺ and Mg²⁺) for 30 min at room temperature and then were incubated for 1.5 h with rabbit anti-myoosin II IgG (used for Western blot analysis) and then with anti-rabbit IgG-HR P-conjugate for 1 h. The primary antibody recognizes the 200-kDa nonmuscle myosin II in immunoblots of Cdx2-transfected IEC-6 cell extracts and does not cross-react with other cytoskeletal proteins. Nonspecific slides were incubated without antibody to nonmuscle myosin II. After three washes in Dulbecco’s PBS, the slides were mounted with Vectashield mounting medium (Vector Laboratories). Slides were viewed through a Zeiss confocal microscope (model LSM410).

RESULTS

Electron microscopy. After the cells were grown in the presence or absence of 5 mM DFMO for 4 days, they were washed with Dulbecco’s PBS and then fixed at room temperature in 2.5% glutaraldehyde-3.2% paraformaldehyde buffered with 0.1 M sodium cacodylate (pH 7.4). Cells were postfixed in 2% osmium tetroxide in the same buffer, dehydrated, and embedded in Epon. Ultrathin sections were examined in an electron microscope.

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[\textsuperscript{3}H]thymidine incorporation to determine the involvement of cell proliferation in this model. There were no significant changes in DNA synthesis within 8 h after wounding in both cell lines (data not shown), indicating that cell division did not participate in this process.

Treatment with IPTG did not affect the migration rates in nontransfected parental IEC-6 cells. In contrast, exposure of stable Cdx2-transfected IEC-6 cells to 4 mM IPTG for 16 days remarkably increased migration after wounding. The migration rates in Cdx2-transfected cells not treated with IPTG were 122 ± 8 at 6 h and 174 ± 12 at 8 h after wounding, respectively. The number of cells migrating over the denuded area in Cdx2-transfected cells treated with IPTG increased to 466 ± 25 at 6 h and 623 ± 36 at 8 h after wounding. In separate experiments, Cdx2-transfected cells had grown to the same degree of confluence but had not received IPTG for 4 days after they were initially treated with 4 mM IPTG for 16 days. The migration rate of Cdx2-transfected cells not treated with IPTG was indistinguishable from those treated with IPTG for 4 days before wounding. To determine the effect of initial cell density on migration after wounding, both parental and Cdx2-transfected IEC-6 cells were plated at different concentrations. As shown in Fig. 2B, there were no...
significant changes in the rates of cell migration between different initial cell densities that ranged from $4 \times 10^5$/cm$^2$ to $8 \times 10^5$/cm$^2$.

Cellular polyamines on migration in Cdx2-transfected cells. This study tested the possibility that cellular polyamines play an important role in the process of Cdx2-transfected cell migration. As can be seen in Fig. 3, ODC mRNA and the enzyme activity were significantly increased at 2 h and peaked 4–6 h after wounding. Maximum increases in ODC activity were approximately 10 times the prewounding control. Increased levels of ODC mRNA and the enzyme activity after wounding were paralleled by increases in the cellular polyamines putrescine, spermidine, and spermine (Fig. 4). Treatment with 5 mM DFMO completely prevented the increased ODC activity and the accumulation of cellular polyamines. Putrescine and spermidine levels were undetectable in the DFMO-treated cells, and spermine levels were substantially lower than those in controls.

Polyamine depletion did not affect the differentiated phenotype in stable Cdx2-transfected IEC-6 cells. There were no significant differences in the morphological characteristics of differentiation between control cells and cells exposed to DFMO for 4 days (data not shown). The level of sucrase-isomaltase mRNA in DFMO-treated cells was identical to that of control cells (without DFMO). On the other hand, "membranous lysosomal bodies" appeared in the DFMO-treated cells (Fig. 5). These membranous bodies were located throughout the cytoplasm in polyamine-deficient cells and could be regularly identified in every experiment. These ultrastructural changes were completely prevented by exogenous spermidine given together with DFMO. The ultrastructure in cells grown in the pres-

![Fig. 3. Ornithine decarboxylase (ODC) mRNA and enzyme activity in stable IEC-6-Cdx2L1 cells at different times after wounding. A: ODC mRNA levels. IEC6-Cdx2L1 cells were grown in DMEM containing 5% dFBS for 4 days, and total cellular RNA was harvested from confluent cell layers that were intact or at various times after wounding. ODC mRNA levels were determined by Northern blotting analysis with the use of a ODC cDNA probe. Hybridization to labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe served as a marker for equal loading of lanes. Three experiments were performed that showed similar results. B: ODC enzyme activity. ODC activity was determined by the methods described in MATERIALS AND METHODS. Values are means ± SE from 6 flasks. * P < 0.05 compared with nonwounded controls.](http://ajpgi.physiology.org/)

![Fig. 4. Effect of α-difluoromethylornithine (DFMO) on ODC activity and cellular polyamine (putrescine, spermidine, and spermine) concentrations in IEC-6-Cdx2L1 cells. Cells were grown in DMEM containing 5% dFBS in the presence 5 mM DFMO for 4 days, and ODC activity and polyamine levels were determined 6 h after wounding. Values are means ± SE from 6 flasks. * P < 0.05 compared with controls at same time point. + P < 0.05 compared with controls at 0 h.](http://ajpgi.physiology.org/)
ence of DFMO plus spermidine was indistinguishable from that of control cells (data not shown). Nontransfected IEC-6 cells treated with DFMO for 4 days exhibited similar ultrastructural changes (data not shown).

Polyamine depletion by treatment with DFMO significantly decreased cell migration in Cdx2-transfected IEC-6 cells (Fig. 6). The number of cells migrating in the DFMO-treated cells was reduced to ~25% of control when counted 6 h after wounding. In the presence of DFMO, exogenous putrescine and spermidine restored cell migration to nearly normal levels. Parental IEC-6 cells also require polyamines for stimulation of cell migration after wounding (Fig. 6, right). None of the polyamines was able to stimulate cell migration over normal levels when added to control cells (data not shown). There was no apparent loss of cell viability in cells either exposed to DFMO or exposed to DFMO plus spermidine (Fig. 7). These results indicate that the migration of Cdx2-transfected IEC-6 cells is polyamine dependent.

Effect of polyamine depletion on nonmuscle myosin II. To determine whether nonmuscle myosin II was involved in the process requiring polyamines for stimulation of cell migration in Cdx2-transfected cells, the formation and the distribution of nonmuscle myosin II were measured in control and in either intact DFMO-treated cells or treated cells 6 h after wounding. In control cells (without DFMO), expression of the nonmuscle myosin II gene increased significantly after wounding (Fig. 8). Nonmuscle myosin II mRNA and protein levels were ~2.5 times prewounding control when measured 6 h following removal of part of the cell layers. On the other hand, depletion of cellular polyamines by DFMO not only significantly decreased basal levels of myosin II in intact layer but also inhibited the increased nonmuscle myosin II expression after wounding (Fig. 8). Nonmuscle myosin II levels in the DFMO-treated cell were ~30% of normal values (without DFMO) in both intact and wounded cell layers. The decrease in nonmuscle myosin II levels was completely prevented by addition of exogenous spermidine (5 µM) in the presence of DFMO. In contrast to influences on nonmuscle myosin II expression, GAPDH mRNA and β-actin protein levels were not altered after wounding in control and DFMO-treated cells (Fig. 8).

To extend the positive findings of depleted nonmuscle myosin II following polyamine depletion, we further explored the cellular distribution of myosin II protein on the migrating edge 6 h after wounding. In control cells (Fig. 9A), nonmuscle myosin II was observed as fine stress fibers in migrating cells. Long stress fibers traversed the cells, and a network of cross-linked myosin fibers was present just inside the plasma membrane. In the DFMO-treated cells (Fig. 9B), the distribution of nonmuscle myosin II stress fibers was sparse and devoid of long stress fiber formation. Addition of exogenous spermidine (5 µM) to the medium...
containing DFMO resulted in maintenance of normal cellular distribution of nonmuscle myosin II (Fig. 9C). Putrescine at a dose of 10 µM was equally effective when it was added to cultures that contained DFMO (data not shown).

DISCUSSION

Intestinal epithelial restitution is a complex process, for it necessitates that differentiated columnar epithelial cells flatten, spread, migrate, and ultimately repolarize (6, 22). The mechanisms by which differentiated epithelial cells change to the flattened phenotype and become motile during restitution are poorly understood. Because of the limitation to study such issues in natural mucosae, due to their complex geometry, heterogeneity, and finite in vitro life span, cultured cell lines are commonly employed to characterize migration events during restitution in detail. Our previous studies (21, 38, 39) and others (6, 7, 19) have established an in vitro model that mimics the early stage of epithelial restitution by utilizing the IEC-6 cell line. Although this model is useful and widely accepted, there are several concerns regarding this system. First, the process of rapid restitution to reseal superficial wounds under physiological conditions is the consequence of differentiated intestinal epithelial cell migration into the defect, rather than of undifferentiated stem cell division (8, 26, 28). The IEC-6 cells, however, originate from intestinal crypt and retain the undifferentiated character of epithelial stem cells (24). Second, the rate of IEC-6 cell migration after wounding is much slower than that of mucosal restitution observed in vivo (19, 28). For these reasons, it is necessary to utilize cultured
differentiated intestinal epithelial cells for identifying the fundamental mechanisms by which intestinal epithelial cells migrate during restitution.

The homeobox gene Cdx2 is an intestine-specific transcription factor expressed at high levels in the intestinal epithelium of adult animals (4, 31). The Cdx2 protein activates transcription of intestinal genes and is responsible for directing differentiation of intestinal epithelial cells (15). Conditional expression of the Cdx2 gene in undifferentiated intestinal epithelial cells results in a significant development of the differentiated phenotype. As can be seen in Fig. 1, the stable Cdx2-transfected IEC-6 cells are associated with morphological and molecular differentiation. These results are consistent with data from others (32), who have demonstrated that forced expression of Cdx2 gene in IEC-6 cells induces multiple characteristics of differentiated intestinal epithelial cells. These include a well-formed columnar layer of cells, polarization with tight junctions, lateral membrane interdigitations, well-organized microvilli at the apical pole, and expression of sucrase-isomaltase. Because of the remarkable similarity that exists between cultured, stable Cdx2-transfected IEC-6 cells and natural intestinal epithelia in vivo, the current study characterizes migration events during restitution by utilizing this differentiated intestinal epithelial cell line.

It is interesting and of physiological significance that the migration of differentiated intestinal epithelial cells after wounding is much quicker than the migration of undifferentiated intestinal epithelial cells (Fig. 2). When measured at 6 or 8 h after removal of part of the cell layer, the rates of cell migration over the denuded areas in stable Cdx2-transfected cells are approximately four times that of parental IEC-6 cells. Because there are no significant increases in DNA synthesis within 8 h after wounding, cell proliferation does not participate in this process. Because mucosal restitution following superficial wounding is a rapid process in vivo (8, 26, 28), these findings suggest that stable Cdx2-transfected IEC-6 cells provide an excellent in vitro model for intestinal epithelial restitution.

The polyamines spermidine and spermine and their precursor putrescine are organic cations found in all eukaryotic cells (11, 23). Our previous studies and others show that cellular polyamines are absolutely required for stimulation of normal IEC-6 cell migration after wounding (20, 38). To confirm that mechanisms operative in undifferentiated intestinal epithelial cells also apply in differentiated intestinal cells, we examined the role of cellular polyamines in the process of epithelial migration in the Cdx2-transfected IEC-6 cells. As shown in Figs. 3 and 4, the process of cell migration after removal of part of the cell layer is associated with a significant increase in ODC gene expression and cellular polyamine levels. Pretreatment with DFMO not only prevents the accumulation of cellular polyamines but also decreases cell migration in differentiated intestinal epithelial cells. Exogenous putrescine or spermidine given together with DFMO completely restores cell migration to normal levels (Fig. 6). These results are similar to those observed in undifferentiated IEC-6 cells (20, 38) and clearly indicate that the differentiated intestinal epithelial cell migration during restitution is also polyamine dependent.

Because depletion of cellular polyamines fails to affect characteristics of differentiated phenotype in Cdx2-transfected cells, the decrease in the migration rate in the polyamine-deficient cells does not result from the alteration of differentiation. Electron microscopic analysis also reveals that “membranous lysosomal bodies” appear within the cytoplasm in the Cdx2-transfected cells exposed to DFMO for 4 days. Although the significance and mechanisms responsible for these membranous lysosomal bodies remain to be elucidated, they are completely prevented when spermidine is given together with DFMO, suggesting that these ultrastructural changes must be related to polyamine depletion rather than to a toxic effect of DFMO. Similar changes are also observed in parental undifferentiated IEC-6 cells treated with DFMO (data not shown).

To further investigate how polyamines modulate cell migration during restitution in differentiated intestinal epithelial cells, the formation and the distribution
of nonmuscle myosin II were examined in Cdx2-transfected IEC-6 cells treated with or without DFMO. Nonmuscle myosin II is an important cellular motor molecule within the epithelial cells of the intestinal mucosa and is implicated in the lamella and lamellipodia of migrating cells (22, 30). When the function of myosin II is inhibited by use of recombination mutants lacking myosin II and by microinjection of myosin II anti-body or antisense RNA (13, 29), cell migration is signifi-
cantly decreased in nonmuscle cells. Figures 8 and 9 clearly show that polyamines are necessary for stimulation of nonmuscle myosin II formation and also are involved in the regulation of nonmuscle myosin II distribution in migrating Cdx2-transfected IEC-6 cells. Depletion of cellular polyamines by DFMO significantly decreases the nonmuscle myosin II formation and results in the reorganization of the cytoskeletal protein.

Although the nature of the molecular mechanisms that decrease the formation of myosin II following polyamine depletion is unknown, data presented in Fig. 8 show that a decreased myosin II protein level is paralleled by a significant decrease in myosin II mRNA in Cdx2-transfected cells exposed to DFMO for 4 days. These results are consistent with other studies that indicated that polyamine depletion prevented the concanavalin A-induced accumulation of mRNAs encoding β-actin and α-tubulin in mouse splenocytes (12) and inhibited the induction of actin and microtubule formation during gastric mucosal healing in vivo (1, 2). It is not clear at present whether a decreased mRNA level for myosin II is due to a decrease in gene transcription or results from alteration of mRNA stabilization.

In summary, these results indicate that stable Cdx2-
transfected IEC-6 cells are associated with a significant development of a differentiated phenotype. These differen-
tiated intestinal epithelial cells migrate over the wounded edge much faster than undifferentiated paren-
tal IEC-6 cells during restitution. Polyamines are re-
quired for the migration of Cdx2-transfected IEC-6 cells in association with their ability to regulate non-
muscle myosin II formation and distribution. Because early mucosal restitution is the function of differen-
tiated polarized columnar epithelial cells in vivo, the stable Cdx2-transfected IEC-6 cells with a highly differen-
tiated phenotype would provide a better in vitro model system to characterize migration events during restitution.

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