Putrescine does not support the migration and growth of IEC-6 cells

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Yuan, Qing, Mary Jane Viar, Ramesh M. Ray, and Leonard R. Johnson. Putrescine does not support the migration and growth of IEC-6 cells. Am. J. Physiol. Gastrointest. Liver Physiol. 278: G49–G56, 2000.—The migration of IEC-6 cells is inhibited when the cells are depleted of polyamines by inhibiting ornithine decarboxylase with α-difluoromethylornithine (DFMO). Exogenous putrescine, spermidine, and spermine completely restore cell migration inhibited by DFMO. Because polyamines are interconverted during their synthesis and catabolism, the specific role of individual polyamines in intestinal cell migration, as well as growth, remains unclear. In this study, we used an inhibitor of S-adenosylmethionine decarboxylase, diethylglyoxal bis(guanilylhydrozone)(DEGBG), to block the synthesis of spermidine and spermine from putrescine. We found that exogenous putrescine does not restore migration and growth of IEC-6 cells treated with DFMO plus DEGBG, whereas exogenous spermine does. In addition, the normal distribution of actin filaments required for migration, which is disrupted in polyamine-deficient cells, could be achieved by adding spermine but not putrescine along with DFMO and DEGBG. These results indicate that putrescine, by itself, is not essential for migration and growth, but that it is effective because it is converted into spermidine and/or spermine.

ornithine decarboxylase; S-adenosylmethionine decarboxylase; cell migration; cell growth; IEC-6 cells; polyamines; α-difluoromethylornithine; diethylglyoxal bis(guanilylhydrozone)

THE GASTRIC AND DUODENAL MUCOSA have the ability to repair themselves rapidly after damage (6, 19), with the repair process consisting of two mechanisms. Mucosal restitution occurs in the early phase by sloughing off of the damaged epithelial cells and migration of remaining viable cells from the area adjacent to, or just beneath, the injured surface to the denuded area. Repair is completed in the later phase by replacement of lost cells through cell division (14). Polymers are absolutely required for the normal repair of gastric and duodenal stress erosions in rats. The inhibition of ornithine decarboxylase (ODC) with α-difluoromethylornithine (DFMO) almost completely prevents healing (34, 35, 38). Healing can be restored by oral administration of putrescine, cadaverine, spermidine, or spermine. Luminal polyamines have been shown to effectively substitute for endogenously synthesized polyamines in both the early and later stages of repair (33, 36).

In our laboratory, a model for migration in cultured rat intestinal epithelial crypt cells (IEC-6) has been developed that mimics early epithelial restitution of gastrointestinal epithelium in vivo. Using this model, we have shown that cells that are almost completely depleted of polyamines after treatment with DFMO lose their ability to migrate (14).

Actin filaments play a critical role in cell locomotion. They are required for both the advancement of the leading edge and the retraction of the trailing edge of the cell during migration (11, 17). In migrating cells, long actin filaments transverse the cells as stress fibers (13, 26). Polyamine deficiency results in the reorganization of F-actin in IEC-6 cells. The most striking features of polyamine-depleted cells are a pronounced actin cortex and great reduction in the number of stress fibers (16).

Exogenous putrescine, spermidine, and spermine completely restore cell migration inhibited by DFMO (15, 37). Because polyamines are interconverted during their synthesis and catabolism (8), the restoration of cell migration in the presence of one specific exogenous polyamine may be due to that particular polyamine or to its conversion to other polyamines. The role of specific polyamines in cell migration, as well as in other processes requiring polyamines, remains unclear. In this study, we blocked the synthesis of spermidine and spermine from putrescine by an inhibitor of S-adenosylmethionine decarboxylase (SAM-e-DC) to investigate the effect of specific polyamines on the migration of IEC-6 cells, as well as cell growth (Fig. 1). We found that putrescine alone was not sufficient for the migration and growth of polyamine-depleted cells, whereas the addition of exogenous spermine restored migration and growth. In addition, we found that the normal distribution of actin filaments required for migration could be achieved by adding spermine, but not putrescine, to polyamine-deficient cells.

MATERIALS AND METHODS

Chemicals and supplies. Biochemicals were purchased from Sigma (St. Louis, MO). L-[1-14C]ornithine and S-[carboxyl-14C]adenosyl-L-methionine were obtained from DuPont NEN (Boston, MA). Matrigel was purchased from Collaborative Research (Lexington/Waltham, MA), Texas Red phalloidin was obtained from Molecular Probes (Eugene, OR). Media, balanced salt solutions, and insulin were purchased from Gibco (Grand Island, NY). Disposable culture ware was purchased from Corning Glass Works (Corning, NY). DL-α-difluoromethylornithine was a gift of the Merrell Dow

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Research Institute (Cincinnati, OH). Diethylglyoxal bis(guanylhydrazone) (DEGBG) was synthesized and kindly provided by Dr. Patrick J. Rodrigues and Dr. Mervin Israel (University of Tennessee Department of Pharmacology). Briefly, aminoguanidine bicarbonate (0.14 M) was dissolved (University of Tennessee Department of Pharmacology). Polyamines and Cell Migration and Growth

Fig. 1. Pathway for polyamine synthesis showing points of inhibition by α-difluoromethylornithine (DFMO) and diethylglyoxal bis(guanylhydrazone) (DEGBG).

### SAMe-DC Activity

Cells were harvested from stock flasks using trypsin/EDTA and plated (day 0) at a density of 1.3 × 10⁶ cells/60 mm dish in DMEM plus 5% dialyzed FBS plus 10 µg/ml insulin, 50 µg/ml gentamicin, and DEGBG. They were maintained in T-150 flasks in a humidified 37°C incubator in an atmosphere of 90% air-10% CO₂. The stock medium was DMEM with 5% heat-inactivated fetal bovine serum (FBS), 10 µg/ml insulin, and 50 µg/ml gentamicin. Depletion of putrescine was assessed by HPLC, as previously described (33). Briefly, after washing the cell monolayers with Dulbecco’s PBS, 0.5 ml of 0.5 M perchloric acid was added to the dish and then the monolayers were frozen at −80°C until all samples were ready for dansylation, extraction, and HPLC. The standard curve encompassed the range from 0.31 to 10 µM. Polyamine content was expressed as nanomoles per milligram of protein.

### Cell Staining

The cells were plated on coverslips and then grown and treated as described for the SAMe-DC activity assay. After the cell monolayers were scratched and allowed to migrate for 8 h, they were fixed in 3.7% formaldehyde for 15 min and then permeabilized with 0.1% Triton X-100 for 15 min. After cells were incubated with Texas Red phalloidin for 45 min at room temperature, they were washed, and the coverslips were mounted on microscope slides. Images were imaged with a Laser Sharp Bio-Rad MRC-1024 laser scanning confocal imaging system (Bio-Rad Laboratories).

### Doubling Time

After growing for 4 days under experimental conditions, cells were taken off by trypsin and subcultured in T-25 flasks with different media. Cell number was counted at 24 and 48 h by Coulter counter, and the doubling time was calculated.

Statistics. All data were expressed as means ± SE. Statistical analysis was performed using ANOVA and appropriate post-hoc testing. Values of P < 0.05 were regarded as significant.

### RESULTS

SAMe-DC activity. SAMe-DC activity was assayed after cells were treated with DEGBG for 4 days. Fig. 2 shows that DEGBG was an effective inhibitor of SAMe-DC, with a 1 mM concentration of DEGBG reducing serum-stimulated SAMe-DC activity to 15% of control.

### Intracellular Polyamines

DFMO (5 mM) completely depleted the cells of putrescine after 4 days and reduced spermidine to 10% of the control level and spermine to 60%. When putrescine was added concomitantly with DFMO in the medium, the intracellular putrescine level was 20-fold higher than control. The addition of putrescine increased spermidine twofold, with 20 µl of 2 N NaOH, which was suspended in a center well above the reaction mixture. The reaction was stopped by the addition of 10% TCA. The trapped 14CO₂ was measured by liquid scintillation spectrometry with a counting efficiency of 95%. Enzyme activity was expressed as picomoles of CO₂ liberated per hour per milligram of protein.
and spermine increased to ~80% of the control. These results show that the presence of putrescine in the medium with DFMO not only increased the intracellular putrescine level but also elevated the spermidine level and maintained the spermine level near that of the control. When spermine was added with DFMO, putrescine, spermidine, and spermine were 145%, 143%, and 85% of control levels, respectively (Fig. 3), showing that exogenous spermine maintained all three polyamines near the control levels.

After the cells grew in medium containing both DEGBG (1 mM) and DFMO (5 mM) for 4 days, intracellular putrescine was undetectable, and spermidine and spermine were reduced to 8.6% and 50% of control, respectively. When putrescine was added to the medium, intracellular putrescine increased 30-fold, but spermidine increased only to 60% and spermine decreased to 30% of control (Fig. 4). Therefore, exogenous putrescine increased intracellular putrescine and did not bring spermidine and spermine to control levels. Under the same conditions, exogenous spermine increased intracellular putrescine to 200% of control and maintained spermidine and spermine at control levels.

When cells were treated with DEGBG for 4 days, intracellular putrescine increased dramatically to ~115 times control levels. Spermidine decreased to 45% of control, and spermine was reduced even further to 20% of control. Exogenous putrescine increased intracellular putrescine further to 138 times control but did not significantly affect spermidine and spermine levels. In cells treated with DEGBG and spermine, intracellular spermidine and spermine increased to control levels, and putrescine was increased to 10 times the control level but remained far below the level with DEGBG alone (Fig. 5).

Cell migration. The number of migrating cells was counted 8 h after the removal of part of the cell monolayer. Administration of DEGBG and DFMO for 4 days significantly decreased cell migration in IEC-6 cells to 40% of control. The presence of exogenous putrescine did not significantly restore cell migration. However, in the presence of exogenous spermine, cell migration was completely restored (Fig. 6).

Migration of cells treated with DEGBG alone was significantly reduced to 44.5% of control. Migration was only 47.8% of control in cells exposed to medium with DEGBG and exogenous putrescine, indicating that putrescine made no significant difference in the migration of these cells. In contrast, the presence of exogenous spermine restored cell migration to control levels (Fig. 7).

Actin staining. The distribution of F-actin was examined in cells on the migrating edge of the monolayer 8 h after initiating migration. In control cells, F-actin was seen in three main areas within the cell (Fig. 8A). Long
stress fibers traversed the cells, a network of actin fibers or actin cortex was located just underneath the cell membrane, and very fine short actin fibers outside the actin cortex extended into lamellipodia. Cells treated with DEGBG or DEGBG plus DFMO appeared to be more spread out. The most obvious feature of these cells was a pronounced actin cortex around the entire cell (Fig. 8B). There were some long stress fibers and short fibers extending into lamellipodia in these cells, but the number of these was greatly reduced compared with control. Some fine punctate staining could also be seen. The appearance of cells treated with DEGBG, DFMO, and putrescine was almost identical to control (Fig. 8C). When spermine was present in the medium, cells treated with DEGBG or DEGBG plus DFMO appeared similar to control cells (Fig. 8D).

Doubling time. The doubling time of cells treated with DEGBG plus DFMO was significantly prolonged. Although the doubling time of cells treated with DEGBG, DFMO, and putrescine was somewhat less, there was no significant difference between these two treatments. The doubling time of cells treated with DEGBG, DFMO, and spermine was almost identical to control (Fig. 9).

**DISCUSSION**

IEC-6 cells treated with DFMO are polyamine depleted, cell migration is inhibited, and exogenous putrescine, spermidine, or spermine completely restores migration (15, 37). In those studies, the effects of specific polyamines on cell migration were not characterized. This study was an attempt to identify the roles of specific polyamines and their relative contributions to cell migration and growth.
Normal levels (Fig. 3). This result suggests that, although the presence of each exogenous polyamine prevents the inhibition of cell migration resulting from polyamine depletion, the conclusion cannot be made that each individual polyamine is able to restore cell migration.

DEGBG is a potent inhibitor of SAMe-DC and has been shown in L1210 cells specifically to inhibit SAMe-DC activity and not to effect the activity of ODC or SSAT (31). Our results show that it effectively blocked SAMe-DC activity in IEC-6 cells as well (Fig. 2). Cells treated with DEGBG and DFMO took up exogenous putrescine but could not synthesize spermidine and spermine from it (Fig. 4). Migration was not restored in cells with high intracellular putrescine but low spermidine and spermine concentrations (Fig. 6). This was true even when the putrescine concentration reached an extremely high level in cells treated with DEGBG alone (Fig. 7). The number of migrating cells treated with DEGBG plus DFMO was not significantly different from that of cells treated with DEGBG plus putrescine. The accumulation of putrescine in cells treated with DEGBG is contributed to by two factors. First, inhibition of SAMe-DC prevents the conversion of putrescine to spermidine and spermine. Second, putrescine was synthesized at a higher rate than normal because we found that treatment of IEC-6 cells with DEGBG increased ODC activity (data not shown).

Complete restoration of cell migration in the presence of exogenous spermine indicates that inhibition of cell migration is not due to the direct effect of DEGBG or DFMO but to the alteration of intracellular polyamines. These results suggest that putrescine itself cannot restore cell migration in IEC-6 cells depleted of polyamines. Putrescine, regardless of its concentration, cannot substitute for spermidine and spermine to support cell migration. Exogenous spermidine (5 µM) increased migration of the cells treated with DEGBG, but it did not completely restore migration as did spermine (data not shown).

The organization of F-actin in migrating cells has been characterized by the formation of long stress fibers transversing the cells (13, 26, 27). We have found that stress fibers are less dense, whereas the actin cortex is greatly increased in density, in polyamine-deficient cells (16). In the current study, putrescine did not restore the normal distribution of F-actin when intracellular spermidine and spermine concentrations were low (Fig. 8).

In the polyamine biosynthetic pathway, the decarboxylation of S-adenosylmethionine yields decarboxylated S-adenosylmethionine (dcAdoMet), which provides an aminopropyl group to putrescine and spermidine for synthesis of spermidine and spermine, respectively (8). In cells depleted of polyamines by DFMO, dcAdoMet is accumulated due to the lack of putrescine and spermidine to accept the aminopropyl group from dcAdoMet. Additional dcAdoMet is produced in those cells because SAMe-DC activity is inversely related to the spermidine and spermine levels (30). Thus DFMO not only causes the depletion of polyamines but also concomitantly causes the accumulation of dcAdoMet (7). On the

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**Fig. 6.** Cell migration. Cells were treated with 1 mM DEGBG, DFMO without or with 10 µM putrescine (PUT) or 5 µM spermine (SPM) for 4 days and counted at 8 h after wounding. Values are means ± SE of 6 observations. *P < 0.05 compared with control.

**Fig. 7.** Cell migration. Cells were treated with 1 mM DEGBG, DEGBG plus 10 µM putrescine (PUT), or DEGBG plus 5 µM spermine (SPM) for 4 days and counted at 8 h after wounding. Values are means ± SE of 6 observations. *P < 0.05 compared with control.
other hand, exogenous polyamines normalize dcAdoMet levels at same time that they restore polyamine levels. First, putrescine and spermidine accept the aminopropyl group to eliminate excessive dcAdoMet. Second, polyamines repress SAMe-DC activity and reduce production of dcAdoMet. Thus the inhibition of cell migration or growth caused by DFMO may be due to either decreased polyamine levels or increased dcAdoMet levels. One study has shown that dcAdoMet inhibits DNA methylation and induces differentiation of teratocarcinoma stem cells (7). However, in cells treated with DFMO and the SAMe-DC inhibitor, there is no accumulation of dcAdoMet, whereas cellular polyamines are depleted (7). Therefore, it is polyamine deficiency, not dcAdoMet accumulation, that accounts for the inhibition of cell migration and cell growth observed in our experiments.

Cell motility depends on dynamic remodeling of the actin cytoskeleton. Integrin receptors play important roles in organizing the actin-containing cytoskeleton and in signal transduction from the extracellular matrix (18). Initially, protrusion of membrane lamellipodia is associated with actin polymerization. The leading edge of the cell subsequently attaches to the substratum through actin-anchored adhesion complexes. Finally, generation of contractile forces is believed to involve a contractile arrangement of actin and myosin (12). Rho, Rac, and Cdc42 GTPases have been shown to regulate the formation of stress fiber and multimolecular focal adhesion complexes (21). In the IEC-6 cell line, the activation of Rho A is essential for cell migration (27). Cell migration is therefore a complex process and requires the involvement of many cellular components and enzymes.

The mechanisms by which polyamines affect cell migration have been investigated in our previous studies. We have found that polyamine deficiency causes reorganization of F-actin and tropomyosin in IEC-6 cells (16) and decreases in the expression of integrin...
subunit α2 and attachment of IEC-6 cells to the extracellular matrix (28). Transforming growth factor-β (TGF-β) stimulates cell migration (5), and polyamines are necessary for the normal expression of the TGF-β gene during migration of IEC-6 cells (39). Gene expression of TGF-β is dramatically stimulated after wounding control cells. Depletion of intracellular polyamines significantly inhibits increased expression of the TGF-β gene in response to wounding and cell migration (5, 39). Another study indicates that polyamines are involved in the regulation of nonmuscle myosin II formation and distribution in IEC-6 cells (37). Parkkinen et al. (23) have found a redistribution of rough ER and the appearance of autophagic vesicles and swollen Golgi apparatus in cells depleted of polyamines. Their observation suggests that polyamines affect the synthesis, processing, and secretion of proteins. Polyamines may have effects on gene transcription, translation, and posttranslational modification of proteins involved in cell migration (28). The proper organization of those proteins, such as in focal adhesion complexes, may also be affected since polyamines may influence the activity of protein kinases involved in this process.

Polyamines are highly charged multivalent cations, and their effectiveness is dependent on the number of positive charges (29). GTPase activity of purified GTP-binding protein (G_α/G_β) from calf brain reconstituted into phospholipid vesicles is stimulated by polyamines. The order of potency is spermine greater than spermidine greater than putrescine (4). Polyamines at physiological concentrations enhance the binding of several proteins (e.g., USF, TFE3, and NF-IL6) to DNA. Putrescine is ineffective, whereas spermine is more potent than spermidine (22). Spermine causes the specific release of chromatin-associated proteins from nuclei of rice (Oryza sativa) seedlings. Spermidine is far less effective and putrescine is essentially ineffective in releasing these proteins (3). The in vitro mechanism by which polyamines affect the protein kinase C activation process has been studied in a reconstituted system consisting of purified enzyme and phospholipid vesicles of varying phosphatidylserine content. Spermine greatly interferes with the association of protein kinase C to liposomes. Spermidine and putrescine are almost ineffective (20). A null mutant in the SPE2 gene encoding SAMe-DC of Saccharomyces cerevisiae has no detectable SAMe-DC, spermidine, or spermine and absolutely requires spermidine or spermine for growth. This requirement cannot be satisfied by putrescine (1). SAMe-DC inhibition causes a marked decrease in spermidine and spermine and a dramatic increase in putrescine. It inhibits growth factor-induced DNA synthesis in primary cultured rat hepatocytes (9) and growth of MCF-7 breast cancer cells (32) and the L1210 cell line (25). Exogenously added spermidine and spermine reverses this inhibition (9, 25, 32). Our results also indicate that putrescine itself cannot sustain normal growth of cells that have low intracellular spermidine and spermine levels (Fig. 9). All of this evidence suggests that putrescine is functionally different from spermidine and spermine.

Our results demonstrate that one exogenous polyamine is converted to other polyamines after being absorbed by IEC-6 cells. After polyamine depletion by DFMO, all polyamines are effective in returning normal function. However, putrescine does not show this effectiveness if its conversion to other polyamines is blocked. Thus, in IEC-6 cells, putrescine itself is incapable of functioning to restore cell migration and growth. These findings suggest that a comparable situation may exist in many other cells and some of the models described in the previous paragraph.

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