Healing of Gastrointestinal Mucosa: Involvement of Polyamines

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Polyamines are involved in the processes of cell migration and proliferation that result in the repair of mucosal lesions. Depletion of polyamines dramatically alters the arrangement of the cytoskeleton, EGF receptor function, the activities of signal transduction proteins, the levels of several protooncogenes, and the expression and cellular content of at least one growth factor involved in these processes.

The mucosa of the gastrointestinal tract has one of the shortest turnover times of any tissue in the body. The lining of the small intestine, for example, replaces itself approximately every 3 days. Stem cells located in the crypts of the intestine or the necks of the gastric gland produce daughter cells that divide several times and then migrate toward the surface. In the case of the small intestine, the newly produced cells move onto a villus and migrate toward the tip, differentiating functionally as they progress. Old cells, sloughed from the tip into the lumen, are replaced by those from below. The normal mucosa maintains a healthy balance between factors that lead to cell loss and those that support or stimulate cell replacement.

The repair of damaged mucosa occurs through two mechanisms (11). The first, termed mucosal restitution, is rapid and consists of the migration of remaining viable cells from areas adjacent to the lesion to cover the denuded area. This process requires an intact lamina propria as a matrix for the cells to migrate over. The second mechanism is the actual replacement of lost cells by the process of cell division. It depends on DNA synthesis, which begins ~12 h after the start of healing. Polyamines are required for both processes (12, 13).

Polyamine biochemistry and physiology

Eukaryotic cells contain and synthesize the polyamines spermidine and spermine and their precursor, putrescine (8). Polyamines alter many cellular functions, but their exact roles at the molecular level remain to be elucidated. We know, however, that their cellular concentrations are highly regulated and that they are essential for normal cell growth and differentiation (8, 10). The first rate-limiting step in polyamine synthesis is the production of putrescine from the amino acid ornithine (Fig. 1). This reaction is catalyzed by ornithine decarboxylase (ODC, EC 4.1.1.17), which has one of the shortest half-lives of any mammalian enzyme and is present at extremely low levels in quiescent cells. Its activity is increased dramatically within a few hours by tissue injury and exposure to a variety of trophic substances including growth factors, hormones, and serum in the case of cultured cells. ODC can be inhibited by \( \alpha \)-difluoromethylornithine (DFMO), which binds covalently and irreversibly to the activated enzyme. DFMO depletes polyamines in vivo and in cultured cells, resulting in the attenuation of growth. That the effects are caused by polyamine depletion is shown by the fact that they are prevented by the addition of exogenous polyamines in the presence of DFMO (8, 13). ODC activity is easily measured by trapping and counting \(^{14}\text{CO}_2\) released from ornithine labeled at the 1-C position. The diamine putrescine is converted to spermidine by the addition of a propylamine group, and spermidine is converted to the tetraamine spermine by the addition of a second propylamine (Fig. 1). The propylamine groups are donated by decarboxylated \( S \)-adenosylmethionine produced from \( S \)-adenosylmethionine by \( S \)-adenosyl-L-methionine decarboxylase (SAMDC, EC 4.1.1.50), the second rate-limiting enzyme involved in polyamine synthesis (8).

Putrescine, spermidine, and spermine contain 2, 3, and 4 amine groups, respectively. With pK values near 10, these amine groups are protonated at physiological pH and bind to negatively
charged molecules such as proteins and nucleic acids. This binding is believed to explain many of the polyamine-related effects at the cellular level. Although similar in some regards to inorganic cations such as Ca\(^{2+}\), the positive charges in polyamines are separated by a fixed-length, flexible carbon chain and are able to span critical distances. Polyamines, for example, can link proteins by serving as substrates for transglutaminase reactions. Polyamines bind to DNA and affect the transcription of a variety of genes including protooncogenes and those that regulate the cell cycle. They have also been shown to alter protein synthesis and the activity of enzymes. Polyamines bind to cell membranes and stabilize them. In so doing they are able to modulate channel activity and influence receptor binding (10).

The cells lining the lumen of the gastrointestinal tract contain ODC and synthesize polyamines, but because their apical membranes are actually "outside" the body they are also exposed to polyamines from a variety of other sources. Bacteria synthesize polyamines in large quantities and release them into the lumen. The normal diet also contains relatively large amounts of these compounds. Sloughed epithelial cells contain polyamines that are released as the cells are digested. Enterocytes contain transporters for polyamines, and polyamine uptake is stimulated by trophic agents (5). Thus the epithelial cells lining the gastrointestinal tract have a ready supply of polyamines in addition to those they synthesize.

**Polyamines and mucosal repair**

We have investigated the relationships between polyamines and the repair of both gastric and duodenal erosions using the rat stress ulcer model. In that model a 6-h period of stress significantly increased ODC activity in both tissues, and enzyme activity remained substantially elevated over that in corresponding controls for 12 h after the period of stress. Peak enzyme activity occurred 4 h after stress. Stress also significantly increased mucosal putrescine, spermidine, and spermine levels, which remained elevated for at least 12 h of the recovery period (12, 13). Intraperitoneal injection of DFMO at the start of the 6-h period of stress and at 8-h intervals thereafter did not affect the degree of lesion formation measured immediately after stress but almost totally prevented healing during the 24-h period of recovery (Fig. 2). In the same animals DFMO also prevented the increases in ODC and polyamine levels normally seen after damage. DFMO inhibited the ongoing repair as soon as 4 h after stress as well as 12 and 24 h later. Orogastric administration of polyamines immediately after the period of stress increased the normal rate of healing and prevented the inhibition of repair caused by DFMO (Fig. 2).

Results were similar for both gastric and duodenal mucosa, and we drew a number of conclusions that applied to both tissues (12, 13). First, increased levels of polyamines provided by ODC are essential to mucosal healing. Second, exogenous, luminal polyamines effectively substitute for endogenously synthesized ones during the repair process. Third, polyamines are essential to both the early phase of mucosal restitution, dependent on cell migration, and the late phase, dependent on cell division.

**Early mucosal restitution: cell migration**

To study the involvement of polyamines in mucosal restitution, we developed an in vitro
model of healing that depended on cell migration (6). A confluent monolayer of IEC-6 cells (a normal intestinal epithelial cell line derived from fetal rat crypt cells) was wounded by scraping with a razor blade, and cell migration was assessed by counting the number of cells that crossed a fixed length of the wound edge in a given period of time. Migration in this model was independent of DNA synthesis, dependent on actin polymerization, and dependent to some extent on extracellular matrix. Significantly, polyamine depletion with DFMO inhibited migration 80%, and exogenous polyamines prevented the decreased migration.

A major question remaining is, why are polyamines essential to migration? Cell migration requires an intact and functioning cytoskeleton. The primary cytoskeletal protein is actin, and its distribution were altered dramatically by polyamine depletion (7). The distribution of other cytoskeletal proteins and their relationship to actin were also significantly different after treatment with DFMO. In normal IEC-6 cells, actin was found in stress fibers traversing the cells.
Nonmuscle myosin II was also found in fibers in close association with actin (Fig. 3). Tropomyosin, a rod-shaped protein that lies along the groove in F-actin and stabilizes it, colocalized with actin (7). In cells depleted of polyamines actin stress fibers were almost absent, and F-actin made up a heavy cortex around the cell. In the same cells, some myosin was found in the cortex but much occurred as separate punctate foci in the cytoplasm (Fig. 3). Supplying exogenous polyamines to DFMO-treated cells resulted in a normal-appearing cytoskeleton. In rat gastric mucosa damaged with hypertonic NaCl there was a significant increase in the formation of F-actin, which was almost totally prevented by polyamine depletion (1). Thus the data obtained regarding the cytoskeleton in cultured cells reflect events in the whole animal. Polyamines are also important for the attachment of IEC-6 cells to the extracellular matrix. Depletion of polyamines led to a specific decrease in the expression of the α2-subunit of integrin (α1 was not affected) that correlated with a decrease in attachment.

In the intestine, several growth factors stimulate the migration of epithelial cells. A number of these, including transforming growth factor (TGF-β), are synthesized by and released from wounded IEC-6 cells (2). The Ras-like small GTPase Rho is essential to a signal transduction pathway that links growth factor receptor activation to the remodeling of the actin cytoskeleton and formation of focal adhesions required for migration. Inhibition of Rho function by a variety of means, including microinjection of C3 toxin from Clostridium botulinum and recombinant dominant negative Rho T19N, inhibited both spontaneous and epidermal growth factor (EGF)-stimulated migration of IEC-6 cells. This was accompanied by disruption of the actin cytoskeleton, so that it closely resembled that seen after polyamine depletion (9). Recent evidence indicates that Rho protein may be decreased significantly in polyamine-depleted IEC-6 cells (Patel et al., unpublished results). TGF-β promotes the healing of damaged mucosa by stimulating cell migration and increasing the production of extracellular matrix. Exogenous TGF-β stimulated migration of IEC-6 cells, and wounding enhanced the expression of TGF-β mRNA. Migration was inhibited by immunoneutralization with anti-TGF-β antibody, indicating that this growth factor may be essential for intestinal epithelial cell migration (2). When TGF-β was added to polyamine-depleted IEC-6 cells at the time of wounding, it completely restored their ability to migrate (15). In addition, the expression of TGF-β mRNA and the amount of TGF-β present in IEC-6 cells were significantly reduced by polyamine depletion. EGF, which also stimulates migration of many cell types including IEC-6 cells, did not restore the ability of polyamine-depleted cells to migrate (4). These data indicate that polyamines are required for epithelial cell migration in association with their ability to maintain levels of TGF-β. Furthermore, because TGF-β-treated cells incubated with DFMO are polyamine depleted, these cells were able to migrate in the absence of polyamines. This suggests that, even though polyamines are able to form actin filaments from monomeric actin in vitro, their effects on the cytoskeleton and migration may be independent of any direct interaction with cytoskeletal proteins.

Cell replacement

Because polyamines are required for the growth of all eukaryotic cells, it is not surprising that the cell replacement stage of mucosal wound healing
is polyamine dependent. As shown in Fig. 2d, there was almost no replacement of gastric mucosal cells 24 h after stress in rats treated with DFMO. Polyamine depletion blocked the increases in protein, RNA and DNA synthesis, and content that normally follow damage (12). Polyamine depletion halted progression through the cell cycle, and DFMO arrested IEC-6 cells in the G1 phase. One of the earliest events triggered in the stress ulcer model is a significant transient increase in the expression of the c-fos protooncogene 2 h into the period of stress (14). At the same time, the level of c-fos protein increased significantly. Stress also significantly increased the expression of the c-myc protooncogene from 4 h after the beginning of the 6-h stress period through the first 4 h of the recovery period. The c-myc protein was elevated significantly during the same time interval. These events were preceded by increases in ODC activity and putrescine levels. Blocking ODC with DFMO totally prevented the increased expression of protooncogenes (14). The expression of c-fos, c-myc, and c-jun, but not junβ, protooncogenes stimulated by serum in IEC-6 cells was also inhibited by polyamine depletion.

The cellular protooncogenes are responsible for the regulation of the cell cycle and are involved in healing as well as normal growth and development. c-myc is the paradigm for two large classes of transcription factors containing either the basic/leucine repeat structure or the basic/helix-loop-helix. The c-myc protein is involved in sequence-specific DNA binding, and c-fos is involved early in the initiation of proliferation. The fos protein functions as a transcription factor in a variety of cell types. c-fos and c-myc, as regulators of transcription, are likely to activate or repress other genes during the healing process. Thus polyamines may be essential to the healing process in part because they are necessary for the expression of these protooncogenes (14).

Investigators have made use of epithelial cell culture models to examine the growth response to wounding in much the same way as they have used them to study migration. As we have discussed, TGF-β stimulates the migration of intestinal epithelial cells and inhibits proliferation. TGF-α, on the other hand, activates the EGF receptor and is a potent mitogen. Like TGF-β, it is synthesized by gut epithelial cells and released during damage. Wounding IEC-6 cells activated two of the mitogen-activated protein kinase (MAPK) pathways (3). The extracellular signal-regulated kinases (ERKs) were phosphorylated within a few minutes of wounding as was c-Jun-NH2-terminal protein kinase (JNK). Activation of ERK1 and ERK2 is believed to be the major pathway mediating the proliferative response to growth factors such as EGF, TGF-α, and insulin. The JNK pathway, also called stress-activated protein kinase (SAPK), is activated by heat shock, ultraviolet (UV) radiation, rapid changes in osmotic pressure, TNF-α, and, to some extent, mitogens. Conditioned medium from wounded IEC-6 cells contained significantly increased amounts of TGF-α compared with medium from confluent IEC-6 cells, and anti-TGF-α antibodies substantially reduced the activation of ERK1 and ERK2 (3). Recent experiments indicate that EGF receptor function is polyamine dependent (4). DFMO prevented the stimulation of both growth and migration by EGF normally seen in IEC-6 cells. In polyamine-deficient cells the intracellular distribution of the EGF receptor and its processing were altered. The prolonged time spent by the receptor near the nucleus, which is characteristic of the EGF-EGF receptor complex and is required for its biological function, did not occur (4). Normally, the EGF receptor follows an endocytic pathway to lysosomes and ultimate degradation.
In control cells the EGF receptor separated itself from the transferrin receptor within 10 min of administration of EGF. The transferrin receptor is constitutively endocytosed and recycled, and many investigators have used it to delineate the endocytic pathway of internalized membrane receptors. In polyamine depleted cells the two receptors remained colocalized, suggesting a failure of normal processing. As shown in Fig. 4, the function of the EGF receptor was also altered in the absence of polyamines. Both receptor phosphorylation and kinase activity were decreased ~50%, whereas there was no change in the amount of receptor protein. Addition of exogenous putrescine to the medium of cultures treated with DFMO prevented these changes (4).

Whether the changes in protooncogenes, signal transduction, and ultimately proliferation are the result of decreased function of the EGF receptor in polyamine-depleted cells is not known. Because polyamines can bind DNA, RNA, and proteins producing conformational changes in them, they theoretically can alter cell function directly at many levels. Although exogenous TGF-β stimulated cell migration in DFMO-treated cultures (15), EGF was not able to stimulate growth of polyamine-depleted cells (4). Thus the defect is not a deficiency in the supply of agonist for the EGF receptor.

Conclusions

Two processes account for the repair of gastrointestinal mucosal lesions. The first, termed early mucosal restitution, is rapid and involves cell migration. The second, cell replacement, occurs later and depends on cell proliferation. Polyamines, multivalent organic cations, are essential to both processes, and their absence alters the amounts or activities of a host of intracellular proteins and enzymes involved in migration and growth. One of the most dramatic effects of polyamine depletion is the disappearance of actin stress fibers from the cytoplasm and localization of F-actin to a heavy cellular cortex. A variety of other stress fibers from the cytoplasm and localization of polyamine depletion is the disappearance of actin, F-actin to a heavy cellular cortex. A variety of other stress fibers from the cytoplasm and localization of polyamine depletion is the disappearance of actin, F-actin to a heavy cellular cortex. 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