Review article

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POLYAMINES AND CELL MIGRATION

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Leeuwenhoek first described polyamines in 1677, but active investigation did not begin until the 1970's. When intracellular polyamine levels are reduced by inhibitors, mutation, or transfection, severe reductions occur in cell division, cell differentiation, and cell migration. These effects are not difficult to demonstrate and measure, and all can be prevented if supplemental exogenous polyamines are supplied. However, linking the overall effects to molecular events remains to be accomplished. In this review, we discuss work (mostly from the last 10 years) that relates to cell migration. Specifically, we have discussed the biology and biochemistry of the polyamines, their transport and regulation, the structure of the cytoskeleton and the mechanics of cell movement. We have also considered four specific processes that polyamines participate in that may affect cell migration significantly. These are: 1) the regulation of intracellular Ca\(^{2+}\) concentration by voltage-gated K\(^{+}\) channels, 2) the maintenance of normal RhoA levels that, with Rac, regulate the assembly of actin stress fibers, focal adhesions, and contractility, 3) the formation of ATP-Mg\(^{2+}\)-polyamine trimers that enhance the phosphorylation activity of ATP toward enzymes in specific signaling pathways and, 4) alterations in the structure of RNA that change translation initiation sites and affect the expression of proteins.

Keywords: putrescine, spermidine, spermine, cytoskeleton, actin polymerization, ornithine decarboxylase, di-\(\alpha\)-difluoromethylornithine, stress fibers, RhoA, microtubules, voltage-gated K\(^{+}\) channels, FAK.

POLYAMINES AND CELL MIGRATION

Introduction

Cell migration is an essential process in embryonic development, wound healing, growth, and metastasis. Migration in bacteria, protozoa, yeast, and eukaryotes involves many processes and molecules in common. In animal cells,
the cytoskeleton provides structure, strength, and direction for migration and is both a sender and a receiver of signals from inside and outside the cell. Cell migration is modulated by a variety of factors including regulatory peptides, metabolic substrates, cytokines, integrins, the extracellular matrix, and polyamines. Although polyamines are necessary for cell migration as well as for growth, differentiation, and other cell functions, we know little of the molecular basis of their actions.

Biology and biochemistry of the polyamines

The polyamines spermidine, spermine, and their diamine precursor putrescine are found in all tissues of almost all living species. Spermine was first described by Leeuwenhoek in 1677 (1), but little progress was made toward understanding the extent of polyamine involvement in biological processes until the 1970s. The reported cellular functions of polyamines now include cell growth (2—6), cell attachment (7, 8), intestinal mucosal maturation (9), cell migration (6, 10, 11), and cell cycle regulation (12). Other reports describe the influence of polyamines on chromatin structure, membrane stability (13, 14), transcription (15—17), the intracellular distribution of F- and G-actin and thymosin β4 (Tβ4) (18), ion channels, and cell signaling (19, 20). Tβ4 is a major regulator of actin polymerization (21). Polyamine excess causes apoptosis (22, 23), while polyamine deficiency can delay it (23, 24). These activities are essential for normal embryonic development, differentiation, cellular maintenance, and wound healing. On the negative side, polyamines also stimulate proliferation and metastasis in cancer cells and are, therefore, a target of therapeutic efforts (5, 25, 26). A comprehensive overview of the polyamine literature from their discovery in the 8—9th centuries to 1998 can be found in Cohen (1).

Putrescine, spermidine, and spermine are organic, aliphatic, cationic amines. The amine groups have pKaS around 10, so that at physiological pH they bind to proteins, DNA, RNA, and other negatively charged molecules. This binding is the source of their biological effects. They are produced in a cyclic reaction sequence starting from ornithine or methionine that results in the formation and interconversion of putrescine, spermidine, and spermine. (Fig. 1). The key regulatory enzymes are ornithine decarboxylase (ODC), which decarboxylates ornithine to synthesize putrescine, and S-adenosylmethionine decarboxylase (AdoMetDC), which decarboxylates S-adenosyl methionine to provide the propylamine groups for spermidine and spermine. In addition to synthesis from ornithine, putrescine can originate from spermidine and spermine by the removal of their propylamine groups. Spermidine synthase, spermine synthase, and polyamine oxidase, an enzyme that oxidatively splits the monoacetyl derivatives of spermidine and spermine, are usually present in
excess and are not rate limiting under physiological conditions. In non-proliferating cells, most of the putrescine comes from spermidine rather than from ODC. ODC activity is low in non-proliferating cells and primarily provides putrescine moieties lost by cells to secretion and degradation. In rapidly dividing cells, such as embryonal cells, gut mucosal cells, and tumor cells, putrescine is synthesized almost entirely by ODC (27).

Fig. 1. The biosynthesis of the major physiologic polyamines and their precursor putrescine. Putrescine has 4 CH groups, spermidine 7, and spermine 10. They carry 2, 3, and 4 positive charges that reflect the strength of their binding ability. The enzymes involved in the reactions are numbered.

Antizyme is an endogenous inhibitor of ODC induced by the excess production of polyamines (28). Antizyme binds to and inactivates ODC and, at the same time, inhibits polyamine uptake (28—30) and stimulates polyamine excretion and degradation by the 26S proteosome (31, 32). Three antizymes of varying abundance and tissue specificity have been described (28, 33, 34).

A variety of drugs that inhibit enzymes at certain stages of polyamine biosynthesis has been synthesized in the search for effective anti-cancer treatments. DFMO (dl-α-difluoromethylornithine) is one of the most useful of
these drugs because it irreversibly inhibits ODC, blocking the production of putrescine from ornithine and inhibiting or reducing any cell functions that require polyamines. If a polyamine is exogenously supplied with DFMO, these functions are maintained at normal levels. Diethylglyoxal bis(guanylhydrazone) (DEGBG), an inhibitor of AdoMetDC, blocks the production of spermine and spermidine from S-adenosyl-L-methionine (35). When IEC-6 cells (a non-tumorigenic intestinal crypt cell from normal rats) are treated with both DFMO and DEGBG, cell growth and cell migration are inhibited, but in this case exogenous spermine, not putrescine, is needed to maintain cell growth and cell migration. The authors concluded that putrescine required further conversion to higher polyamines before it could restore cell growth and migration (36). Other reported actions of polyamine synthesis inhibitors include prevention of mitogen-induced accumulation of mRNAs for β-actin and α-tubulin (37), DNA and protein synthesis reduction, and induction of a persistent defect in pH homeostasis due to the lowering of the pH set point of the Na⁺/H⁺ exchanger (38). These effects may also have significant impact on cell migration and growth.

In order to define some of the structural requirements of polyamines, we tested analogs of spermidine, spermine, and putrescine for their ability to support migration in DFMO treated IEC-6 cells. All analogs of spermidine with the general formula \( x - 3 \) (referring to the numbers of carbon atoms on either side of the central nitrogen), where \( x \) varied from 2 to 12, competed for entry into the cells. However, in addition to spermidine (\( x = 4 \)), only compounds for which \( x = 2, 3, \) or 6 supported migration. This suggests that separation of the charged N atoms by more than 6 C atoms produces a compound that cannot effectively interact with the cytoskeleton. Spermine analogs, \( 3 - x - 3 \), for which \( x = 3, 6, 9, \) or 12, competed for entry into the cells, but only compounds for which \( x = 3 \) or 6 supported migration, and none was as effective as spermine (\( x = 4 \)) (39). In another study using analogs of putrescine (a diamine with 4 carbons), all diamines with 4—10 carbon atoms competed with putrescine for its specific carrier, but only putrescine could support migration (40).

Polyamine transport in mammalian cells is specific, saturable, energy-dependent, carrier-mediated, and may require RNA and protein synthesis (5). Transport activity is increased under growth-promoting conditions and decreased when exogenous polyamines are added. Many cells have a common transporter for polyamines as well as separate transporters for spermidine and spermine (41—43). IEC-6 cells and colon cancer LoVo cells take up significantly increased amounts of putrescine within 1 h after stimulation with serum or asparagine while, at the same time, inhibiting polyamine release (44, 45). Using cell culture inserts, we found that the amount of polyamine taken up or released depended on whether it was added to the apical or basal side of the
cell. In IEC-6 cells, putrescine uptake was greater from the apical than from the basal side. When intracellular polyamine concentration was reduced by inhibiting ODC, putrescine uptake did not increase, but its release was inhibited (45). LoVo cells, on the other hand, took up putrescine more readily from the basal than from the apical side (44). Putrescine uptake by both cell lines was Na+-independent (44, 45).

The electrochemical gradient across the plasma membrane (E_m) is a function of Na^+, K^+, and Cl^- concentration gradients and relative membrane permeability. Voltage-gated K^+ channels (K_v) are a major factor determining E_m in a variety of cell types, especially non-excitible cells (46, 47). Intracellular Ca^{++} is maintained by Ca^{++} influx through Ca^{++} channels in the plasma membrane and by Ca^{++} release from intracellular stores (48, 49). Ca^{++} influx depends on the E_m or driving force of Ca^{++} across the plasma membrane. When K^+ channels are stimulated or increased in number, E_m becomes hyperpolarized and Ca^{++} influx increases. The cytoplasmic free Ca^{++} concentration rises, leading to cell contraction and cell migration. Inhibition or loss of voltage-gated K_v channels causes hypopolarization and decreases Ca^{++} influx. Loss of Ca^{++}, whether inside or outside the cell, severely inhibits cell migration (50, 51). IEC-6 cells do not express voltage-gated Ca^{++} channels. Instead, the activity of voltage-gated K^+ channels (K_v) controls the membrane potential that regulates cytoplasmic free Ca^{++} concentration. The stimulation of cell migration by polyamines in IEC-6 cells involves the expression of K^+ channels. Wang and co-workers reported that the depletion of cellular polyamines by DFMO resulted in decreased Kvl.1 channel expression, membrane depolarization, decreased cytosolic Ca^{++} concentration, and inhibition of cell migration. Exogenous spermidine reversed the inhibitory effects of DFMO on K_v channel gene expression, membrane depolarization, and intracellular Ca^{++} concentration and also restored cell migration to control levels. Decreasing intracellular Ca^{++} concentration by removing extracellular Ca^{++} or by depolarizing the cell membrane also inhibited cell migration. They proposed that polyamine-dependent intestinal cell migration after wounding may be due, at least partially, to enhanced K_v channel (Kvl.1) expression, membrane hyperpolarization, and increased cytoplasmic Ca^{++} concentration (19).

The structure of the cytoskeleton and the mechanics of cell movement

β-Actin is the major structural protein of the cytoskeleton in most cells. It is present in cytoplasm in monomeric form (G-actin) and in filamentous form (F-actin) at a concentration of ~1000 μM (52). Polymerization of G-actin monomers into F-actin requires an intricate series of G-actin sequestration, monomer nucleation, filament severing, and uncapping of filament ends (53).
F-actin, as bundled into stress fibers, provides strength and rigidity to the cytoskeleton as well as attachment sites for the focal adhesion proteins and integrin receptors that link the extracellular matrix to the actin cytoskeleton. The interaction of cells with extracellular matrix proteins or growth factors controls cell migration by activating mitogen-activated protein kinases ERK1 and ERK2 and molecular coupling of the adapter proteins p130cas (CAS) and c-Crk2. During cell migration, ERK and CAS/Crk operate as distinct signaling pathways that facilitate actin-myosin motor assembly and actin membrane ruffles, respectively (54).

A serendipitous insight into the hardware of cellular movement came from an early study of the “rocket-like” motion of Listeria monocytogenes (55). These bacteria express surface proteins that recruit actin nucleation factors from the host cell. The nucleation factors accumulate around the bacterium, initially in a symmetrical cloud. As the actin filaments attach and release at a significant off-rate, the symmetry is broken and the bacterium is propelled through the cell’s cytoplasm at the front of a “rocket” on a burst of polarized actin (56, 57). In this way, the bacterium proceeds through cell after cell without encountering immune attack. Similar rocketing movements have been reported for viruses (58), endosomes (59), vesicles, and various unidentified structures (60). The reconstruction of bacterial rocketing in vitro has shown that lamellipodial and filopodial cell movement in animal cells mimics bacterial rocketing to a surprising degree. Using purified proteins of Listeria and Shigella in cell free extracts, Loisel found that actin, activated Arp 2/3 (actin related protein 2/3) complex, ADF/cofilin, and capping protein, but not myosin, were sufficient for motility. Free energy released by ATP hydrolysis linked to actin polymerization drove the actin-based propulsion and did not require myosin (61).

In general, epithelial cells form wide, wave-like lamellipodia, while fibroblastic cells construct long slender filopodia. Both are protrusions of cellular membrane that are instrumental in cell migration but differ internally. Lamellipodia have dense short dendritic actin filaments branching from Arp2/3 nucleation sites on their outer borders, while filopodia form long parallel stress fibers that extend the length of the protrusion. In both cell types, actin polymerization is the engine of cellular movement. As in bacterial rocketing, a relatively small number of proteins and phospholipids are sufficient for cell migration (62). The protein N-WASP, a homolog of WASP (Wiskott-Aldrich syndrome protein), regulates actin polymerization by activating Arp2/3 (63, 64). Arp 2/3 is a stable complex of 7 subunits conserved across eukaryotic phyla (65). N-WASP and the Arp2/3 complex together connect signal transduction pathways directly to the stimulation of actin polymerization (66). GTP-Cdc42, a Rho family member, recruits N-WASP, to the cell membrane (67) and to the side of an actin filament (65, 68) where it activates the Arp 2/3 complex. Whether N-WASP/WASP recruits Arp2/3 to the lamellipodia or whether an
unknown protein is interposed is a question which has not been answered (69). The activated Arp 2/3 complex nucleates actin monomers for polymerization into new actin filaments (70). The new filaments branch from the side of the original actin filament at a 70° angle, forming a dense dendritic array of actin filaments on the leading edges of the lamellipodia (71). A constant supply of actin monomers is needed for rapid elongation of actin for lamellipodial protrusion. Older filaments are depolymerized by ADF/cofilins, and profilin catalyzes the exchange of ADP for ATP, recycling the pieces back to the pool of actin monomers. The unpolymerized monomers are bound to profilin and thymosin β4 (Tβ4) and released for elongation onto new barbed ends (72). As the filaments polymerize from their barbed ends in opposition to the capping proteins (73), the membrane is pushed outward (74, 75). N-WASP is tightly regulated by its constitutively active output domain (VCA) and the two regulatory binding domains, Cdc42 and phosphatidylinositol-4,5-bisphosphate (PIP₂). Until there is a signal for migration, the two regulatory domains hold the VCA-Arp2/3 complex in an inactive conformation (63). Cooperative activation by both Cdc42 and PIP₂ is required to begin strong actin polymerization (63, 76).

The arrangement of actin filaments in filopodia varies from that in lamellipodia. The Arp2/3 complex is not localized to filopodia, nucleation occurs only once, and the actin filaments are bundled in parallel and do not form a branching network. Nevertheless, actin polymerization is the source of force for extension. Gelsoolin severs the older actin filaments and the barbed ends are capped, producing an excess of free pointed ends. ADF/cofilin dissociates the severed segments into monomers that augment the supply for association with the remaining barbed ends. In addition, the intrinsic low rate of filament treadmilling is accelerated by the synergistic action of cofilin and profilin. The filaments are bundled by fascin and α-actinin for strength and coordination. As growth occurs at the barbed ends, the bundled filaments are thought to push against the membrane by an elastic Brownian ratchet mechanism (75, 77).

In eukaryotic cells Cdc42, Rac, and Rho, members of the Ras superfamily of small GTPases, cooperate to induce the formation of lamellipodia or filopodia. Rac1 stimulates the formation of membrane ruffles and lamellipodia (78, 79). Cdc42 stimulates the formation of filopodia. RhoA regulates the assembly of actin stress fibers, focal adhesions, and contractility (80). Cdc42, Rac1, and RhoA also regulate a signal transduction pathway linking growth factor receptors to the formation of stress fibers and focal adhesions (78). Focal adhesions develop around actin stress fibers where integrins contact the extracellular matrix under the cell body (80). When cells are treated with Clostridium botulinum exoenzyme C3, RhoA is ribosylated, actin stress fibers are lost, and cell detachment and inhibition of cell migration results (81, 82).
Recent studies have shown that integrin-mediated adhesion to the extracellular matrix regulates the activity of the Rho family in turn (83—86). The integrin family of transmembrane adhesion receptors links components of the extracellular matrix to the actin cytoskeleton and mediates tension in adherent cells. The strength of the adhesions and the organization of the cytoskeleton are determined by the degree of tension exerted. As the cells migrate, focal adhesions alternately attach to the extracellular matrix at the front of the cell and detach at the rear as the cell contracts to move forward (87, 88). RhoA is activated by engaging with integrins depending on the length of the engagement, the type of integrin, and the degree of integrin clustering (89).

Cox has shown that α5β1 integrin-mediated signaling to RhoA, Cdc42, and Rac1 on fibronectin regulates a stop signal for cell migration in CHO (Chinese hamster ovary) cells (90). Cell migration stops when the ratio of activity between these three small GTPases is no longer optimal for protrusion and polarization of the cell. This occurs depending on the density of the substrate. Cdc42 and Rac1 are required for fibronectin stimulated cell protrusion and polarization. Their activation is biphasic in that it parallels optimum cell polarization and protrusion for migration. RhoA activity, on the other hand, increases to a plateau with increasing substrate density. As RhoA activity rises, it down-regulates Cdc42 and Rac1 activity, inhibiting cell polarization and protrusion, and ultimately stopping cell migration. In addition, Rac1 can directly inhibit Cdc42 activity, causing stable rather than transient protrusion (90). Cell migration can be seen as a response to the activation and deactivation of these proteins, the balance of activity between them, and the nature and density of the extracellular matrix. The original stimulus to migrate—morphogenesis, development, spreading, cytokines, metastasis, etc—is overlaid on the separate functions of the small GPTases.

Investigators have tried various substrates in attempts to measure the degree and direction of force exerted by cells during migration (91—95). Recent technological improvements in the elastic substrate method have made it possible to produce spatially resolved measurements of the variable traction and directions of force exerted by single motile cells. Studies of migrating 3T3 fibroblasts grown on elastic collagen-coated polyacrylamide sheets containing fluorescent beads show that most of the force for movement is radially distributed from the cell perimeter toward a region near the cell nucleus. The direction of force is reversed in the anterior region of the cell (96). The propulsive force for fibroblast locomotion (~0.2 dyn), is imparted to the substratum within 15 μm of the leading edge. These observations show that the lamellipodium of the fibroblast can exert intense traction force while the cell body and posterior seem to be mechanically passive
and are pulled forward entirely by anterior force (97). Strong mechanical force in the anterior region is believed to play a role in cytoskeletal signaling as well as in locomotion. Inhibitors of actin and myosin II show that actin-myosin II interactions generate these forces. Treatment of the cells with cytochalasin D disrupts actin filaments, causes complete relaxation of force, and stops protrusion of the leading edge. Myosin II inhibitors cause 80—90% relaxation of force but do not stop forward protrusion which continues under the pressure of actin polymerization (96).

The role of microtubules in cell migration seems unclear as different results have been reported for different cell types. Microtubules in fish fibroblasts regulate the dynamics of focal adhesions, apparently by targeting them directly to deliver relaxing signals to promote turnover and initiate either protrusion or retraction. The relaxing signals themselves are unknown but may include regulators of the Rho family (98, 99). In melanoma cells and melanoblasts, microtubules are required for forward movement of the cell body, retraction of the tail, and modulation of cell adhesion, although lamellipodia and ruffling can be formed without microtubules (100). In 3T3 (mouse) fibroblasts, the depolymerization of microtubules with nocodazole has no effect on cell movement or protrusion of a leading edge (96). In IEC-6 cells, we found that treatment with C3 to inhibit stress fiber formation also severely shortens the length of microtubules as well as migration (McCormack, unpublished data).

Other mechanisms of lamellipodial protrusion such as hydrostatic pressure (101) or recycling of the plasma membrane (102) have been reported for other cells. Madin-Darby canine kidney epithelial cell monolayers close wounds by forming lamellipodia that depend on Rac and phosphoinositide rather than Rho or Cdc42 (103). T84 cells (a colon cancer cell line) first retract, then extend lamellipodia drawn by purse strings of actin filaments to close wounds (104), and wound closure is similar in embryonic tissue (105).

**Polyamine roles in cell migration**

We became interested in the involvement of polyamines in cell migration when we discovered that wound healing in the gastrointestinal tract of rats was seriously inhibited by polyamine deficiency resulting from treatment with DFMO. In rat gastric and intestinal mucosa, damage caused by swimming stress (106, 107), corticosterone administration (108), or hypertonic saline (109, 110) could be repaired only slowly, if at all, under conditions of polyamine deficiency. (Fig. 2) Needing a more accessible system, we turned to a normal rat intestinal crypt cell line, IEC-6, and a colon cancer cell line, Caco-2, for further experiments involving cell migration in culture. For that purpose, we devised a quantitative cell migration assay in which
confluent monolayers were treated with DFMO for 4 days to lower polyamines, wounded by a razor scratch, then reincubated for 3—6 hours to allow migration into the denuded area (11). (Fig. 3). In order

Fig. 2. The effect of DFMO on repair of the oxyntic gland mucosa after hypertonic saline damage. Rats were treated intragastrically with NaCl (3.4 molar) (A), NaCl + DFMO (B), or NaCl + DFMO + spermidine and killed at successive hours later. The oxyntic gland mucosa was removed, fixed, and stained for F-actin with rhodamine phalloidin. The sections shown are from rats killed at 4 h. Recovery from damage was rapid in (A) and (C) but had not begun in (B). From: Banan et al, Am J Physiol 1996, 271: G893.

to avoid the complication of cell proliferation that begins ~12 hours after wounding, we chose to study only the early restitution period of wound repair (111). Migration was reduced in DFMO-treated groups by ~80% and ~50% in IEC-6 cells and Caco-2 cells, respectively. The cells showed sparse, disorganized stress fibers throughout the cytoplasm, a heavy, relatively smooth actin cortex without lamellipodia, and a flattened contour (6, 45). This microscopic appearance has been observed previously in polyauxotrophic CHO cells (112), and we came to recognize it as characteristic of polyamine depleted cells. Significantly, supplementation with putrescine or spermidine prevented the effects of DFMO. (Fig. 4). Hoping to explain the severe effects of polyamine deficiency on cell migration, we began to investigate a number of cellular actions related to migration in DFMO treated cells. We explored the effects of polyamine deficiency on the EGF receptor, RhoA, myosin II, tropomyosin, β-tubulin, focal adhesion kinase, cell attachment, and cell spreading. In addition, we determined the structural attributes that allowed polyamine analogs to support migration in polyamine deficient cells.
Fig. 3. The ability of putrescine, spermidine, and spermine to promote migration in polyamine depleted cells. Using the cell migration assay described in reference 11, we compared the polyamines with respect to maintaining migration in DFMO treated cells. The same area immediately after the monolayer was scratched and 6 hours later is shown. Treatments were: a and b, controls; c and d, DFMO; e and f, DFMO + putrescine; g and h, DFMO + spermidine; i and j, DFMO + spermine. From: McCormack et al, Am J Physiol 1993, 264: G367.
Fig. 4. The migrating edge of IEC-6 cells stained with Texas Red phalloidin. The cells are shown at 3 h of migration. The abundance of F-actin stress fibers is obvious in the control and DFMO/putrescine groups, as are cell polarization and lamellipodia. The DFMO group shows the scarcity of interior stress fibers along with the heavy cell cortex typical of polyamine depleted cells.
EGF, as well as other growth factors, is able to induce changes in cell shape and stimulate migration. Growth factor binding to receptors causes receptor dimerization followed by phosphorylation of specific tyrosine residues in the cytoplasmic domains of the receptors. The phosphorylated residues then become attachment sites for Src homology 2 (SH2) domain-containing signaling proteins. These signaling complexes coordinate programs required for shape changes during growth, attachment, and migration. The Crk-associated substrate p130Cas (Cas) and the Cas-Crk complex are mediators of focal adhesion kinase (FAK)-mediated cell migration (113). We have shown that EGF binding increases EGF receptor tyrosine phosphorylation and kinase activity in IEC-6 cells in 2 minutes, and cytoplasmic and nuclear staining of Tß4 in IEC-6 cells in 10 minutes. By 30 minutes, the increase has disappeared. None of these changes occurs in DFMO treated cells (114). Other consequences of DFMO in EGF stimulated cells are the divergence of the recycling pathway of the EGF receptor from its normal route, heterogeneity in the distribution of cytoplasmic G-actin, diminished stress fibers, and thickening of the cell cortex. All are changes detrimental to cell migration (18).

Since we knew that RhoA is essential for migration in IEC-6 cells (8), we were interested in testing the effect of polyamine deficiency on RhoA in our cells. We found that the level of RhoA protein and the migrating ability of the cells decreased in parallel over the 4 days of DFMO treatment. The decrease in both was entirely preventable by concomitant exogenous putrescine. Neither the mRNA nor the half-life of RhoA was altered by DFMO, but the synthesis of RhoA was significantly reduced. We thought that perhaps activated RhoA could restore migration in polyamine deficient cells, so we transfected IEC-6 cells with constitutively active RhoA. DFMO almost entirely prevented migration in the transfected cells although RhoA activation was not affected. In mock-transfected cells, DFMO inhibited cell migration and blocked RhoA activation as well. These results show, for the first time, that polyamines are necessary for an event downstream from RhoA, as well as for the functioning of RhoA (Ray, unpublished).

Specific myosins transport Golgi-derived vesicles, organelles, pigment granules, etc. along actin filaments in a specific and coordinated process. Myosin II may be a vesicle motor (115). In normal IEC-6 cells, myosin II was prominently localized with the stress fibers and cortex of control and DFMO treated cells supplemented with putrescine. In DFMO treated cells, however, myosin II had dissociated from F-actin and scattered throughout the cytoplasm in many small foci (116). Cdx2-cells (a transfected differentiating IEC-6 cell) also required polyamines to maintain a normal distribution of myosin II (117). Tropomyosin, on the other hand, continued to remain associated with F-actin in polyamine deficient cells although the distribution of
F-actin itself presented the usual picture of few stress fibers and a heavy cortex (118).

Polyamines are essential for the healing of gastric and duodenal mucosal damage. Early stages of mucosal healing are due primarily to the migration of adjacent cells into areas of denuded lamina propria and not to cell proliferation. Using rats damaged by intragastric 3.4 molar salt solution, we observed microtubules by immunofluorescence during the healing process. In control rats, microtubule density increased around and below the damaged mucosa at 2 and 4 hours after damage and had returned to normal by 10 hours. In DFMO treated rats, microtubules were markedly reduced at all time points, and, as in all other experiments involving polyamine deficiency, administering a supplemental polyamine with DFMO maintained the control condition (110). The importance of microtubules in cell migration may vary in different cell types. In BHK (hamster kidney) cells, for instance, polyamine deficiency had no effect on the pattern of microtubules (119). At retracting edges, microtubules make multiple contacts at focal adhesions, preceding contact release and cell edge retraction. The microtubules may control the cell’s polarity by releasing localized doses of relaxing signals at focal adhesions to retard or reverse their development (99). The interrelationship between microtubules, actin filaments, and the motors that move organelles and vesicles on them is not yet established. In general, the current concept is that long-range movement of vesicles occurs on microtubules and short-range movement on actin filaments (115).

FAK (focal adhesion kinase) is a cytoplasmic protein tyrosine kinase needed for attaching the cytoskeleton to the extracellular matrix. FAK controls important biological processes including cell spreading, migration, apoptosis, and cell cycle kinetics through integrin transmembrane receptors (120–123). Integrin attachment to the extracellular matrix induces tyrosine phosphorylation and enzymatic activation of FAK (124, 125). The carboxy terminus of FAK contains binding sites for paxillin and talin, actin binding proteins of focal adhesions (126, 127). This region of FAK, the focal adhesion targeting sequence (FAT), determines the location of focal adhesion sites (128). Paxillin binding has been considered necessary for focal adhesion targeting of FAK, but a recent study of embryonic chick cell FAK mutants has shown that paxillin binding is not required for FAK targeting in those cells (129). In a study of focal adhesion signaling in polyamine depleted IEC-6 cells, we found that cell attachment was delayed, cell spreading was reduced, focal adhesion sites were few, and stress fibers were sparse compared to control cells. Also, the phosphorylation of Tyr^925, the paxillin binding site in the focal adhesion targeting domain of FAK, and the subsequent phosphorylation of paxillin were decreased and delayed in DFMO treated cells. Immunocytochemistry showed that the strong colocalization of F-actin and FAK on the outer edges of cells
during early attachment was weak in DFMO treated cells and, as the polyamine depleted cells began to spread, the disparity in FAK and F-actin between them and control cells increased (130). These results strongly suggest that the severe reduction of cell migration in DFMO treated cells is traceable, at least in part, to inadequate focal adhesion sites caused by polyamine deficiency.

What processes do polyamines affect during cell migration?

Four processes in which polyamines could have decisive effects are as follows: 1) regulation of intracellular $\text{Ca}^{++}$ concentration through voltage-gated $\text{K}^+$ channels, 2) modulation of phosphorylation and dephosphorylation activity, 3) inhibition of cell migration by adhesion-dependent signaling and, 4) control of RNA translation.

1) Polyamines influence the plasma membrane potential by regulating voltage-gated $\text{K}^+$ channels. Voltage-gated $\text{K}^+$ channels are a major factor in determining the membrane potential of non-excitatable cells. In polyamine deficiency, these channels are inhibited or lost. This causes hypopolarization of the cell membrane and reduces the influx of $\text{Ca}^{++}$, a stimulus for cell contraction and migration (19, 131, 132). This may account for some part of the decrease in migration that occurs in DFMO treated cells.

2) Adhesion-dependent signaling may regulate cell migration through the Rho family of GTPases. On fibronectin, the signal is generated through $\alpha_5\beta_1$ integrin to Cdc42 and Rac1. Their activation parallels optimum cell polarization and protrusion for migration. RhoA activity, on the other hand, continues to increase with increasing substrate density until, at some point, it inhibits Cdc42 and Rac1 and down-regulates cell polarization and membrane protrusion (90). The balance between Cdc42 and Rac1 on one hand and RhoA on the other provides a way to regulate cell migration in relation to the substrate. Polyamine levels, finely adjusted to current cellular requirements by ODC, antizymes, growth factors, and transport, add a way to regulate responsiveness to current needs for migration. We have found that polyamine deficiency causes a loss of RhoA protein but no loss in Rac1 protein. Since RhoA regulates the assembly of actin stress fibers, focal adhesions, and contractility, cell migration was also severely reduced (Ray, unpublished).

3) Polyamines may influence cell migration by binding to ATP. ATP is an integral part of signal transduction cascades that involve protein phosphorylation. These signal cascades direct cytoskeletal remodeling as needed for cell migration or mitosis. Cations, including $\text{Mg}^{++}$, enforce conformational changes in ATP that are important for enzyme recognition. ATP, $\text{Mg}^{++}$, and polyamines (3, 133), which are, of course, divalent and polyvalent cations, are present in animal cells at comparable concentrations.
Gunther and coworkers have demonstrated that polyamines interact with ATP and with ATP-Mg\(^{++}\). Using NMR analysis, they have shown that polyamines, especially spermine, bind to ATP-Mg\(^{++}\) to form a ternary complex that causes a conformational shift in ATP. The shift strongly enhances the phosphorylation activity of ATP toward enzymes in signaling pathways for growth and differentiation, apoptosis inhibition, and transcriptional and translational control, i.e. protein kinase A, protein kinase C, and casein kinase 2 (134).

4) Because most intracellular polyamines exist in a polyamine-RNA complex in the cell, they can influence translation by altering the structure of RNA (135). Polyamines strongly stimulate the synthesis of oligopeptide-binding protein (OppA) at the level of translational initiation (136). Measurement of the sensitivity of three OppA RNA species to RNAses showed that specific binding sites for polyamines in mRNA, tRNA, and rRNA had changed position after binding polyamines. The changes mean that the polyamines had either stabilized or loosened the RNA structures depending on the position of the polyamine binding sites (135, 136). Such shifts in RNA structure could determine what protein or peptide instructions are translated and, therefore, which will be expressed. Our investigation of RhoA has lead us to suspect that the loss of RhoA protein in polyamine deficient cells is due to a downstream event that depends on polyamines, perhaps a failure of translation (Ray, unpublished).

In conclusion, polyamines are needed for cell migration because:

Polyamines modulate cell membrane potential by affecting the voltage-gated K\(^+\) channels that determine the driving strength of Ca\(^{++}\), a stimulus for cell contraction and migration.

Polyamines are involved in the regulation of Rho family GPTases that determine lamellipodial protrusion and cell polarization during migration. Polyamines form ATP-Mg\(^{++}\)-polyamine trimers that stimulate many-fold the phosphorylation of certain kinases in important signaling cascades.

Polyamines bind strongly to RNA, altering its structure sufficiently to change translation initiation sites and perhaps affect the expression of proteins important for cell migration.

The biological responses to polyamine withdrawal described in many reports over the past 30 years still need to be related to fundamental mechanisms such as the above before we fully understand the processes involved in normal cell migration. Then we can apply that knowledge to medical problems in which cell migration has gone awry, for example, misdirected embryonic development, wounds that refuse to heal, tumor metastasis, infection by microorganisms, and inappropriate immune response.
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