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Research Article

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Adenine Nucleotides Stimulate Migration in Wounded Cultures of Kidney Epithelial Cells

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Abstract

Adenine nucleotides speed structural and functional recovery when administered after experimental renal injury in the rat and stimulate proliferation of kidney epithelial cells. As cell migration is a component of renal regeneration after acute tubular necrosis, we have used an in vitro model of wound healing to study this process. High density, quiescent monkey kidney epithelial cultures were wounded by mechanically scraping away defined regions of the monolayer to simulate the effect of cell loss after tubular necrosis and the number of cells that migrated into the denuded area was counted. Migration was independent of cell proliferation. Provision of adenosine, adenine nucleotides, or cyclic AMP increased the number of migrating cells and accelerated repair of the wound. Other purine and pyrimidine nucleotides were not effective. Arginine-glycine-aspartic acid-serine peptide, which blocks the binding of extracellular fibronectin to its cell surface receptor, completely inhibited migration in the presence or absence of ADP. Very low concentrations of epidermal growth factor ($K_{0.5} \sim 0.3$ ng/ml) stimulated migration, whereas transforming growth factor-$\beta 2$ was inhibitory ($K_i \sim 0.2$ ng/ml). Thus, adenosine and/or adenine nucleotides released from injured or dying renal cells, or administered exogenously, may stimulate surviving cells in the wounded nephron to migrate along the basement membrane, thereby rapidly restoring tubular structure and function. (*J. Clin. Invest.* 1992. 90:288–292.) Key words: acute renal failure • transforming growth factor-β • epidermal growth factor • heparin • extracellular matrix

Introduction

It has been known for many years that the injured nephron can regenerate. After acute tubular necrosis, surviving cells at the edges of the wound and uninjured embryonic rest cells proliferate and migrate to reepithelialize the nephron (1–4). The factors that control this process of epithelial wound healing are poorly understood. We have used cultures of high density, quiescent, nontransformed monkey kidney epithelial cells of the BSC-1 line to simulate the renal tubular epithelium (5), identify compounds that regulate cell growth, and define their mechanisms of action (6). This experimental strategy led us to suggest that the salutary effect of infused adenine nucleotides in rats after acute toxic, ischemic, or obstructive renal injury (7) could result from the capacity of these agents to stimulate DNA synthesis (8–10). Infusion of ATP with $MgCl_2$ was recently reported to initiate DNA synthesis in the rat kidney after ischemic injury (11). In monkey renal cells in culture, provision of ADP stimulated expression of early growth response genes (Egr-1, NAK-1, c-*fos*, and c-*myc*), protooncogenes, and other genes associated with progression of the cell cycle (9, 12–14). ADP also signaled rapid release of a platelet-derived growth factor (PDGF)[1] B chain homodimer-like protein, which if released in vivo could act in a paracrine manner, as renal epithelial cells do not respond to this peptide as a mitogen (15).

To study the repair process in the injured nephron, we have turned our attention from the proliferative response during regeneration to cell migration by using mechanically wounded high-density, quiescent monolayers of BSC-1 cells as a model system. A portion of the monolayer was physically removed, and migration of cells into the denuded area was measured. Holley and co-workers previously used this model of in vitro healing to study the dynamics of DNA synthesis at the edge of the wound (16). Similar models have been used to study wound repair in cultures of corneal endothelial cells (17, 18), vascular endothelial cells (19, 20), and fibroblasts (21). We have observed that when a wound is made in high-density renal epithelial cultures, cells migrate into the denuded area and restore the integrity of the monolayer, as is observed in regenerating renal tubules after an injury. Migration was markedly stimulated by low concentrations of exogenous adenine nucleotides that did not initiate cell proliferation.

Methods

Nontransformed, subcloned African green monkey kidney epithelial cells (BSC-1 line), Madin-Darby canine kidney (MDCK) cells, and BALB/3T3 fibroblasts were grown in DMEM as described previously (8, 9, 22). Cells were seeded in 60-mm dishes and grown to high density in DMEM containing 1% calf serum and 1.6 μM biotin. This medium was then replaced with DMEM containing 0.01% serum and 16 μM biotin, and the cultures were used 1 d later. Wounds were made by gently removing narrow strips of cells from the monolayer with a sterile 200-μl disposable plastic pipette tip so that the extracellular matrix (ECM) apparently remained intact. 10 edge-to-edge wounds were made per 60-mm culture dish; the strips of displaced cells were removed with a Pasteur pipette. Specific reagents were added immediately after making the wound, and the extent of cell migration into the denuded area was assayed at designated times thereafter. To assay cell

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1. *Abbreviations used in this paper:* ECM, extracellular matrix; EGF, epidermal growth factor; FGF, fibroblast growth factor; FSBA, fluorosulfonylbenzoyladenosine; IGF, insulin-like growth factor; MDCK, Madin-Darby canine kidney; PDGF, platelet-derived growth factor; RGDS, arginine-glycine-aspartic acid-serine; TGF, transforming growth factor.

migration, a rectangular wound area was demarcated at a site chosen at random in the culture, and the total number of cells in it were counted in a light microscope at a magnification of 100. The size of wounds used for study was calculated as follows: the base (b) of the wound was defined by aligning it with the maximal diameter of the microscope field (1.70 mm), and its height (h) was measured at 0.57±0.01 (SE) mm in 10 wounds, so that the area of the rectangle (b × h) under study was 0.97 mm^2, i.e., about 1 mm^2. Each value for migration was the mean number of cells in at least 10 wounds having the size defined above; 5 in each of two different cultures. SE was less than 4%, a value too small to be displayed on the figures.

The following growth factors were purchased from Collaborative Research Inc., Bedford, MA: PDGF, insulin-like growth factor (IGF)-I, IGF-II, acidic fibroblast growth factor (FGF), and basic FGF. Epidermal growth factor (EGF) was purchased from Amgen Biologicals, Thousand Oaks, CA, and transforming growth factor (TGF)-type β2 from R&D Systems, Inc., Minneapolis, MN. Adenosine, adenine nucleotides, and cyclic 3',5'-AMP; guanine, thymine and uridine nucleotides, ribose phosphate, and 5'-*p*-fluorosulfonylbenzoyladenosine were obtained from Sigma Chemical Co., St. Louis, MO.

The data were compared by Student's unpaired *t* test.

Results

When a wound was made in a high density, quiescent culture, cells at the perimeter migrated towards the center so that by day 2 the wounded area was completely filled in. Migrating cells displayed a spindle shape and were flatter than those in the monolayer that were distant from the wound. These differences in cell morphology permitted us to define the edges of the wound and count the number of cells migrating into the denuded area. Preliminary experiments showed that by day 1 about 30 cells migrated into a wound having an area of 1 mm^2, and by day 2 about 110 cells. We previously noted that addition of ADP to cultures of BSC-1 cells stimulated DNA synthesis (half-maximal concentration, ~ 20 μM) (8) and rapidly induced changes in cell shape and in the distribution of the intermediate filament cytokeratin-8 (23). Since the change in cell shape suggested an effect of ADP on adhesion of cells to their substratum that could influence cell movement, the effect of ADP on migration of BSC-1 cells into the wound was examined. When ADP was added to high-density, quiescent cultures to achieve a final concentration of 200 μM, which is optimal for stimulation of DNA synthesis (8), the number of cells in a defined wound increased by more than threefold at 24 h compared to control, and in most instances the wound was completely filled in at that time (data not shown). Exogenous ADP stimulated cell migration in a concentration-dependent manner with a half-maximal concentration of 2 μM (Fig. 1 *A*). At a concentration of 1 μM, the increased number of cells in the wound at 12 and 24 h was not the result of mitogenesis because this low concentration of ADP did not stimulate [^{3}H]-thymidine incorporation into DNA (8). Moreover, cells labeled with bromodeoxyuridine and stained with a fluorescent antibody to the nucleotide using the method of Martin et al. (24) did not demonstrate cells synthesizing DNA at the edges of the wound. Analysis of the time course of cell migration revealed that stimulation by ADP was evident 12 h after wounding the monolayer (Fig. 1 *B*). Adenosine, AMP, cAMP, and ATP were as effective as ADP in stimulating migration (Fig. 2). Other purine and pyrimidine nucleotides such as GDP, GTP, TDP, TTP, UDP, UTP, CDP, and CTP had no effect, nor did adenine or ribose phosphate alone, or together. ADP also stimulated migration into wounds made in monolayer cultures of canine kidney epithelial cells of the MDCK line prepared under similar conditions, but not in cultures of mouse fibroblasts (BALB/3T3 cells).

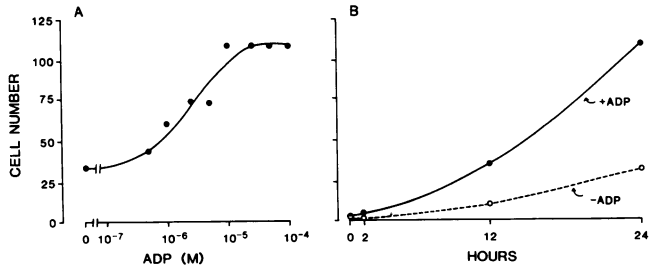

Figure 1. Effect of ADP on migration of BSC-1 cells into wounds in high density quiescent cultures. Cells were scraped from the monolayer to inflict a wound, as described in Methods. (*A*) Effect of different concentrations of ADP on cell migration into a wound. (*B*) Time course of ADP stimulation of cell migration. ADP (10 μM) was added immediately after wounding, and the number of cells migrating into the wound was counted 2, 12, and 24 h later. Values are the mean number of cells counted in each of 10 wounds in two cultures. SE was less than 4% of the mean so that measures of variance are not apparent on the figure.

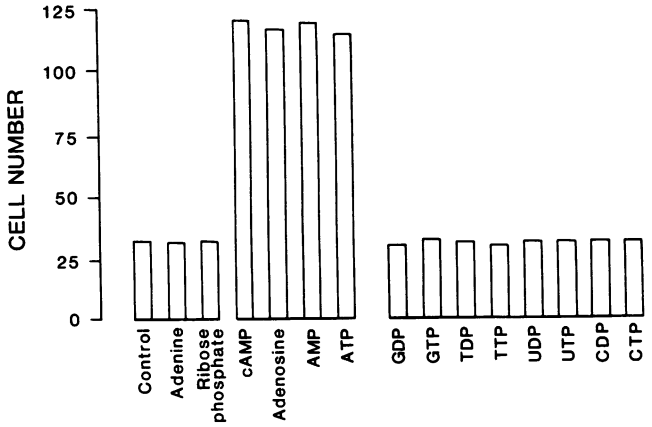

Figure 2. Effect of adenine, ribose phosphate, adenosine, and purine and pyrimidine nucleotides on cell migration into wounds made in monolayers of BSC-1 cells. Each compound (10 μM) was added to the culture medium, and the number of cells that migrated into a wound that was 1 mm^2 in size was counted 24 h later. Values are means for 10 different wounds in two cultures; SE was less than 4%.

Because growth factors are known to influence wound healing (25–27) and tumor cell invasion and migration (28) in vivo and in vitro, we evaluated the capacity of several of these peptides to affect migration in BSC-1 cells. Specified concentrations of EGF, acidic or basic FGF, IGF-I or -II, or PDGF were added to a culture immediately after wounding, and migration of cells into the wound was assayed 1 d later. EGF (0.15–8 ng/ml) had a striking stimulatory effect on cell migration. Even at a concentration of 0.15 ng/ml, EGF nearly doubled the number of cells in the wound ($P < 0.001$), whereas 0.8 ng/ml maximally increased migration by three- to fourfold (Fig. 3). In contrast, basic FGF (0.5–12 ng/ml) stimulated migration maximally at 4 ng/ml (146%) ($P < 0.001$), and IGF-I (2.5–40 ng/ml) at 20 ng/ml (45%) ($P < 0.001$) (data not shown). PDGF (1–8 U/ml) and IGF-II (0.5–12 ng/ml) did not stimulate migration, nor did acidic FGF (2–12 ng/ml) with or without heparin (1–100 μg/ml). Next, we investigated the effect of TGF-β2, an autocrine growth inhibitor produced by BSC-1 cells (29) that

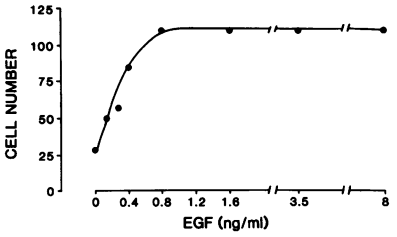

Figure 3. Effect of EGF on cell migration into wounds made in cultures of BSC-1 cells. EGF was added to monolayer cultures immediately after wounds were inflicted, and the number of cells migrating into the denuded area was counted 24 h later. Values are means for 10 different wounds in two cultures; SE was less than 3%.

affects kinetic behavioral properties of diverse types of cells (30, 31). Fig. 4 *A* depicts the marked inhibitory effect of TGF-β2 on migration of BSC-1 cells (half-maximal concentration ~ 0.2 ng/ml). Moreover, ADP-stimulation of cell migration was totally abolished by this autocrine factor (Fig. 5).

Cell movement is dependent in part on adhesion of plasma membrane receptors (integrins) to specific ECM proteins such as fibronectin, laminin, and vitronectin (32). As repair of kidney tubular cell injury appears to require an intact basement membrane (3), injury-induced migration would be expected to depend on an interaction between the cell surface and ECM proteins. To test this hypothesis, we examined the effect of the synthetic peptide arginine-glycine-aspartic acid-serine (RGDS) that competes with extracellular fibronectin for its plasma membrane receptor and might thereby inhibit cell attachment and migration (32, 33). RGDS peptide at a concentration of 2 μg/ml inhibited migration by 45%, and at 5 μg/ml inhibited by more than 80% ($P < 0.001$) (Fig. 4 *B*). At a concentration of 5 μg/ml, RGDS peptide totally prevented ADP-stimulated migration (Fig. 5). The appearance of migrating cells in the presence of RGDS peptide differed from cells in its absence. Cells exposed to the peptide had irregular outlines, and appeared condensed.

Heparin modulates the action of specific growth factors, expression of growth-regulatory genes, and production of ECM components (34–37), and stimulates migration of bovine capillary endothelial cells in vitro (38). The effect of heparin on migration of BSC-1 cells was ascertained by adding specified concentrations to wounded cultures and assaying migration 1 d later. At a concentration of 2 μg/ml, heparin inhibited cell migration into the wound by 50%; increasing the concentration up to 100 μg/ml did not augment this inhibitory effect ($P < 0.001$) (Fig. 4 *C*). Enhancement of cell migration by ADP was totally reversed by heparin at a concentration of 2 μg/ml (Fig. 5).

To further characterize the mechanism by which ADP stimulates migration, we tested the hypothesis that the effect of the nucleotide is mediated by a receptor on the cell surface, as has been proposed for platelets in which ADP induces changes in adhesion and shape (39). In platelets, these responses to ADP appear to be mediated by a membrane receptor that can be blocked by 5′-*p*-fluorosulfonylbenzoyladenosine (FSBA), an analogue of the nucleotide that binds covalently to the ADP receptor (40). When FSBA (100 μM final concentration) was added with ADP to wounded cultures, total inhibition of ADP-induced cell migration was observed (Fig. 5). FSBA had no effect on migration in the absence of ADP nor when migration was stimulated by EGF. Thus, it appears that the capacity of FSBA to block nucleotide stimulation of migration was mediated by preventing ADP binding to the cell surface.

Discussion

The results indicate that adenosine, adenine nucleotides, and cAMP stimulate migration of renal epithelial cells into wounds made in monolayer cultures. Each of these compounds can speed migration so that the denuded area is completely filled in with cells 1 d after wounding, compared with 2 d in control cultures. The stimulatory capacity of adenine nucleotides appears to be nucleotide-specific because other purine (guanosine) and pyrimidine (thymidine, uridine, cytosine) nucleotides did not exhibit this effect. The adenosine moiety seems critical for accelerated migration because adenosine was as effective as its derivatives containing one (AMP, cAMP), two (ADP), or three (ATP) phosphate residues, and neither adenine nor ribose phosphate had any effect. The capacity of ADP to stimulate migration appeared relatively cell-type specific as it was observed in renal epithelial cells of both monkey and canine origin, but not in fibroblasts.

Adenine nucleotides, particularly ADP, are the most potent agents yet defined as stimulators of DNA synthesis in BSC-1 cells (8). The results of the present study indicate that 10- to 20-fold less ADP is sufficient to maximally stimulate migration than is required for proliferation. That ADP (1 μM) could double the rate of migration without initiating mitogenesis was shown by demonstrating that cells at the edges of the wound and within it did not exhibit increased labeling with bromodeoxyuridine. Thus, the rapid closure of epithelial cell wounds after exposure to the low dose of ADP used in this study appears to result from the capacity of the nucleotide to stimulate migration not proliferation. For EGF, the half-maximal concentration required to stimulate migration (0.4 ng/ml) (Fig. 3) is also much lower than that needed for stimulation of DNA synthesis (6 ng/ml) (41). In epithelial cells from rat liver, EGF stimulation of migration is associated with inhibition of DNA synthesis (42).

As BSC-1 cells constitutively release PDGF and TGF-β2 (15, 29), we were particularly interested in finding out if peptide growth factors contribute to control of cell migration. The

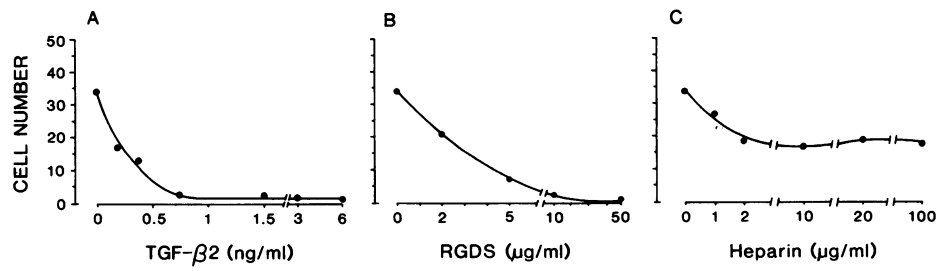

Figure 4. Effect of TGF-β2 (*A*), RGDS peptide (*B*), or heparin (*C*) on migration of BSC-1 cells into wounds made in monolayer cultures. Each compound was added immediately after creating the wound, and the number of cells migrating into it was counted after 24 h. Values are means for 10 different wounds in two cultures; SE was less than 3%.

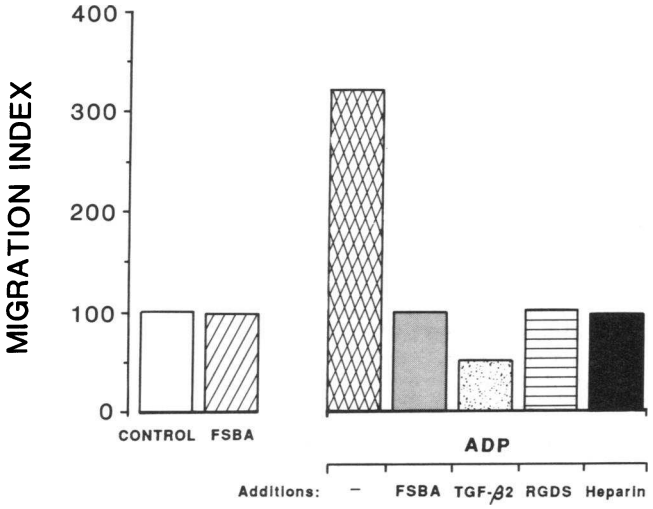

Figure 5. ADP-stimulated migration of BSC-1 cells: effect of different compounds. Wounds were made in high-density, quiescent cultures, and each of the following agents was added at time 0 together with ADP (10 μM): FSBA (100 μM), TGF-β2 (1.5 ng/ml), RGDS peptide (5 μg/ml), or heparin (2 μg/ml). The extent of cell migration into the wound was assessed 24 h later as the migration index which was calculated by the following formula: 100 × (number of cells in wound to which an addition was made ÷ number of cells in control wound). SE was less than 4%.

results indicated that EGF was as potent as adenosine and adenine nucleotides, basic FGF and IGF-I were much less stimulatory, and PDGF, IGF-II, and acidic FGF had no effect. TGF-β2 was a very potent inhibitor of migration (half-maximal concentration ~ 0.2 ng/ml) both in the absence of ADP (Fig. 4) and in its presence (Fig. 5). In contrast, the half-maximal concentration for TGF-β2 is 20-fold higher for inhibition of growth (4 ng/ml) (41) in confluent cultures of BSC-1 cells than for migration. Although EGF and TGF-β are known to regulate migration in other types of cultured cells (19, 43–46), the mechanisms by which they act are not known.

Cell migration depends on adhesion of cells to their substratum, which includes roles for binding of ECM proteins to their cell surface receptors (integrins), the cytoskeleton, plasminogen activator, and plasminogen activator inhibitor. As migration of BSC-1 cells is easily inhibited by the RGDS peptide that competes with fibronectin for its receptor, it is possible that fibronectin-integrin binding may participate in migration both in the presence and absence of ADP, as appears to be the case in other types of cells. TGF-β has been shown to modulate cellular production of matrix proteins, plasminogen activator, and plasminogen activator inhibitor (30, 47), and EGF stimulates transcription of integrins in 3T3 fibroblasts (48). In rat liver epithelial cells, EGF-induced migration is associated with altered fibronectin and laminin gene expression (49), and ECM proteins appear to modulate migration (50). In addition, activation of fibronectin receptor function has been demonstrated in keratinocytes during cutaneous wound healing (51), and specific integrin subunits were detected in migrating, but not resting, keratinocytes (52).

That ADP binds to a specific receptor on the cell surface and thereby signals cell movement is suggested by the observation that the ADP analogue FSBA inhibited nucleotide-induced migration (Fig. 5). We have previously reported that ADP induces rapid reorganization of cytokeratin-8 filaments and alters cell shape (23), which suggests a direct effect. Alternatively, the effect of ADP on migration might be indirect. ADP could induce production and/or release of an autocrine motility, or scatter factor (53) by BSC-1 cells that would stimulate migration, which is suggested by our previous observation that ADP initiates rapid release of PDGF B chain-like protein (15).

Infusion of adenine nucleotides or EGF can speed recovery of injured kidney tubules in rats with experimental acute renal failure (7, 54–56). The present study suggests that the beneficial effects of these agents could be mediated in part by their capacity to stimulate cell migration along the tubular basement membrane, thereby promoting rapid reepithelialization of the injured nephron. EGF or TGF-β released from intact neighboring renal cells or delivered by the circulation could also modulate migration. The results also suggest that adenosine, AMP, cAMP, ADP, and/or ATP liberated by injured or dying cells could speed repair of the nephron by stimulating migration of surviving cells. Higher extracellular concentrations of adenine nucleotides achieved by infusion or cellular release could stimulate migration of surviving cells from the edges of the wound to replace detached necrotic cells, and also initiate DNA synthesis to speed the regenerative process.

Acknowledgments

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