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Roles of Extracellular Matrix and Cytoskeleton in Intestinal Epithelial Restitution

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The intestinal mucosa maintains a barrier that limits exposure of the body to potentially injurious luminal contents. In this role, the epithelium is subject to attack by infectious, pharmacologic and other chemical agents from the luminal (apical) surface. The epithelium may also be damaged by inflammatory cells as they traverse intercellular junctions while migrating from the interstitium to the intestinal lumen. Thus, epithelial injury may range from damage to intercellular junctions to erosion and ulceration. Healing of mild epithelial injury may only require reassembly of intercellular junctions. In contrast, more severe injury requires spreading, migration and proliferation of epithelial cells to adequately seal the wound and restore barrier function. In the latter case, healing of mucosal wounds has been characterized as a coordinated process (fig. 1) in which epithelial cells at the edge of the defect alter their shape and spread across the wound. The observation that these processes of spreading and migration can be regulated by matrix proteins likely reflects distinct differences in matrix composition between denuded basement membrane and ulcerated mucosa from a teleologic standpoint. Healing can also be accelerated by growth factors in a manner that is largely independent of their mitogenic properties, since part epithelial proliferation only contributes significantly to healing if the wound cannot be resealed in the first 24 h. Ultimately, as migrating cells spread and cover the damaged surface, they must also form intercellular junctions to restore barrier function. Cell shape, migration and junction assembly depend on

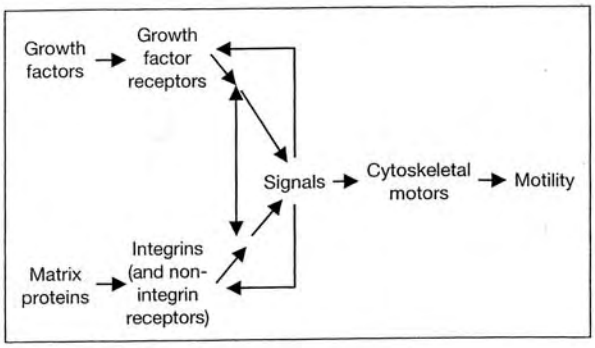

Fig. 1. Growth factors and matrix proteins are altered in mucosal injury. Multiple mechanisms, including direct effects of cell wounding, release of chemotactic factors from injured tissue, and migration of inflammatory cells into injured tissue with growth factor secretion may lead to increased concentrations of growth factors at sites of injury. Wounding both disturbs the architecture of the tissue matrix and may denude the basement membrane, thus exposing the interstitial matrix below. Growth factors and matrix proteins each act on intestinal epithelial cells via specific receptors that initiate intracellular signal transduction cascades. These ultimately regulate cytoskeletal motors that provide the force necessary for cell motility. However, it has now become clear that additional complexity and cross talk resides in the interactions between growth factor- and matrix-initiated signal cascades and the ability of such signals to feed back to and alter the expression, organization and activity of the growth factor and matrix receptors themselves.

an intact cytoskeleton. While each of these events is critical to ultimate healing of mucosal wounds, the focus of this chapter will be limited to the roles of the extracellular matrix and cytoskeleton in migration and restoration of barrier function.

In vivo Processes and in vitro Models

As noted above, migrating epithelial cells abandon their columnar shape and assume a flattened squamoid appearance. At the simplest level, this shape change allows cells to cover a larger surface area and, thus partially compensate for cell loss following mucosal injury. In conjunction with the shape change, these cells also exhibit decreased expression of differentiation markers. This was initially interpreted as dedifferentiation. However, the process might be better described as a phenotypic shift, or redifferentiation, towards a specialized migratory phenotype that reorganizes membrane, cytoskeleton and matrix receptors [1] and also displays altered matrix-dependent intracellular signaling patterns [2].

One in vitro model of intestinal epithelial injury that has been well characterized involves superficial injury of guinea pig ileal mucosal sheets mounted in Üssing chambers [3]. Treatment of the mucosal surface with low concentrations of nonionic detergent for 5 min caused denudation of the epithelium at villous tips without destruction of the basement membrane or crypt epithelium. As a result of this injury, resistance to passive ion flow decreased significantly and flux of the extracellular markers mannitol and inulin increased 3- to 5-fold immediately after injury [3]. The villus tips were again covered by columnar absorptive cells within 2 h after injury, a time course that is incompatible with enhanced cell proliferation. Analysis of the structural events occurring during recovery showed that absorptive cells shouldering the foci of denudation rapidly changed shape after injury: they became flattened and sent cell projections over the denuded basement membrane. By 60 min after injury, cells from opposite shoulders of the denudation abutted, thus resealing the defect. Paralleling these structural changes, transepithelial resistance, potential difference, and mannitol and inulin fluxes returned toward control values. These data show that focal epithelial discontinuities in the small intestine may be rapidly resealed. This resealing correlates with epithelial cell shape change and migration but occurs too rapidly for cell division to be significantly involved. Such reparative processes may substantially limit the deleterious physiologic impact of superficial forms of intestinal injury.

Further studies in the isolated mucosal sheet model using functional antibodies to matrix proteins and receptors have suggested a role for specific matrix proteins in the regulation of restitution [4, 5]. However, it is difficult to do more fundamental mechanistic studies in such whole tissue models. Thus, experimental models using cultured cell models have been used to further analyze migration. However, mechanistic studies of enterocyte migration have often lagged behind studies of fibroblast or inflammatory cell migration. In part, this is due to technical difficulties inherent in the use of well-differentiated intestinal epithelial cell lines. We have focused on Caco-2 cells, derived from a human colonic adenocarcinoma, as a useful model. Specifically, we have used a Caco-2 BBe subclone that displays a microvillus brush border with a full range of small intestinal hydrolases [6], tight junctions [7], polarized protein sorting [8], and physiologic ion and drug transport [9–13]. Caco-2 cells resemble enterocytes morphologically and functionally and are frequently used to model normal human small intestinal epithelium [14–18]. Our laboratories have studied this Caco-2 BBe subclone to elucidate mechanisms by which matrix and cytoskeleton influence enterocyte biology [19, 20]. Others have studied cell migration using human tumor-derived T84 [21] and HT29 [22] cell lines or nontransformed rat intestinal epithelial cell lines, such as IEC-6 [23].

Direct and Independent Matrix Effects

The earliest phase of intestinal epithelial restitution involves rapid migration and spreading of epithelial cells to fill an experimental wound. Cell proliferation is also an important component of the healing process, but lags behind cell migration. Thus, the earliest phase of healing, restitution, occurs before proliferation can contribute to the process, and many reductionist studies directed toward understanding mucosal healing have therefore focused on epithelial sheet migration.

For instance, using a 'fence' assay, in which Caco-2 cell migration outward from a defined starting area is measured over 6 days, the effects of various substrates and growth factors was determined [20]. Although initial attachment and spreading of Caco-2 cells on surfaces coated with collagen types I and IV and laminin were similar, cell proliferation and migration on collagen I-coated surfaces was significantly greater than on the other substrates [20]. These data suggest that disruption of the basement membrane and direct exposure of the epithelial cell basal surface to collagen I, a dominant collagen in the interstitium beneath the basement membrane, may trigger a maximal signal for migration that is not achieved on basement membrane-derived matrix components such as laminin.

Although restitution occurs prior to and independently of cell proliferation, even this early phase of wound healing can be accelerated by mitogens such as epidermal growth factor [20], hepatocyte growth factor [21], fibroblast growth factors [23], and insulin-like growth factor-1 [22]. This subject is covered in more detail elsewhere in this volume.

An interesting additional hypothesis is that the effects of growth factors and matrix proteins are interactive rather than simply additive. That is, we [20, 24] and others [25] have suggested that matrix proteins not only directly affect gut epithelial cell motility but also influence the response to growth factors. Consistent with this hypothesis, epidermal growth factor was able to further stimulate Caco-2 migration over laminin in a fence migration assay, but migration over collagen I-coated surfaces did not increase [20]. This matrix-dependent difference in the ability of epidermal growth factor to enhance Caco-2 cell migration was not due to differences in cell proliferation, since epidermal growth factor stimulated proliferation equivalently on both laminin and collagen I and since the differential effects of epidermal growth factor on migration over laminin and collagen I persisted when migration studies were performed in the presence of mitomycin C to block cell proliferation. Instead, the effect appeared to be caused by laminin- and EGF-dependent co-modulation of $\alpha 2$ integrin subunit expression and organization at the lamellipodial edge of the migrating cells [20]. The effects of transforming growth factor-β_1 on Caco-2 cell migration over

laminin and collagen I also diverged, similarly to those of epidermal growth factor [24]. Transforming growth factor-β_1 stimulated migration over laminin but inhibited migration over collagen I [24], although the mechanism for this effect appeared independent of the $\alpha 2$ integrin subunit. Thus, differential regulation of migration over various matrices is not as simple as maximal stimulation by collagen I and graded stimulation by laminin. Just as growth factors alter the expression and organization of matrix receptors, so it seems also likely that matrix proteins alter the expression and function of some growth factor receptors and their downstream signals (fig. 1) [25]. Indeed, in many cell types, including epithelial cells, protection from apoptosis by tonic growth factor signaling seems to require matrix adhesion [26, 27].

Integrins

Differences in migration rates over different matrixes are, in part, determined by cell-matrix interactions. In the intestine and elsewhere, integrins are specific mediators of these interactions. In response to experimental wounds created by mechanical injury of cultured monolayers, T84 intestinal epithelial cells synthesize several laminin isoforms and deposit laminin 5 in the matrix that extends into the wound [28]. This is accompanied by intracellular redistribution of the laminin-binding $\alpha 3b1$ integrin and $\alpha 6$-containing integrins [28]. Moreover, blocking antibodies targeted against components of laminin 5, or $\alpha 3$-, $\beta 1$- and $\alpha 6$-containing integrins markedly inhibited cell migration and wound closure [28]. Thus, laminin and $\alpha 3$-, $\beta 1$- and $\alpha 6$-containing integrins are critical to wound healing in this T84 cell model [28].

In addition to laminin, migration over collagen types I and IV, the primary collagens of the interstitium and basement membrane, respectively, is important both biologically and experimentally important to our understanding of epithelial wound healing. However, the laminin-binding $\alpha 3$-, $\beta 1$- and $\alpha 6$-containing integrins do not mediate cell attachment to collagen. This falls to the $\alpha 2\beta 1$ and $\alpha 1\beta 1$ integrins. When the $\alpha 1$-, $\alpha 2$- and $\beta 1$-containing integrins were evaluated in Caco-2 cells, a decrease in surface $\alpha 1$ integrin subunits was induced by epidermal growth factor in cells cultured on collagen I [20]. In contrast, surface pools of $\alpha 1$ integrin subunits increased following treatment with epidermal growth factor when cells were cultured on laminin [20]. Additionally, although quantitative analysis did not reveal significant changes in the distribution of $\alpha 2$ integrin subunits between surface and intracellular pools, morphological analysis demonstrated marked differences in $\alpha 2$ integrin subunit organization at the leading edge of cells migrating over collagen I or laminin. Antibody blockade of $\alpha 2$ integrin prevented epidermal growth factor-mediated stimulation of

migration over laminin, but did not inhibit migration over laminin in the absence of epidermal growth factor or over collagen I in either the presence or absence of epidermal growth factor. Thus, epidermal growth factor appears to exert a matrix-specific effect on enterocyte migration by modulating both expression and subcellular distribution of α2 integrin [20].

Signal Transduction Processes

When bound to extracellular ligands, integrin receptors cluster and activate intracellular signaling cascades. The best characterized of these is activation of the tyrosine kinase focal adhesion kinase (FAK), which co-localizes with clustered integrins and cytoskeletal proteins to form focal adhesion complexes. Although the biochemistry of FAK activation is incompletely understood, it does appear to require the cytoplasmic domain of a β integrin subunit, focal adhesion assembly and association with stress fibers. We have recently shown in the Caco-2 cell model that activation of specific β1 heterodimers with functional antibodies directed against individual α integrin subunits differentially affects Caco-2 cell proliferation, differentiation and signaling [29]. However, it remains unclear whether these observations reflect true differences in the activity of integrins with different α subunits or simply differences in their cellular localization and linkages to downstream signaling molecules within the cell.

Once activated, FAK autophosphorylation occurs and FAK also associates with and phosphorylates other molecules including adapter proteins, e.g. paxillin and Shc, and src kinases, e.g. c-src and Fyn. In turn, c-src phosphorylates FAK, forming an SH2-binding site for Grb2. This complex series of phosphorylation events initiates a series of downstream signaling events that appear to vary among cell types, but which eventually lead to activation of the mitogen-activated protein kinase (MAPK) cascades [30].

When tyrosine kinase activation was compared in cultures of confluent contact-inhibited Caco-2 cells and subconfluent migrating Caco-2 cells with lamellipodia, total tyrosine kinase activity was increased in migrating cells and these increases were proportionally greater in the detergent-insoluble (membrane and cytoskeletal) fraction than in the detergent-soluble (cytosolic) fraction [31]. Interestingly, although the proportion of intracellular FAK and paxillin that is phosphorylated increased in cells migrating across matrix proteins, total protein pools of FAK and paxillin decreased substantially. Thus, the actual amount of phosphorylated FAK and paxillin in migrating Caco-2 cells was less than that present in confluent contact-inhibited Caco-2 cells. Similarly, ERK1 and ERK2 were activated while total protein content was changed following adhesion to collagens I or IV or laminin. The patterns of activation and expression of these

signal proteins in migrating cells compared with contact-inhibited confluent cells varied in a matrix-dependent manner [2]. As might be expected, the reorganization of these proteins associated with Caco-2 cell motility is also matrix-dependent [32]. The pattern of activation observed in Caco-2 cells migrating over collagen is consistent with recent descriptions of ERK activation during gut mucosal wound healing in vivo [33]. In addition, immunofluorescent microscopic evaluation suggests that motility is also associated with a reorganization of intracellular FAK away from the lamellipodial edge. Thus, Caco-2 motility seems to be associated with regulation of FAK at three different levels – phosphorylation, total protein content and subcellular distribution. The biological relevance of this FAK and ERK activation is also apparent, as expression of a dominant negative FAK construct decreased Caco-2 motility and inhibited ERK2 activation [2]. Additionally, pharmacological ERK inhibition with PD98059 inhibited Caco-2 motility. Thus, the FAK-ERK pathway appears to be critically involved in regulating Caco-2 cell migration [2, 30]. However, the complexities of spatial changes in FAK activation remain poorly understood.

Actomyosin Contraction

Although intracellular signaling processes and membrane receptors that mediate cell-matrix interactions are critical to cell migration, the movement of cells over a surface ultimately requires the generation of force. Numerous studies in a broad range of cell types have demonstrated the essential role of actomyosin contraction in this process. In epithelial cells, actomyosin contraction is principally mediated by conventional type II myosin. Each myosin II molecule is composed of two heavy chains, two essential light chains and two regulatory light chains (MLC). Phosphorylation of MLC at serine 19 by myosin light chain kinase triggers a conformational shift in myosin that leads to actomyosin contraction. Thus, MLC phosphorylation is a biochemical marker of actomyosin contraction. MLC phosphorylation is also a likely target of at least some of the signal transduction pathways described above. For example, myosin light chain kinase can be phosphorylated by MAP kinases ERK1 and ERK2 [34]. MAP kinase phosphorylated myosin light chain kinase is more sensitive to calmodulin activation [34]. Thus, via activation of myosin light chain kinase, MAP kinase activation can increase MLC phosphorylation and, potentially, increase cell migration rates.

MLC phosphorylation has been studied in motile fibroblasts using a polyclonal antibody that specifically recognizes MLC phosphorylated at serine 19 [35]. Phosphorylated MLC was distributed in a polarized pattern related to the direction of cell movement, with increased MLC phosphorylation near membrane ruffles at the leading edge and in a trailing region that included the

nucleus [35]. This suggests that actomyosin contraction at both poles of migrating fibroblasts is involved in cell migration [35]. Similarly, phosphorylated MLC was also concentrated in actomyosin bundles at the leading edge of migrating sheets of renal epithelial cells [35]. These observations suggest that actomyosin filaments at the leading edge may be involved in force production during epithelial cell migration. This is consistent with the observation that polyamine-depleted cultures of IEC-6 intestinal epithelial cells have a markedly disrupted distribution of myosin II and reduced migration over experimental wounds [36]. However, that interpretation contrasts sharply with previous conclusions that the primary role of myosin II in fibroblast cell migration is at the rear of the migrating cells [37]. How can these disparate conclusions then be resolved? Do the discrepancies reflect cell-specific differences, or is the role of myosin II in cell migration still incompletely understood? Better understanding of these issues will be critical to our understanding of epithelial cell migration and wound healing in general.

To further evaluate the role of myosin II in intestinal epithelial cell migration, we sought to determine whether enhanced MLC phosphorylation, and therefore actomyosin contraction, is sufficient to accelerate intestinal epithelial cell migration. We established clonal populations of Caco-2 cells that express a truncated MLC kinase catalytic domain construct [38] under the control of the tetracycline-regulated inducible expression system [39]. The tMLCK construct (tMLCK) lacks the regulatory/inhibitory domain of MLC kinase [38]. Thus, the kinase is active when the protein is expressed and does not require Ca^{2+} and calmodulin for activation [38]. In preliminary studies we showed that washout of doxycycline led to increased MLC kinase activity and MLC phosphorylation in cell lysates [40]. We have used the fence migration assay described above to directly evaluate the effect of tMLCK expression on Caco-2 cell migration over collagen I. To avoid the potential that tMLCK expression might alter cell attachment, Caco-2 cells were allowed to attach within the fence overnight in the presence of doxycycline (to prevent tMLCK expression). After cell attachment, the fence was removed and the cells transferred to media with or without doxycycline to repress or induce tMLCK expression, respectively. tMLCK expression reduced the surface area covered by Caco-2 migration by $10 \pm 3\%$ ($p < 0.02$, fig. 2). This change is quantitatively similar to the TGF-α-induced inhibition of cell migration over collagen I [24]. Although this quantitative similarity between the effects of tMLCK expression and TGF-α treatment is likely coincidental, it is interesting to note that available data do support a role for actomyosin contraction as an intermediate in the effects of TGF-α on endothelial monolayer integrity [41].

Immunofluorescent microscopy was used to evaluate actin filaments at the leading edge of Caco-2 cells with or without tMLCK expression after 1 day of

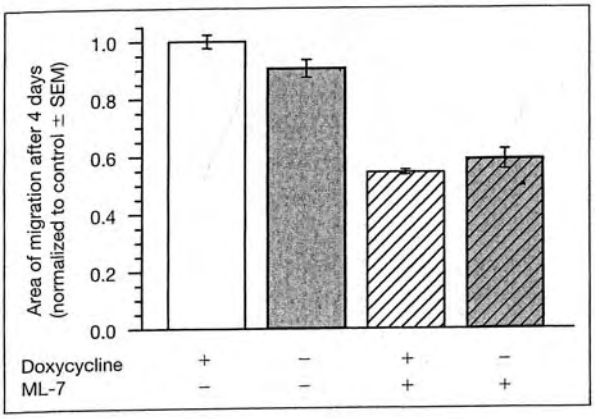

Fig. 2. Effects of tMLCK expression and MLC kinase inhibition on Caco-2 migration. Cell migation was allowed to proceed for 4 days after the induction (or repression) of tMLCK expression and addition (or not) of 10 μM ML-7. Migration area for each sample was then determined. Mean area ± SEM (n = 7) is shown. For the migration in the absence or presence of doxycycline (both without ML-7), the difference in migration was significant ($p < 0.02$). Differences in migration in the absence or presence of doxycycline (both with ML-7) were not statistically significant.

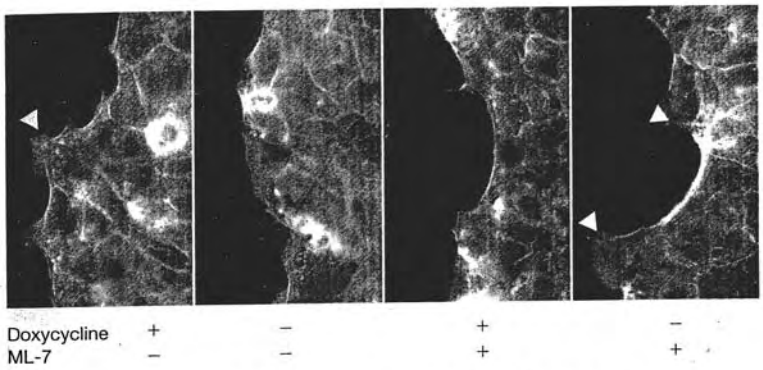

Fig. 3. Effects of tMLCK expression and MLC kinase inhibition on actin distribution in migrating Caco-2 cells. Fluorescent photomicrographs of actin distribution at the leading edge of migrating cells 1 day after the initiation of migration, tMLCK expression (by washout of doxycycline) and addition of 10 μM ML-7. Arrows indicate lamellipodia.

migration. In the absence of tMLCK expression, lamellipodia were easily identified, as were long stress fibers parallel to the leading edge (fig. 3). In sharp contrast, lamellipodia were almost entirely absent in cells expressing tMLCK. Moreover, the number of stress fibers parallel to the leading edge was markedly

decreased. These observations suggest that regulated actomyosin contraction and relaxation is necessary for extension of lamellopodia. If true, this would be at odds with other systems, for example *Dictyostelium*, where myosin II is specifically absent from protrusive structures and myosin I is considered to be of greater import [42]. However, it would be consistent with the idea that myosin II is critically involved in the assembly of actin filaments and lamellopodia at the leading edge of migrating epithelial cells [43].

To further evaluate the role of tMLCK expression in Caco-2 cell migration, we evaluated migration and actin assembly in cells with or without tMLCK expression that were also treated with the MLC kinase inhibitor ML-7. ML-7 inhibited cell migration over collagen I both in the absence and presence of tMLCK expression (fig. 2). However, migration was inhibited to a greater extent in cells without tMLCK expression, such that migration of cells with or without tMLCK expression was comparable when ML-7 was present (fig. 2). Thus, differences in cell migration induced by tMLCK expression are abolished when both tMLCK and the endogenous MLC kinase are inhibited. Addition of ML-7 to cultures without tMLCK expression not only limited forward progress of the leading edge, but also led to the development of large gaps within the surface over which the cells have migrated.

The morphology of the migrating monolayers also supports the idea that MLC kinase inhibition with ML-7 may partially reverse the effects of tMLCK expression. Lamellipodia at the leading edge of migrating cells were poorly seen in cells with tMLCK expression, but addition of ML-7 to cells expressing tMLCK restored lamellipodia and, to some degree, stress fibers assembly at the leading edge. This result supports the hypothesis that the effects of tMLCK expression on cell migration are at least partly due to excessive and inappropriate MLC phosphorylation.

Thus, actomyosin contraction, mediated by myosin II, appears to be critical to migration of intestinal epithelial cells. The result that both inhibitors of MLC kinase, e.g. ML-7, and activators of MLC phosphorylation, e.g. tMLCK, suggest that neither is sufficient to activate cell migration. In contrast, it appears that a complete cycle of actomyosin contraction and relaxation is necessary for cell migration, and lamellipodium formation, to occur. Thus, for example, tMLCK expression may prevent cell migration by inducing a rigor-like contraction of the cytoskeleton and thereby preventing force generation and leading edge assembly.

Conclusions

Intestinal epithelial biology is profoundly affected by complex interactions between the extracellular matrix and the actin cytoskeleton. In this chapter we

have focused on the roles of these effectors, both in concert and separately, in cell motility and mucosal wound healing. Although the mechanisms by which these processes occur remain incompletely understood, it is clear that integrin-mediated cell-matrix adhesion is a central event. In addition to providing the mechanical coupling between the cell and the substratum, binding of integrins to extracellular matrix components may activate signaling cascades necessary for cell migration to proceed. Examples include activation of focal adhesion kinase and MAPK. However, differing subcellular localizations of integrin-matrix binding interactions during motility are likely to also provide more complex spatiotemporal signaling than is presently appreciated. It is also likely that bidirectional interactions between myosin II-triggered actomyosin contraction and integrin function and distribution remain to be defined. Thus, the interactions between extracellular mediators, transmembrane proteins and cytoskeletal components are likely to jointly influence cell phenotype during restitution. This represents an important area for further study.

Acknowledgements

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