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An epithelial cell destined for apoptosis signals its neighbors to extrude it by an actin- and myosin-dependent mechanism

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Background: Simple epithelia encase developing embryos and organs. Although these epithelia consist of only one or two layers of cells, they must provide tight barriers for the tissues that they envelop. Apoptosis occurring within these simple epithelia could compromise this barrier. How, then, does an epithelium remove apoptotic cells without disrupting its function as a barrier?

Results: We show that apoptotic cells are extruded from a simple epithelium by the concerted contraction of their neighbors. A ring of actin and myosin forms both within the apoptotic cell and in the cells surrounding it, and contraction of the ring formed in the live neighbors is required for apoptotic cell extrusion, as injection of a Rho GTPase inhibitor into these cells completely blocks extrusion. Addition of apoptotic MDCK cells to an intact monolayer induces the formation of actin cables in the cells contacted, suggesting that the signal to form the cable comes from the dying cell. The signal is produced very early in the apoptotic process, before procaspase activation, cell shrinkage, or phosphatidylserine exposure. Remarkably, electrical resistance studies show that epithelial barrier function is maintained, even when large numbers of dying cells are being extruded.

Conclusions: We propose that apoptotic cell extrusion is important for the preservation of epithelial barrier function during cell death. Our results suggest that an early signal from the dying cell activates Rho in live neighbors to extrude the apoptotic cell out of the epithelium.

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Background

Apoptosis is essential for both shaping an embryo and maintaining homeostasis in adult tissues. It can be distinguished from necrosis by the distinct sequence of morphological changes that result from activation of apoptotic-specific proteases, the procaspases [1]. These changes include plasma membrane blebbing, cytoplasmic shrinkage, and nuclear condensation and fragmentation. Typically, nearby cells rapidly phagocytose apoptotic cells to remove them from a tissue [2].

Apoptosis has generally been studied in single cells in culture. Much less is known about the process in an intact tissue such as an epithelium. Simple epithelia, consisting of one or two layers of cells, cover most embryos and organs, providing a protective barrier. During embryonic development and throughout life, large numbers of cells within such epithelia go through apoptosis. Apoptosis could interrupt the barrier function of the epithelium in at least two ways: it may cause the epithelium to become leaky, and phagocytic clearance of the dying cells could create gaps in the epithelium. We find, however, that the barrier is maintained, even when a large proportion of epithelia cells become apoptotic. How can the epithelium maintain its function as a barrier in the face of massive cell death? We show that apoptotic cells are extruded from both embryonic epithelia in vivo and MDCK cell monolayers. Using the MDCK model system, we show that a dying cell signals to its neighbors to activate an actin- and myosin-dependent contractile mechanism that both extrudes the dying cell and prevents the formation of a gap when the dying cell exits the epithelium.

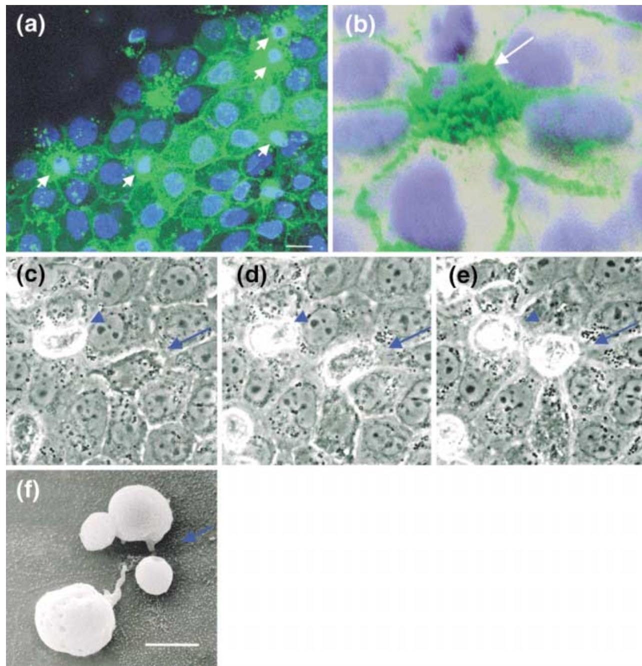
Results

Apoptotic cells are extruded from a simple epithelium in vivo and in a tissue culture model

To investigate how apoptotic cells in an embryonic epithelium are removed, we stained the plasma membranes and nuclei of the epidermis of an embryonic day 8 (E8) chick limb with DiI and Hoechst dye, respectively, and examined the tissue by confocal microscopy. Many apoptotic cells could be detected by their condensed nuclei and blebbed plasma membranes (Figure 1a, arrows). A three-dimensional reconstruction from a series of confocal sections showed that these apoptotic cells were extruded out of the plane of the epithelium (Figure 1b). We obtained similar results when we examined E13 mouse limb (data not shown). Thus, apoptotic cells are removed from these simple epithelia in vivo by extrusion.

To study apoptotic cell extrusion in more detail, we used

Figure 1



Apoptotic cells are extruded from chick embryonic epithelium and an MDCK monolayer. **(a,b)** Confocal fluorescence images of plasma membranes (green) and DNA (blue) from the epidermis of a day E8 chick limb stained with DiI and Hoechst dye, respectively. (a) Arrows indicate apoptotic cells with condensed nuclei and blebbing membranes. (b) 3D reconstruction showing an apoptotic cell with condensed chromatin and convoluted, blebbed plasma membrane (arrow), which is being extruded out from the epithelium. **(c–e)** Apoptosis induced in a MDCK monolayer with short-wave UV light. Phase contrast micrographs from a phase time-lapse movie showing cells at 60 min (c), 80 min (d), and 100 min (e) after UV treatment. The cell indicated by the blue arrow contracts ([c], early), blebs ([d], mid), and becomes phase bright ([e], late). The surrounding cells stretch inward beneath the dying cell. The blue arrowhead points to an apoptotic cell in the later stages of apoptosis, which is still attached to the monolayer at 100 min (e) but will eventually detach into the medium. **(f)** Scanning electron micrograph of cells at ~100 min after UV treatment, showing four apoptotic cells at late stages of extrusion. Each apoptotic cell remains tethered to the epithelium by a stalk (blue arrow). Scale bars, 10 μm.

an in vitro model of a simple epithelium—a monolayer of MDCK cells. We treated the monolayer with short-wave UV light to induce apoptosis. This induced about 10% of the cells to become apoptotic by 90 min, and, by 12 hr, all of the cells were apoptotic. In a time-lapse movie, the apoptotic cells could readily be seen to extrude from the apical surface of the monolayer (Figure 1c–e; Movie 1 in the Supplementary material available with this article online). As a dying cell was extruded into the culture medium, the surrounding cells contracted inward so that no gaps appeared in the monolayer. The first detectable stage of apoptosis was cell darkening (Figure 1c, arrow), followed by membrane blebbing and cell contraction (Figure 1d, arrow), giving the cell a phase-bright appearance as it came out of the plane of the monolayer (Figure 1e, arrow). We will refer to these sequential morphological changes as early (Figure 1c), mid (Figure 1d), and late (Figure 1e) stages of apoptosis. The apoptotic MDCK cells initially extruded on a long stalk (Figure 1f) and then pinched off into the medium. In vivo, apoptotic cells did not extrude on long stalks but were pinched off earlier in the extrusion process. Once initiated, the early to late stages of extrusion took ~40 min, with another 40 min for the cells to pinch off from the monolayer.

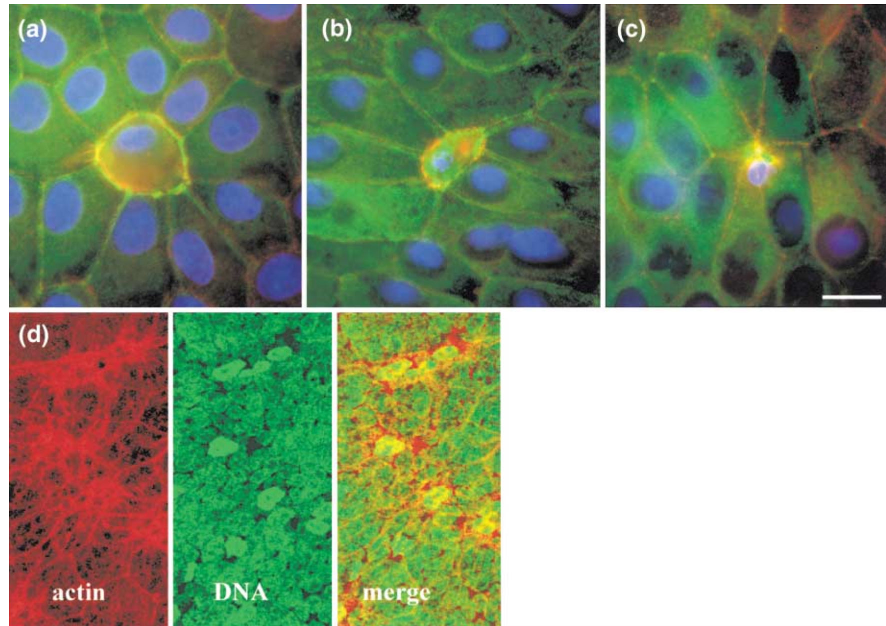
Actin and myosin form rings around apoptotic cells

We stained UV-irradiated MDCK monolayers with actin (red) and myosin (green) antibodies and Hoechst dye to observe DNA (blue) and viewed the cells by fluorescence microscopy. When a cell became apoptotic, the nucleus condensed and fragmented, and the actin staining increased at the interface of the dying cell and its live neighbors, forming a ring, which we refer to as the "apoptotic actin ring" (Figure 2). As extrusion progressed from early to late stages of apoptosis, actin and myosin staining increased around and below the dying cell, and this ring was apparent even at early stages of apoptosis, when the nucleus was just beginning to condense (Figure 2a). During mid and late stages (Figure 2b,c), the ring constricted, as the surrounding live cells closed in under the dying cell. In this and all subsequent figures, the focus for visualizing the DNA is in the plane of the apoptotic cell. Thus, early in apoptosis (Figure 2a), when the nucleus was just starting to condense, the nucleus of the apoptotic cell lay in the same plane as its neighbors. Later (Figure 2b,c), as the apoptotic cell was extruded apically, the nuclei of the surrounding cells were out of the plane of focus. Similarly, rings of actin formed around extruded apoptotic cells in the epidermis of day E8 chick embryo limbs (Figure 2d) and day E13 mouse embryo limbs (data not shown).

Actin/myosin contraction drives extrusion

To test whether actin polymerization and contraction are required for the extrusion of apoptotic cells, just after UV-irradiation, we treated MDCK monolayers either with the actin-polymerization-blocking drugs latrunculin A or cytochalasin D or with drugs that block myosin contraction, the Rho-kinase inhibitor Y-27632 or the myosin light chain kinase inhibitor ML-9. Figure 3a,d shows an extruded control apoptotic MDCK cell by phase contrast and fluorescence microscopy, respectively. When UV-irradiated MDCK monolayers were treated with latrunculin A (Figure 3b,e), Y-27362 (Figure 3c,f), cytochalasin D, or ML-9 (data not shown), the actin cables disassembled, and the apoptotic cells were not extruded from the monolayer. Myosin was also disrupted by Y-27632 treatment (Figure S1). Moreover, the nucleus of the control apoptotic cell was always above the plane of the nuclei of surrounding cells (Figure 3d), whereas, when extrusion was blocked

Figure 2



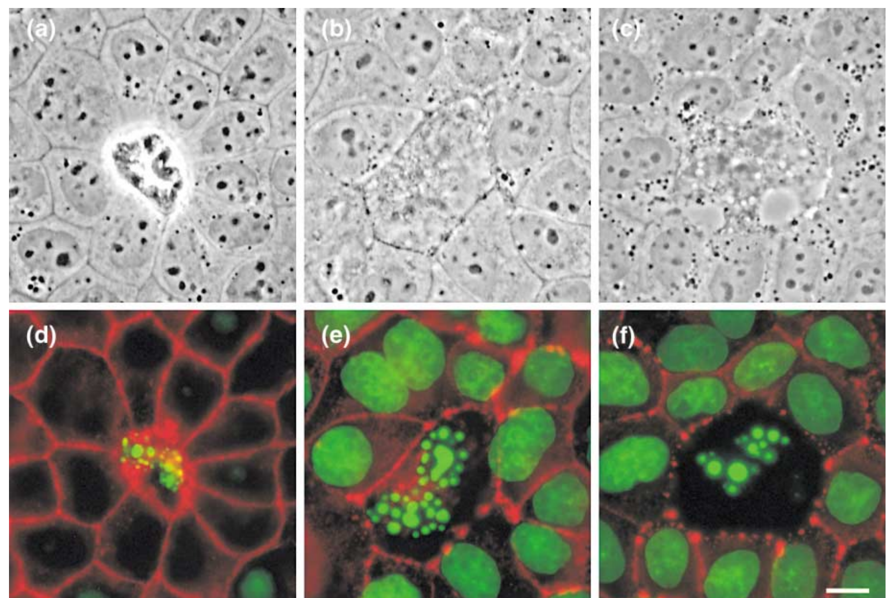
Actin and myosin assemble into a ring during extrusion of apoptotic cells. Triple staining of myosin II (green), actin (red), and DNA (blue) shows that actin and myosin II colocalize (yellow) at the interface between the apoptotic cell and its neighbors at **(a)** early, **(b)** middle, and **(c)** late stages of apoptotic cell extrusion. For (a–c), actin and myosin were photographed in the plane of the ring, while DNA was photographed in the plane of the apoptotic nucleus. Note that an apoptotic nucleus moves out of the plane of the other nuclei, as it is extruded from the monolayer. **(d)** Confocal images of actin (red) and DNA (green) in epithelium of an E8 chick limb. Note that actin accumulates into ring structures around condensing apoptotic nuclei. Scale bars, 10 μm.

with drugs, all nuclei were in the same plane (Figure 3e,f). We used the out-of-plane position of apoptotic nuclei to score the percentage of extruded apoptotic cells with each drug treatment. From apoptotic cells analyzed in three separate experiments with each drug treatment, only 4% extruded in the presence of latrunculin A (n = 3442), cytochalasin D (n = 1300), or Y-27632 (n = 3361); 14% extruded in the presence of ML-9 (n = 448); and 96% extruded in control cultures (n = 3177). Thus, actin filament formation and actin/myosin contraction are required for extrusion of apoptotic cells from a monolayer.

Barrier function is maintained during extrusion

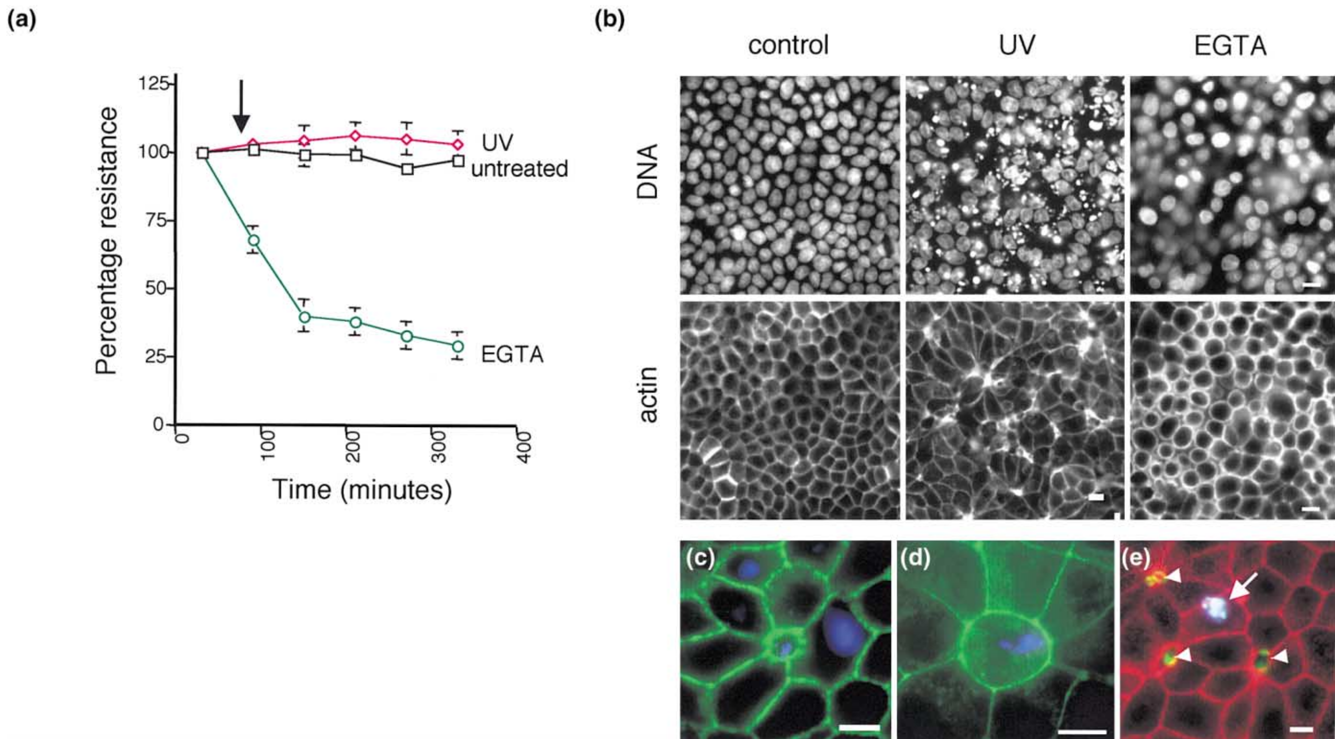
To test whether the barrier function of the monolayer was maintained during the extrusion process, we measured the electrical resistance of UV-treated or untreated MDCK monolayers grown on filters. The monolayers treated with UV maintained the same electrical resistance as untreated

Figure 3



Actin polymerization and contraction are required for apoptotic cell extrusion. **(a–c)** Phase contrast and **(d–f)** fluorescence micrographs of UV-treated MDCK cells treated with DMSO (control, [a,d]), latrunculin A (b,e), and Y-27632 (c,f). The actin (red) was photographed in the plane of the actin cable, while the DNA (green) was photographed in the plane of the apoptotic nucleus. Note that with either drug the apoptotic cells fail to contract, and the surrounding cells do not change their shape in response to the apoptotic cell. Scale bars, 10 μm.

Figure 4



The barrier function of an MDCK monolayer is maintained during extrusion of apoptotic cells. **(a)** Electrical resistance with time after UV-treatment (n = 10), no treatment (n = 10), or with EGTA (n = 3). Arrow indicates when EGTA was added. **(b)** Fluorescence micrographs of the monolayers after 330 min. The nuclei are stained with Hoechst dye and actin with TRITC-phalloidin. Note that electrical resistance is maintained in the UV-treated cells even when most of the cells are apoptotic. **(c,d)** UV-treated MDCK monolayers stained with Hoechst dye for DNA (blue) and β-catenin (green) (c) or occludin (green) (d) to label adherens junctions and tight junctions, respectively. **(e)** The UV-irradiated MDCK cells labeled with the cell-impermeable nuclear dye Sytox. Note that this dye (blue) does not label cells in late stages of apoptosis (arrowheads), unless they have been completely extruded from the monolayer (arrow). Scale bars, 10 μm.

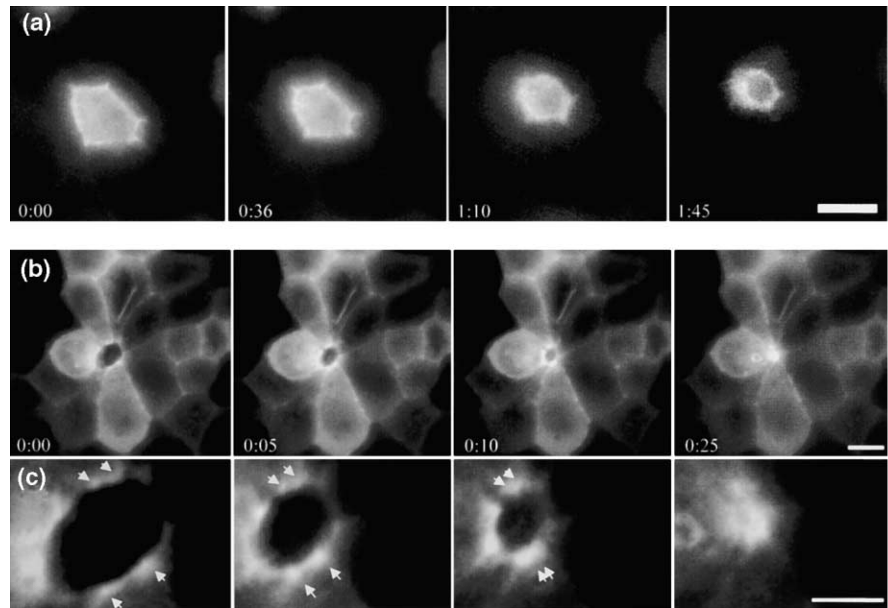
monolayers, even after 6 hr, when a large proportion of the UV-treated cells had become apoptotic (n = 10; Figure 4a). To sustain this high resistance, the live cells in the UV-treated monolayers appeared to stretch as much as two cell diameters to close the spaces left by the dying cells (compare DNA and actin staining in control versus UV-treated panels in Figure 4b). When cell junctions were disrupted by treatment with EGTA, however, the electrical resistance declined rapidly (Figure 4a, circles, and Figure 4b, EGTA panels).

To examine how the barrier function of the MDCK monolayer might be maintained during cell extrusion, we studied the distribution of three cell-cell junction proteins. The localization of β-catenin (Figure 4c) and of occludin (Figure 4d) and ZO-1 (data not shown) to the interface of the apoptotic cell and its neighbors suggests that both adherens and tight junctions, respectively, are maintained as an apoptotic cell was squeezed out of the monolayer (also see reference [3]). Although junctions between live and dying cells are apparently maintained, the plasma membrane of the apoptotic cell must also remain intact throughout the extrusion process for the barrier function of the monolayer to remain intact. To determine when plasma membrane integrity is lost, we stained UV-treated MDCKs with the cell-impermeable dye Sytox. The dye only labeled apoptotic cells that had been completely extruded from the monolayer (Figure 4e), suggesting that the plasma membrane of the dying cell remains intact throughout the extrusion process.

The apoptotic actin ring forms and contracts in both the apoptotic cell and its neighbors during extrusion

From the cell staining described above, we found that apoptotic cell extrusion requires the formation and contraction of an actin and myosin ring between the dying cell and its neighbors. However, from these studies it is unclear whether the actin ring formed in the apoptotic cell, its neighbors, or both or which cells provide the contractile force for extrusion. To determine in which cells this ring is formed, we examined UV-treated MDCK monolayers expressing a GFP-actin fusion protein [4]. As

Figure 5



An apoptotic actin ring forms and contracts in both the apoptotic cell and its neighbors during extrusion. **(a)** Time-lapse stills from a movie of a UV-treated MDCK monolayer expressing GFP-actin only in apoptotic cell. Time is in hours and minutes. **(b)** Time-lapse stills from a movie of a UV-treated MDCK monolayer expressing GFP-actin only in neighboring cells but not in the apoptotic cell itself. Time is in minutes. **(c)** Enlargement of the actin ring around the apoptotic cell shown in (b). The arrows indicate cell-cell junctions that can be seen as "spots" of actin, which move inward over time. Scale bars, 10 μm.

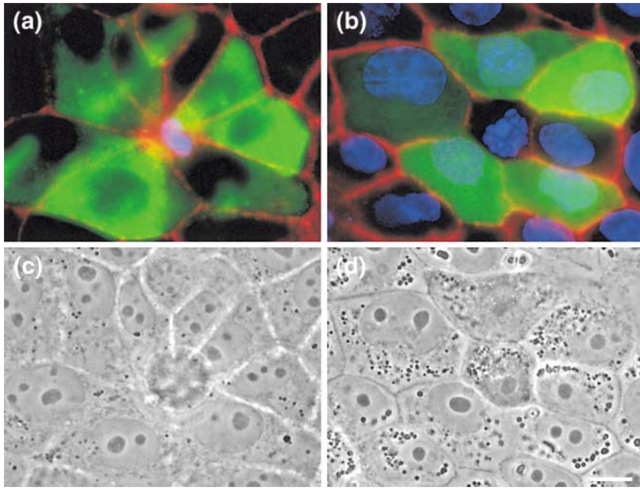
GFP-actin is not expressed in all cells in this line, we were able to follow extrusion in cases where only the apoptotic cell (Figure 5a) or the live neighbors (Figure 5b,c) expressed GFP-actin. In this way, we could show that the actin ring formed at the live/dead cell interface in both the apoptotic cell and its surrounding neighbors. By following cells in time-lapse movies of GFP-actin MDCK monolayers, we found that the actin ring slowly condensed in the apoptotic cell during the extrusion process (Figure 5a; Movie 2). This is consistent with previous reports that show that actin and myosin contract during apoptotic cell blebbing [5–7]. By contrast, the actin ring in the neighboring cells contracted completely and more than four times as fast during extrusion (Figure 5b,c; Movie 3). This is best seen in high-powered views of the live/dead cell interface shown in Figure 5c, where the two cell-cell junctions between the apoptotic cell and its neighbor can be seen as bright spots (arrows) that have moved inward over a 10 min period. Thus, the neighboring cells seem to be drawing into the place of the dying cell's exit by the contraction of their actin rings, rather than by extension of lamellipodia. This finding raised the possibility that the live cells actively extrude the dead cell.

Contraction of the live neighbors is required for extrusion

Although we have shown that extrusion requires actin/myosin contraction, because the myosin inhibitors used were cell permeable we were unable to determine whether contraction is required in the apoptotic cell, its live neighbors, or both. To determine whether actin/myosin contraction in the neighbors is required for extrusion, we injected *Clostridium botulinum* C3 exoenzyme into the neighboring cells of an early apoptotic cell (visualized by phase microscopy). The C3 toxin specifically inhibits Rho, a small GTPase that regulates the actin/myosin cytoskeleton [8, 9]. Extruded cells could be seen by phase microscopy. By staining nuclei with Hoechst dye and actin with rhodamine-phalloidin, we could also score extrusion by whether the apoptotic nucleus was in the same plane as the nuclei of its live neighbors. As shown in Figure 6, C3 toxin injection completely blocked extrusion (Figure 6b,d; n = 9), whereas injections of BSA did not (Figure 6a,c; n = 5). Thus, Rho activity is required in the neighboring cells for extrusion.

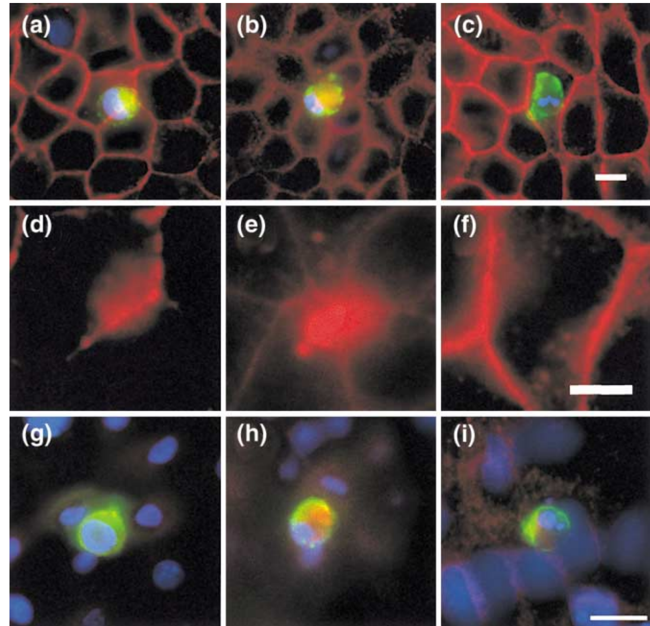
Injection of C3 toxin into the apoptotic cell did not block extrusion (data not shown; n = 15), indicating that Rho activity in the apoptotic cell is not required for extrusion. It has been shown, however, that Rho-associated kinase-I (ROCK-I), which is downstream of Rho, is cleaved and activated by caspases during apoptosis. Thus, C3 would not be expected to block this activation of ROCK-I [6, 7]. To overcome this problem, we treated UV-irradiated MDCK monolayers with the caspase inhibitor Z-Val-Ala-Asp-fmk (z-VAD-fmk), which has been shown to block the ROCK-I-dependent activation of myosin that is responsible for membrane blebbing during apoptosis [5–7]. As we discuss later (see Figure 8a), treatment with z-VAD-fmk had no effect on the extrusion process. This finding suggests that ROCK-I-dependent myosin contraction in the apoptotic cell is not required for extrusion. While the role of actin/myosin contraction in the dying cell is unclear, contraction of actin and myosin in the live neighbors is essential for extrusion.

Figure 6



Contraction of neighboring cells is essential for extrusion. UV-treated MDCK monolayers in which apoptotic cell neighbors were injected with **(a,c)** BSA or **(b,d)** C3 toxin. In (a,b), actin is stained with phalloidin (red), and DNA is stained with Hoechst (blue); dextran (green) indicates which cells were injected. The actin and dextran were photographed in the plane of the actin cable, while the DNA was photographed in the plane of the apoptotic nucleus. Note that, in contrast to BSA injections, in C3 injected cells, the nucleus of the apoptotic cell (blue) is in the same plane as its neighbors, indicating that extrusion has failed. (c,d) Phase contrast pictures of (a,b), respectively. Scale bar, 10 μm.

Figure 7



Early apoptotic cells induce actin filament formation on contact with live MDCK monolayers. **(a–c)** Alexa-488-Lectin-labeled cells (green) were added to an intact MDCK monolayer. After 90 min, the cells were stained with TRITC-phalloidin to label actin (red) and Hoechst dye for nuclei (blue) to indicate whether the added cell was live or in early or late stages of apoptosis. **(d–f)** Enlarged views of (a–c), showing actin in the focal plane of the monolayer. Note that added live cells ([a,d]; n = 2100) and late apoptotic cells ([c,f]; n = 250) did not produce actin cables in the contacted monolayer. In contrast, added early apoptotic cells induced actin filament formation in the contacted monolayer ([b,e]; n = 620). The out-of-focus actin staining in (d,e) is filamentous actin present in the added cells that are in a different focal plane; the added late apoptotic cell (f) no longer contains actin filaments and did not produce these shadows. **(g–i)** Myosin immunofluorescence (red) of alexa-488-Lectin-labeled cells (green) added to an intact MDCK monolayer, where nuclear staining with Hoechst (blue) indicates when added cells are (g) live or in (h) early or (i) late stages of apoptosis. Scale bar, 10 μm.

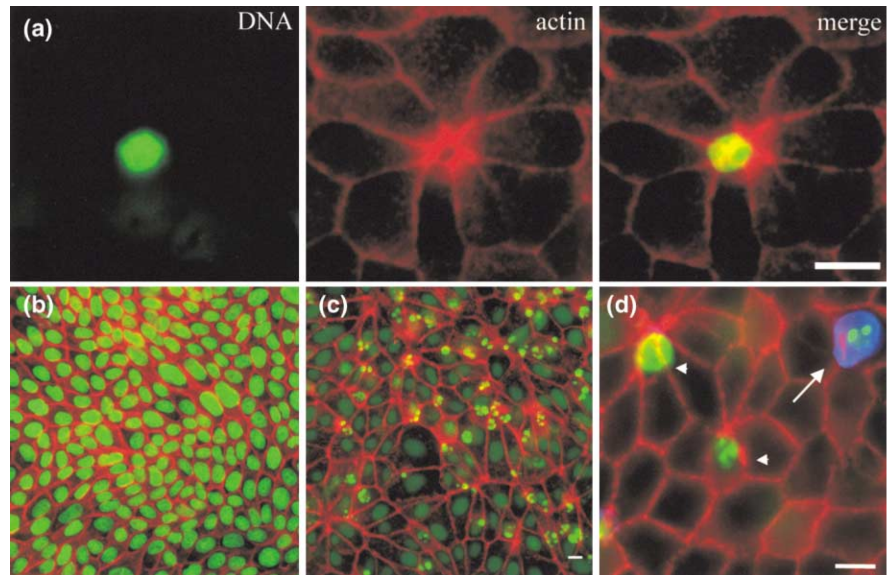
Early apoptotic cells signal their neighbors to form actin cables

What signals the formation of the apoptotic actin ring in the neighboring cells during the extrusion process? One possibility is that the neighbors sense a mechanical pull from the dying cell [10]. Another is that the dying cell produces a signal that induces the formation of the actin ring in its neighbors. To help distinguish between these two models, we added fluorescently labeled apoptotic cells to an intact MDCK monolayer and analyzed whether they induced the formation of actin cables in the living monolayer. Because only some of the labeled cells were apoptotic, we also labeled them with a DNA stain to assess their state of apoptosis: whereas live cells had large intact nuclei, early apoptotic cells had intact condensed nuclei, and late apoptotic cells had condensed and fragmented nuclei. As shown in Figure 7, early apoptotic cells induced an accumulation of actin filaments in the cells they contacted (Figure 7b,e), whereas live cells (Figure 7a,d) and late apoptotic cells (Figure 7c,f) did not: 91% of early apoptotic cells (n = 620) induced actin in the monolayer; only 3% of late apoptotic cells (n = 250) and 4% of live cells (n = 2100) did so. In addition, myosin patches also accumulated in live monolayers in contact with early apoptotic (Figure 7g) but not live cells (Figure 7h). We do not think the actin and myosin induction represents an early stage of phagocytosis because phagocytosis was never seen by time-lapse movies when early apoptotic cells were added to monolayers: early apoptotic cells adhered to the monolayer for 40–100 min and then floated away (data not shown). When added to monolayers expressing GFP-actin, the actin cables appeared dynamically and started to disassemble as the cells floated away (Figure S2). Thus, early apoptotic cells directly signal the cells they contact to induce actin and myosin cables.

The signal for extrusion is early in the apoptotic pathway, before caspase activation

To help determine when in the death process the extrusion signal is produced, we used a variety of compounds to block or assay different stages of apoptosis. Z-VAD-fmk is a cell-permeable peptide that irreversibly inhibits many caspases, including some of those responsible for apoptosis [11]. Addition of z-VAD-fmk to UV-treated

Figure 8



The signal to induce actin cables is an early event in apoptosis. UV-irradiated MDCK monolayers were treated with various inhibitors and then stained with TRITC-phalloidin for actin (red) and Hoechst dye for nuclei (green). Each experiment was repeated three times. **(a)** Treatment with z-VAD-fmk blocks nuclear condensation and fragmentation (DNA panel) but does not block actin ring formation (actin panel) or extrusion. **(b)** Treatment with the K^+ channel inhibitor 4-AP just before UV irradiation blocks extrusion and all other visible signs of apoptosis. **(c)** 4-AP treatment has no effect if added directly after UV treatment. **(d)** UV-treated monolayers labeled with Annexin V to label cell surface PS (blue, arrow). Note that PS is only exposed when apoptotic cells have been completely extruded; even apoptotic cells in late stages of extrusion that are still attached to the monolayer do not stain ([b], arrowheads). Scale bars, 10 μm.

MDCK cells blocked nuclear condensation and fragmentation, membrane blebbing, and cell shrinking, but, surprisingly, it did not block either the formation of actin cables or the extrusion process (Figure 8a). This remarkable finding suggests that the production of the signal for extrusion preceded both caspase activation and the appearance of the morphological hallmarks of apoptosis.

Phosphatidylserine (PS) exposure is a key cell-surface early signal used by apoptotic cells to induce their phagocytosis [12, 13]. We explored whether PS exposure might also be a signal for actin cable formation and extrusion. When irradiated MDCK monolayers were labeled with fluorescent Annexin V, which binds to PS, we found that only apoptotic cells that had been completely extruded from the monolayer were labeled (Figure 8b). The only treatment able to block actin cable formation and extrusion was 4-aminopyramidine (4-AP), a K^+ channel inhibitor, which blocked all visible signs of apoptosis (Figure 8c). As previously reported by others [14, 15] K^+ channel activation apparently happens within seconds of exposure to UV, as addition of 4-AP seconds after UV treatment had no affect on apoptosis (Figure 8d). Therefore, the signal to extrude an apoptotic cell is a key event that occurs before most previously described steps in the apoptosis pathway.

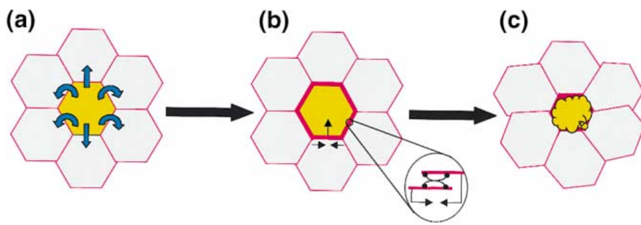
Discussion

Our studies reveal a mechanism for clearing apoptotic cells that has received little attention. We show that apoptotic cells are removed from simple avian and mammalian epithelia, both in vivo and in vitro, by extrusion rather than phagocytosis. Chick embryo retinal pigmented epithelium [16] and embryonic mouse vestibular sensory epithelium [17] apparently clear apoptotic cells by a mechanism that looks similar to the extrusion process we describe here. Similarly, reports on programmed cell death in the epithelia of *Drosophila* embryos suggest that these cells may also be cleared by extrusion [18–20] (our unpublished data). Extrusion may be the mechanism by which apoptotic cells are shed from the epithelial monolayer lining adult intestine [21]. Therefore, it seems likely that extrusion could provide a general mechanism for removing apoptotic cells from simple epithelia.

Our electrical resistance studies show that epithelial barrier function can be maintained even when a large proportion of the cells in an epithelium becomes apoptotic. Maintenance of this barrier is likely due to the dynamic readjustment of adherens and tight junctions during the extrusion process (also see [21]). Our resistance results differ from those of Peralta Soler et al., who found that TNF-induced apoptosis caused the resistance of LLC-PK epithelial monolayers to drop by 50% after 2 hr [22]. In their monolayers, apoptotic cells were expelled basally, became trapped between the monolayer and the filter, and were then phagocytosed by their neighbors. It is, therefore, possible that the disruption of barrier function resulted from the phagocytosis occurring within their monolayer. If so, it may explain why apoptotic cells are cleared from simple epithelia by extrusion rather than by phagocytosis.

While apoptotic cells are often removed from simple epithelia by extrusion, it seems likely that in vivo the extruded cells would eventually be engulfed. This is supported by our studies and those of [3], where cell-surface PS, a signal for apoptotic cell phagocytosis, is not exposed

Figure 9



Model for how apoptotic cells may be extruded from an epithelium. **(a)** An early signals to its neighbors. **(b)** This signal initiates the formation of actin/myosin cables in the basolateral region of the neighboring cells; at the same time, cables also assemble within the dying cell itself, as a result of the apoptotic process. The cables may contract like a muscle sarcomere (inset). **(c)** Rho-mediated actin cable contraction within the neighboring cells drives apical extrusion of the apoptotic cell and simultaneously draws the neighboring cells inward to fill the gap that would have been left by the extruded cell. While contraction of the apoptotic cell itself is not sufficient to drive extrusion, it may also contribute to the force of extrusion.

until apoptotic cells are completely extruded from the monolayer. Moreover, we never see macrophages engulfing apoptotic cells within the embryonic chick or mouse epidermis. Similarly, acridine orange staining of *Drosophila* embryos shows that phagocytosis of apoptotic cells occurs only in intertissue spaces [18], suggesting that the dying cells are first extruded from the tissues. Because human amniotic fluid is primarily composed of epithelial cells and macrophages [23, 24], we expect that apoptotic epithelial cells are also phagocytosed after extrusion during human embryogenesis. There are some reports that phagocytosis occurs within an epithelium [2, 25, 26], but the apoptotic bodies in the phagocytes appear to be in late stages of apoptosis, and it is difficult to exclude the possibility that the cells were first extruded and then phagocytosed. It will be important to determine whether apoptotic cells are always extruded from simple epithelia before they are phagocytosed. Since programmed cell death in epithelia is extensive during development and a damaged barrier would be detrimental for tissue function, apoptotic cell extrusion may turn out to be an essential developmental process.

Based on our findings, we propose the following model for how apoptotic cells are extruded from an epithelium (Figure 9). A cell destined for apoptosis signals to its neighbors to form an actin and myosin ring at the live/dead cell interface. In addition, an actin ring also forms at this interface within the apoptotic cell itself, presumably as a result of caspase activation (Figure 9a). Rho-mediated contraction of the actin/myosin ring within the live neighbors squeezes the apoptotic cell out of the epithelium (Figure 9b) and simultaneously closes the gap that would have been left by the exiting apoptotic cell (Figure 9c). The actin and myosin filaments in the ring may contract by a sliding mechanism as they would in a sarcomere or cytokinetic ring (reviewed in [27]) (Figure 9b, inset). Although the contraction of the actin ring in the apoptotic cell may also contribute to the force of extrusion, it is not sufficient to drive extrusion.

What signals the formation of actin cables in the cells surrounding an apoptotic cell? From our studies adding labeled apoptotic cells to MDCK monolayers, we conclude that an early-expressed, nondiffusible signal from the apoptotic cell induces actin cable formation in the cells that contact the apoptotic cell (Figure 9a). This apoptotic cell addition experiment may not perfectly model how an extruding apoptotic cell signals to its neighbors within an epithelium during extrusion, although it should provide a useful system to begin to identify the extrusion signal. One difference, for example, is that the added apoptotic cells induce actin cables to form on the apical surface of the monolayer instead of along the basolateral side of neighboring cells as during normal extrusion. Could the cables induced by the added apoptotic cells simply reflect early stages of cell-cell adhesion or phagocytosis? We think not, because the addition of live or late-staged apoptotic cells did not induce cables. In addition, although the induced actin cables appeared to be quite dynamic in time-lapse movies, they never led to phagocytosis of the apoptotic cell; instead, the cables disassembled after the apoptotic cell floated away. Interestingly, actin cables could only be induced if the monolayer was confluent; they could not be induced in islands of MDCK cells (our unpublished data). Thus, a monolayer may need to be intact and polarized to respond to the extrusion signal.

Another reason for thinking that the apoptotic cell addition model may be a useful model for normal apoptotic cell extrusion is that the kinetics of actin cable formation are similar in the two processes. Added apoptotic cells induce actin cables as early as 60 min after UV-treatment, which is when the first actin rings are formed in UV-induced monolayers. Moreover, the nuclear morphology of an added apoptotic cell that is capable of inducing actin cables is the same as that of an apoptotic cell that is actively extruding from a monolayer. Late-stage apoptotic cells, which would correspond to postextruded apoptotic cells, rarely produce actin cables when added to a monolayer. For all of these reasons, we suspect that an early apoptotic cell signals to the live cells it directly contacts which, within an epithelium, would cause the accumulation of actin and myosin filaments along the basolateral surface surrounding the apoptotic cell.

How and where do these actin and myosin cables form (Figure 9b)? Because latrunculin A and Y-27632 mainly disrupt actin cables at the interface between the apoptotic cell and its neighbors but not the cortical actin filaments of the monolayer, we infer that actin filament turnover

may be more dynamic at this interface. Therefore, de novo actin polymerization may be an important first step for apoptotic actin ring formation, and myosin interaction with actin may stabilize the actin cable formed. In our studies, neighbors contract beneath apoptotic cells to extrude them apically. This could result from formation of an actin ring, or basket, on the basolateral surface and contraction of this actin both circumferentially and basally underneath the extruding apoptotic cell. Alternatively, the actin ring could form basally and contract circumferentially. High-resolution 4D confocal and electron microscopy, as well as further drug studies, will be needed to better elucidate the mechanism for extrusion. From inspection of apoptotic cells in cultured LLC-PK monolayers [22] and some *Drosophila* epithelia [20, 28], it appears that these cells are extruded basally. Basal extrusion may depend on contraction of apical cortical actin rings, as occurs when epithelial cells contract apically to cause an epithelium to invaginate and form tubes during development [29]. What signals epithelial cells to form and contract actin cables basally when extruding an apoptotic cell apically and to contract apically when extruding an apoptotic cell basally are interesting questions for future work.

Which cells are important for extrusion? A model proposed by Mills et al. suggested that the contraction of a blebbing apoptotic cell alone could act to drag epithelial cell neighbors into the space vacated by the dying cell [30]. From inhibition of actin/myosin contraction within apoptotic cell neighbors with C3 toxin, we find that contraction in these cells is essential for extrusion. The role of actin/myosin contraction in the apoptotic cell remains unclear. Although we have been unable to directly inhibit apoptotic cell contraction by C3 injection, two observations suggest that this contraction does not play an essential part in extrusion. Movies of GFP-actin show that the contraction in the apoptotic cell takes greater than four times as long as the contraction in the neighboring cells. Additionally, contraction in the apoptotic cell looks different for that in the neighboring cells: the actin condenses over the entire apoptotic cell surface and loops out in blebs. Furthermore, inhibition of caspases with z-VAD-fmk, which has been shown to block myosin activity in the apoptotic cell [6, 7], does not block extrusion. Thus, although we cannot rule out whether apoptotic cell contraction contributes to the force of extrusion, we expect that it may simply help compact the apoptotic cell as its neighbors squeeze it out.

The actin/myosin cables formed in the cells surrounding an apoptotic cell look similar to various types of "actin purse strings" ([31–33] and reviewed in [34]) that contract to help close wounds or other gaps in epithelia during embryonic development. It is possible that wound healing uses the same contraction pathway as apoptotic cell extrusion, as apoptotic or necrotic cells may also be required for the formation of the actin/myosin purse string in wounded epithelia monolayers (our unpublished data). In both cases, a signal produced by dying cells may induce a dynamic rearrangement of actin, myosin, and adherens and tight junctions to rapidly reseal the epithelium.

While previous work has focused on the importance of phagocytosis for clearing apoptotic cells in tissues, our findings emphasize the importance of extrusion for removing apoptotic cells from simple epithelia. By removing the dying cell and repairing the gap that might have formed from the dying cell's exit, the extrusion process maintains the barrier function of the epithelium. The process involves a novel mechanism in which the apoptotic cell signals its immediate neighbors to reorganize their actin cytoskeleton and actively extrude the dying cell. Future work will be needed to identify the signal and to elucidate exactly how actin and myosin mediate extrusion.

Materials and methods

Embryo staining

E8 chick or E13 mouse forelimbs were rinsed in PBS and fixed for 2 hr in 4% formaldehyde in PBS. After rinsing in PBS, the limbs were permeablized in 0.5% Triton X-100 in PBS for 30 min, blocked in AbDil (2% bovine serum albumin in PBS) for 30 min, and stained with 1 μg/ml Hoechst 33342 (Sigma) and either 0.1 μg/ml DiI (Molecular Probes) or 0.25 μg/ml TRITC-phalloidin and for 1 hr, followed by 3× 30 min washes with PBS + 0.1% Triton X-100 (PBST).

Cell culture, injections, and staining

To induce apoptosis, MDCK monolayers were treated with short-wave UV light for 3 min, using a Stratalinker (Stratagene). A plasmid encoding both actin and enhanced green fluorescent protein (EGFP) (gift from Gerard Marriott) was used to transfect MDCK cells, using a Lipofectamine Plus kit (Gibco-BRL). Individual UV-treated MDCK cells were injected with either 0.2 mg/ml C3 toxin or 1 mg/ml BSA, plus 5 mg/ml 50 kDa lysine-fixable Texas red dextran to mark the injected cells. In some experiments, MDCK cells were treated with 5 μM Latrunculin A (a gift from Miranda Sanders, University of California at Santa Cruz, California), 1 μM Cytochalasin D (Sigma), 200 μM Y-27632 (a gift from Yoshitomi Pharmaceutical Industries), 100 μM ML-9, 50 μM zVAD-fmk (Oncogene), 2 mM 4-AP (Sigma), or 0.4% DMSO (control) just after UV treatment (unless otherwise indicated). Drug doses were first determined by titration and were dependent on the source and age of the drug.

To stain for actin and DNA, cells were rinsed with PBS, fixed with 4% formaldehyde in PBS for 20 min, permeablized for 5 min with 0.5% Triton in PBS, blocked with AbDil for 5 min, and then stained with 0.25 μg/ml TRITC-phalloidin (Molecular Probes) and 1 μg/ml Hoescht 33342 in AbDil for 20 min followed by three washes with PBST. For immunofluorescence, cells were fixed as above except for a short permeablization step prior to fixation using 0.1% Triton X-100 in PBS containing 1 μg/ml unlabeled phalloidin (Sigma) for 60 s, followed by incubation with β-catenin, occludin, ZO-1 (all Zymed), or nonmuscle-myosin II (Biogenesis) antibodies. Monolayers of MDCK cells were incubated for 10 min in complete media prior to fixation with 1 μM Sytox Green (Molecular Probes) to stain cells that had lost plasma membrane integrity.

Microscopy

Fluorescence micrographs were obtained using a Nikon eclipse e800 microscope and captured using a SynSys cooled charge-coupled device

(CCD) camera (Roper Scientific). Time-lapse movies were made using an Axiovert microscope (Zeiss) and captured using a Micromax CCD camera (Roper Scientific). Metamorph software (Universal Imaging) was used to control the camera and to process images. Confocal micrographs were obtained using a TCS-SP microscope (Leica), and Z series were reconstructed into a 3D image using Bit Plane software (Imaris). All images were further processed using Photoshop (Adobe) and Powerpoint (Microsoft) software.

Scanning Electron Microscopy

Fixed UV-treated MDCK monolayers were rinsed in 0.1 M Na cacodylate, postfixed in 1% osmium tetroxide in 0.1 M Na cacodylate and rinsed further in 0.1 M Na Cacodylate. After dehydration in a graded ethanol series, specimens were critical point dried and sputter coated with gold before viewing on a Jeol 5410 scanning electron microscope.

Measurements of electrical resistance

MDCK cells were grown to confluence on Transwell filters (3413, Costar), and resistance measurements were made using a Millicell-ERS meter (Millipore). Prior to EGTA treatment, transepithelial resistance was typically 900 Ohms/cm^2. Measurements were taken just after UV treatment and every 60 min thereafter. In some experiments, we added EGTA to 2 mM in complete DMEM 60 min after the first reading.

Addition of apoptotic cells to monolayers

To make early apoptotic cells, MDCK cells were trypsinized, resuspended in 1 ml of DMEM, plated in a 35 mm dish, and treated with short-wave UV for 3 min. Unirradiated cells were used as controls. For old apoptotic cells, supernatants containing apoptotic cells from overgrown MDCK monolayers were used. Cells were transferred to a microfuge tube and centrifuged for 1 min at 3000 rpm in a microfuge (Eppendorf). Cells were fluorescently labeled by resuspending them gently in 50 μl DMEM containing 40 μg/ml Alexa 488-snail Lectin (Molecular Probes) for 15 min and washing three times with 1 ml of DMEM. The labeled cells were then added to confluent MDCK monolayers cultured on glass coverslips and incubated for 90 min, rinsed with PBS, and fixed and stained with rhodamine-phalloidin and Hoechst dye. For cells added to a GFP-actin monolayer, UV-treated cells were added as above, except that they were not labeled.

Supplementary material

Supplementary material including phase and fluorescence movies of apoptotic cell extrusion as well as two figures showing myosin disruption by Y-27632 treatment and movie stills from Figure 7 is available at http://images.cellpress.com/supmat/supmatin.htm.

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