Balance of life and death in alveolar epithelial type II cells: proliferation, apoptosis, and the effects of cyclic stretch on wound healing

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Crosby LM, Luellen C, Zhang Z, Tague LL, Sinclair SE, Waters CM. Balance of life and death in alveolar epithelial type II cells: proliferation, apoptosis, and the effects of cyclic stretch on wound healing. Am J Physiol Lung Cell Mol Physiol 301: L536–L546, 2011. First published July 1, 2011; doi:10.1152/ajplung.00371.2010.—After acute lung injury, repair of the alveolar epithelium occurs on a substrate undergoing cyclic mechanical deformation. While previous studies showed that mechanical stretch increased alveolar epithelial cell necrosis and apoptosis, the impact of cell death during repair was not determined. We examined epithelial repair during cyclic stretch (CS) in a scratch-wound model of primary rat alveolar type II (ATII) cells and found that CS altered the balance between proliferation and cell death. We measured cell migration, size, and density; intercellular gap formation; cell number, proliferation, and apoptosis; cytoskeletal organization; and focal adhesions in response to scratch wounding followed by CS for up to 24 h. Under static conditions, wounds were closed by 24 h, but repair was inhibited by CS. Wounding stimulated cell motility and proliferation, actin and vinculin redistribution, and focal adhesion formation at the wound edge, while CS impeded cell spreading, initiated apoptosis, stimulated cytoskeletal reorganization, and attenuated focal adhesion formation. CS also caused significant intercellular gap formation compared with static cells. Our results suggest that CS alters several mechanisms of epithelial repair and that an imbalance occurs between cell death and proliferation that must be overcome to restore the epithelial barrier.

pulmonary alveolar cells; type II pneumocytes; mechanical stretch; mechanical ventilation; ventilator-induced lung injury; wound healing; focal adhesion; morphometry

MECHANICAL VENTILATION in the presence of acute lung injury can cause additional injury (5, 17, 57, 63), highlighting the importance of studies on cell responses to lung distension and wounding. Because of their central role in repair following injury, it is important to understand how primary rat alveolar type II (ATII) cells respond to mechanical stretch. The current study was undertaken to characterize and quantify how cyclic stretch (CS) alters the balance between proliferation and apoptosis during repair following injury to primary ATII cells.

ATII cells secrete surfactant (43, 44), serve as progenitor cells for alveolar type I (ATI) cells, replenish ATII and ATI cell populations after lung injury (38, 60), and play a role in immune responses, e.g., migration of polymorphonuclear leukocytes (7), respond to signals of neighboring cells or circulating immune cells (65), and migrate and spread in response to injury (61). Previous studies have indicated that ATII cells begin to proliferate 24 h after injury (3, 36, 45, 47, 66) and are stimulated to undergo apoptosis in response to CS (17, 18, 28, 29, 34, 51). It is believed that stem cell niches are located throughout the lung, where pluripotent, but quiescent, cells reside (26, 33, 48, 49). ATII cells may exist as stem cell populations that respond to injurious stimuli, similar to Clara cells of the airway (25, 48), by expanding in a hierarchical manner, producing daughter cells whose progeny are responsible for replenishing the resident ATI and ATII cell populations (39, 40, 64), although ATII cells have recently been shown to be capable of division and repopulation as well (27).

Few studies have examined how CS regulates epithelial repair following injury. Assessing cell injury by a live-dead assay, Tschumperlin et al. (58, 59) studied the effects of deformation frequency, duration, and amplitude on ATI cell viability. Members of this group then studied the effects of stretch on actin architecture, intracellular ATP levels, and tight junction proteins (6) in ATII cells cultured for 5 days, when they were described as phenotypically type I-like. Several recently published studies with alveolar cell spreading and migration as an end point (2, 21, 23) demonstrate that keratinocyte growth factor facilitates wound repair and promotes cell motility without stimulating proliferation and show that H2O2 induces apoptosis, thus inhibiting repair. We previously studied lung epithelial cell migration on laminin, elastin, and collagen matrices and the involvement of signaling of the focal adhesion proteins focal adhesion kinase (FAK), Rac, and Tiam1 in scratch-wounded cells (8, 10–12, 14, 15, 62). The present study investigated the properties of wound cells undergoing CS to understand how cells spread, migrate, reorganize the cytoskeleton, proliferate, and undergo apoptosis and repair. This will contribute to our understanding of how cell and tissue injury occurs with the overdistension that may occur during mechanical ventilation and how repair is disrupted by it.

MATERIALS AND METHODS

Cell isolation, culture, and morphology. Primary ATII cell isolation, including reagents and their sources and the use of the Flexercell strain unit, are described in detail elsewhere (8), except FBS was obtained from Biowest (Miami, FL) and was not heat-inactivated. Proliferation was quantified using the Quick Cell Proliferation Assay Kit (Biovision) and a Click-iT ethidium deoxyuridine (EdU) Alexa Fluor high-throughput imaging [high content screening (HCS)] assay (Invitrogen). Apoptosis was measured using an ApopTag in situ oligoligation (ISOL) dual-fluorescence apoptosis detection kit (Chemicon/
Millipore). Actin, vinculin, and focal adhesion staining was performed using the FAK100 actin cytoskeleton/focal adhesion staining kit (Millipore).

ATII cells were isolated from Sprague-Dawley rats according to the methods of Dobbs et al. (16) and plated and cultured as previously described (8). The animal use protocol was approved by the Institutional Animal Care and Use Committee of the University of Tennessee Health Science Center. Earlier experiments determined that primary ATII cells require an RFL6 (rat fetal lung fibroblast, American Type Culture Collection) cell-derived matrix to maintain attachment. The method of Dobbs et al. (16) was followed (see below). Membranes were applied, and images were obtained using a confocal microscope (model LSM5, Zeiss). Images from ×25 and ×40 fields were collected and compared for differences in location and occurrence of green vinculin and red F-actin fluorescent proteins and yellow focal adhesions, where F-actin and vinculin were colocalized, with each treatment. ImageJ was used to analyze images.

Cell proliferation and apoptosis. To assess proliferation, we used the Click-IT EdU Alexa Fluor high-throughput imaging (HCS) assay at 0, 2, 12, and 24 h, according to the manufacturers’ suggested protocol. This assay incorporates EdU to determine the number of cells in the S phase of replication. Ten fields per slide were recorded using a Zeiss LSM5 confocal microscope (×25 objective), and the number of positive-staining cells was counted. For wounded conditions, images were collected near and far from the wound edge, defined as <30 cells (near) and >30 cells (far) from the wound edge. Cell proliferation was reported as the proliferative index (PI, %, where PI = number of S phase-staining cells/total number of cells × 100).

We confirmed the identity of ATII cells in the S phase by immunofluorescence staining using an ATII cell-specific antibody, anti-rat RTII70 (kind gift of Dr. L. Dobbs, University of California, San Francisco), in cells stained using Click-IT EdU. To stimulate apoptosis, we treated cells with sulindac sulfone (7 μM) for 16 h prior to wounding and maintained apoptosis in the medium following wounding. For stimulation of apoptosis, the ApopTag kit was used to assess apoptosis (see below).

Apoptosis was measured using established morphological criteria, including cell shrinkage with loss of intact plasmalemma and cellular processes, detachment from the surrounding cells, loss of cytoplasm, and chromatin condensation, nuclear margination (forming crescents or halos), and/or the presence of apoptotic bodies (35), and by an ApopTag ISOL dual-fluorescence apoptosis detection kit. Ten fields per slide for unwounded cells and 20 total fields for wounded cells [10 near (within 10 cells of the wound edge) and 10 far (11–20 cells from the wound edge)] were analyzed using a ×40 objective + ×2 zoom. Microscope settings were kept constant for all readings, and the gain difference between the red and green channels (488 and 543 nm, respectively) was kept to ≤200 units. Confocal images, including positive and negative controls, were captured using a Zeiss LSM5 microscope. Results are reported as the apoptotic index (AI, %, where AI = number of apoptotic cells/total number of cells × 100).

Statistical methods. Student’s t-tests, one-, two-, and three-way ANOVA with comparisons against control (St/U), or pair-wise comparisons (Holm-Sidak method) were performed using SigmaStat version 3.5 software. P < 0.05 was considered significant.

RESULTS

CS slowed wound repair and altered the morphology of wounded monolayers. To examine how CS affected the morphology of ATII cells during wound repair, confluent cultures were exposed to CS following wounding, and phase-contrast images were collected from 0 to 24 h. Figure 1, A–N, shows the morphology of ATII cells for each treatment. St/U cells were confluent and displayed a typical cobblestone appearance at all times (Fig. 1, A–D), but CS/U cells developed gaps between cells that increased markedly from 2 to 24 h (Fig. 1, I–K). These gaps can be seen more prominently in fluorescence micrographs of F-actin stained with TRITC-phalloidin (Fig. 2 compared with Fig. 3). After wounding, cells at the wound edge of static monolayers formed a more orderly front at the wound edge (Fig. 1, E–G), while cells at the wound edge of CS...
monolayers showed less organized wound fronts at the leading edge (Fig. 1, L–N). While wounds were closed by 24 h under static conditions (Fig. 1, H and O, solid line), CS significantly impaired wound closure (Fig. 1, N and O, dashed line). Cells at the wound edge migrated at a rate of \( \frac{110}{14} \) m/h, as calculated from width measurements over time under static conditions, and the initial \( \frac{500}{15} \) µm gap was nearly closed by 24 h (Fig. 1O). CS severely inhibited migration, with wounds closed by only 5–10% of the initial width at 24 h. Small, rounded, highly light-refractive cells growing in suspension or loosely tethered to the substrate could be seen but did not appear to contribute to wound closure.

**Wounding and CS altered cytoskeletal and focal adhesion structures.** To compare structural changes in cells following wounding and in response to CS, we examined the distribution of F-actin and vinculin. In St/U cells, typical patterns of cortical actin, stress fibers, and punctate vinculin distribution were observed and did not markedly change over 24 h (Fig. 2A; see low-magnification images of all conditions in Supplemental Fig. S1 in Supplemental Material for this article, available online at the Journal website). Immediately after wounding, the appearance of actin and vinculin was similar to that in unwounded cells (Fig. 2B), but by 2 h, vinculin staining at the wound edge was prominent and F-actin appeared to reorganize with increased stress fibers (Fig. 2C). At 12 h, a thickened band of actin could be clearly seen parallel to the leading edge of the wound (Fig. 2D). Prominent colocalization of actin and vinculin, indicated by yellow “matchstick”-like or pinpoint foci, was seen at the wound edge at 12 h (Fig. 2D). By 24 h, most wounds had completely closed and exhibited staining patterns similar to unwounded cells, with perhaps less actin organization in the cells where the wounds closed (Fig. 2E). In CS/U cells, substantial gaps were seen after 2 h (Fig. 3A), and cortical actin appeared to be disrupted compared with static cells. Fewer stress fibers were observed at 2 and 12 h (Fig. 3, A and B) but appeared to increase after 24 h of stretch (not shown). In CS/W cells, large gaps appeared near the wound edge at 2, 12, and 24 h (Fig. 3, C–E; also see Supplemental Fig. S1). In gaps that formed, strong F-actin staining was observed along the periphery of the cells (Fig. 3C). Although stress fibers were observed at 2 h, few were observed at 12 h. At these later times, vinculin staining and colocalized actin and vinculin staining were less prominent in CS cells at the wound edge than in static cells.

**CS inhibited spreading in wounded cells.** To directly compare cell spreading in monolayers following wounding, we
measured cell area for individual cells within 650 μm of the wound edge (near) and compared them with unwounded cells and cells between 650 and 1,300 μm from the wound edge (far). Figure 4 shows that cell area did not increase in unwounded cells, whether under static or CS condition. However, in static cells near the wound edge, cell area significantly increased at 12 and 24 h. Cell area also increased significantly between 650 and 1,300 μm from the wound edge, but not to the same extent as in cells closest to the wound edge. Although some increase in area was observed in cells exposed to CS, these increases were significantly less than those in static cells.

**CS caused gap formation in wounded cells.** As shown in Fig. 3, CS caused the formation of intercellular gaps. To quantify this, we measured the percent area covered by gaps. Figure 5 shows that very few gaps were present in St/U or St/W monolayers. When unwounded cells were exposed to CS, there was an initial significant increase in gap area at 2 h, but by 12 and 24 h the gaps were smaller and appeared to be repaired (see Supplemental Fig. S1). At 24 h, there was no significant difference in the percent area covered by gaps between CS/U and control (St/U) cells. In wounded monolayers, however, CS caused a significant increase in gap area by 2 h that transiently

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**Fig. 2.** Wounding caused changes in distribution of F-actin (red) and vinculin (green). Focal adhesions are indicated by overlap (yellow). A: St/U at 0 h. B–E: St/W at 0 h (B), 2 h (C), 12 h (D), and 24 h (E).
decreased at 12 h but significantly increased at 24 h (compare Fig. 3, C–E).

CS decreased proliferation and increased apoptosis. To determine the effect of CS on cell proliferation following wounding, we quantified the number of cells incorporating EdU. Figure 6A shows EdU incorporation in cells close to the wound edge at 24 h, while cells far from the wound edge showed little EdU incorporation (Fig. 6B). Figure 6, A and B, also demonstrates positive staining (anti-RT1I70) for ATII cells. To quantify these results, we counted the number of EdU-positive cells relative to the total number of cells in each field. Figure 6C shows significantly increased EdU incorporation in St/U and St/W cells near the wound edge after 12 and 24 h. By 24 h, EdU incorporation was similar in St/U cells, static cells more than one field distant from the wound (not shown), and CS/U cells. Wounding stimulated a significant increase in EdU incorporation within 30 cells of the wound edge in static and CS cells. By 24 h, EdU incorporation was significantly lower in cells exposed to CS than in static cells. When we measured incorporation in wounded monolayers greater than one field away from the wound edge, there was no difference compared with unwounded cells. For comparison, we manually counted the total number of viable cells for each condition following trypsinization. As shown in Fig. 6D, the
It is well established that, during the initial stages of wound repair, epithelial cells near the wound edge spread and migrate to cover the exposed area (32). We previously examined signaling pathways (FAK, phosphatidylinositol 3-kinase, JNK1, RhoA, Rac1, and Tiam1) involved in wound closure in a scratch-wound model of injury in primary rat alveolar (11, 12) or airway epithelial (10, 13, 15, 52, 54) cells, and we consistently measured decreased migration in cells exposed to CS. In the present studies, using multiple scratch-wounding, we again observed that CS inhibited cell migration by ~90% (Fig. 1). As we previously observed with 16HBE14o− cells (52), ATII cells near the wound edge increased in size and spread into the wound area in static cells (Fig. 4), and CS prevented the increase in cell size. We have not previously examined the role of CS in proliferation or apoptosis in wound closure of primary ATII cells. In one of our studies (11), we did not measure cell proliferation, because others had reported that ATII cell proliferation was minimal within the first 3 days of culture (50). In previous studies with 16HBE cells, we measured wound closure prior to substantial cell proliferation or we compared cell counts from monolayers with a single scratch wound and found no difference (10, 53). In one study, we treated 16HBE cells with mitomycin C to inhibit proliferation, and we observed small differences in wound closure after ~15 h (52). However, when we treated ATII cells with mitomycin C in the present study (data not shown), there was substantial toxicity, and we could not distinguish the effect of inhibition of proliferation from toxicity. Instead, using EdU, we measured the proliferation index, which enabled us to distinguish changes that were occurring primarily at the wound edge. Such localized changes may not be detectable by measurements of total cell numbers or by biochemical assessments. We used a similar approach to examine apoptosis near the wound edge, which has not previously been investigated. The current study confirms that CS not only impaired ATII cell migration and spreading, but it also altered the balance between proliferation and cell death.

We observed that, in wounded monolayers exposed to CS, substantial gaps appeared between cells and increased in area over time (Fig. 3; see Supplemental Fig. S1). These results support previous observations that CS impairs tight junction formation and cell-cell attachment (6). Others observed paracellular Rho kinase-mediated gap formation after CS in endothelial cells (4). We found that gaps formed in unwounded cells exposed to CS appeared to repair between 12 and 24 h (Fig. 3).
and Fig. 5), while gaps in multiply wounded monolayers continued to increase in size over time. We speculate that the presence of multiple wounds weakened the mechanical integrity of the monolayer and prevented remodeling and repair. This also suggests the importance of paracellular communication during repair that was disrupted by multiple scratch wounds.

Actin filament reorganization is a prerequisite to cell migration, vesicle and organelle transport, and cytokinesis and is induced after microtubule disruption with wounding. In the present study, wounding caused cells to spread at the margins of the wound, migrate into the margins, and redistribute actin filaments within the cell, changing their location and thickness. These changes were significantly altered by CS. Rho signaling plays a key role in cell adhesion, motility, and contraction, which are mediated by actin cytoskeletal reorganization such as we observed. Others (9) showed that microtubule depolymerization activates Rho signaling during aortic smooth mus-
cle contraction and gap formation in endothelial cells (see above), in accordance with previous reports of RhoA activation during migration (10), but a later study showed reduced proliferation in adult rat ATII cells in response to sustained stretch. We believe that these processes are involved in the observed response to wounding or CS, as shown by others (4, 10, 11, 14, 15, 22).

Our results demonstrate that wounding stimulated cell proliferation near the wound edge and that CS partially inhibited this response (Fig. 6). Proliferation also occurred in unwounded cells, and CS inhibited EdU incorporation. Early studies with fetal ATII cells suggested that CS stimulated proliferation (37, 56). We previously found increased bromodeoxyuridine labeling in cultured, immortalized human airway epithelial cells (16HBE14o−/H11002) that were cyclically stretched (52). We also previously examined wound closure (52) in vitro in 16HBE14o− cells by applying a single scratch wound and 20% CS for up to 48 h, and we measured wound closure and cell spreading, density, and proliferation. In our previous study (52), cell density was lower near the wound edge, and CS inhibited wound closure. With use of lactate dehydrogenase release and Trypan blue uptake as indicators, negligible cell death was measured over 48 h. This suggests a difference in the response of an immortalized cell line vs. primary cells or airway vs. alveolar cells. It is clear that primary ATII cells are much more sensitive to CS and could be expected to have a higher rate of cell death under similar conditions. In a study of human A549 adenocarcinoma cells, McAdams et al. (41) found that 18% CS at the rate of 30 cycles/min significantly increased proliferation over 72 h, without altering cell viability over time. In our studies, we have been unable to stretch primary ATII cells with ≥20% CS without observing significant cell detachment from the Silastic membrane. Growing cells on a cell matrix deposited by fetal rat lung fibroblasts improved the maintenance of cell attachment with ≤20% CS. We do not know how closely the matrix composition deposited by the fibroblasts matches that of native lung tissue, but lung fibroblasts do contribute to the matrix in vivo. Although the composition of the matrix is likely to affect proliferation and apoptosis, a comparison of the effects of individual matrix materials would be difficult because of the poor adherence of cells during CS.

We found that wounding resulted in an immediate increase in apoptotic cells (Figs. 7 and 8), which was likely a response to the wounding process. However, by 24 h, the apoptotic index in unstretched, wounded cells had decreased to the level of unwounded cells. Using 22% CS at the rate of 3 cycles/min, Edwards et al. (17, 18) showed that CS induced apoptosis in primary rat ATII cells after 1 h. Later, using conditions different from those used in the present study, Hammerschmidt et al. (29) measured increased apoptosis in primary rat ATII cells after CS. They examined the response of cells after initial

![Fig. 7. CS stimulated changes in morphology indicative of apoptosis. A: St/U at 0 h. B: St/U at 12 h. C: St/W ≥10 cells from the wound edge (white line indicated by arrow shows wound edge) at 12 h. D: St/W >10 cells from wound edge at 12 h. E: CS/U at 12 h. F: CS/W ≥10 cells from the wound edge (wound edge not shown) at 12 h. Thin arrows, apoptotic cells; thick arrow, apoptotic body. Images were obtained using confocal microscopy, CR590 and FAM fluorescence staining, ethidium bromide nuclear counterstaining, and 2-channel excitation at 488 and 543 nm, with ×40 objective + ×2 zoom.](image-url)
culture for only 24 h (compared with our condition of 48 h of culture) and stretched cells at frequencies of 40 and 60 cycles/min (compared with 10 cycles/min in the present study). They also used area strains of 30% and 40%, comparable to ~14% and 18% linear strain (compared with our use of 15% biaxial strain). The extent of deformation of alveoli in vivo has not been definitively determined, but some estimates suggest that normal tidal volume breathing causes ~4% linear distension (19, 20). We examined higher levels of stretch, because the amount of stretch in an injured lung will vary throughout injured and uninjured regions. Some air-filled regions may be overdistended because of differences in compliance compared with fluid-filled regions, and this can lead to increased levels of stretch (~15 to 25% in injured lungs). Perlman and Bhattacharyya (46) measured a linear strain of ~14% in alveolar epithelial cells in an uninjured isolated lung inflated from 5 to 20 cmH2O (80% total lung capacity), but they also noted substantial heterogeneity. It is important to recognize that the study of Hammerschmidt et al. did not include wounding. Since conditions and regimens differed, direct comparison is difficult, but they reported a background (nonstretched) level of apoptosis of ~10%, which is similar to our results. Apoptosis increased with increasing frequency or change in surface area, as did necrosis. In the present studies, CS caused increased apoptotic indexes through 24 h in all CS groups. Also, as mentioned previously, the apoptotic index was defined as the ratio of positive cells to total cells in the field, so either a decrease in positive cell numbers or an increase in total cells would cause the apoptotic index to decrease. In fact, an increase in cells near the wound edge of static cells was observed at 24 h [see Fig. 6, St/W (0–30 cells) at 24 h]. We interpret this to mean that the number of apoptotic cells was probably at least as great at 24 h as at 12 h, but increased proliferation at 24 h in St/W may have decreased the apparent apoptotic index.

After wounding, unstretched cells migrated into the wound, spread, and proliferated, and there was an initial burst of apoptosis. Actin was redistributed to the wound edge, and actin fibers reorganized. When CS was applied, cells did not spread normally but remained smaller and more densely packed. Proliferation decreased in these cells compared with unstretched cells. Large intercellular gaps appeared in stretched monolayers, and these resealed in unwounded cells but continued to enlarge in wounded cells. CS also stimulated apoptosis at the wound edge that increased with time. Stimulation of apoptosis impaired wound closure in a manner similar to CS. CS interfered with wound repair mechanisms by decreasing proliferation and by stimulating apoptosis. CS altered the balance between proliferation and cell death. We conclude that wound repair thus depends on a delicate and sensitive balance between these two opposing processes, and ultimately restoration of epithelial homeostasis depends on a combination of both.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.


