5-Lipoxygenase and cyclooxygenase regulate wound closure in NIH/3T3 fibroblast monolayers

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Wound healing involves multiple cell signaling pathways, including those regulating cell-extracellular matrix adhesion. Previous work demonstrated that arachidonate oxidation to leukotriene B4 (LTB4) by 5-lipoxygenase (5-LOX) signals fibroblast spreading on fibronectin, whereas cyclooxygenase-2 (COX-2)-catalyzed prostaglandin E2 (PGE2) formation facilitates subsequent cell migration. We investigated arachidonate metabolite signaling in wound closure of perturbed NIH/3T3 fibroblast monolayers. We found that during initial stages of wound closure (0–120 min), all wound margin cells spread into the wound gap perpendicularly to the wound long axis. At regular intervals, between 120 and 300 min, some cells elongated to project across the wound and meet cells from the opposite margin, forming distinct cell bridges spanning the wound that act as foci for later wound-directed cell migration and resulting closure. 5-LOX inhibition by AA861 demonstrated a required LTB4 signal for initial marginal cell spreading and bridge formation, both of which must precede wound-directed cell migration. 5-LOX inhibition effects were reversible by exogenous LTB4. Conversely, COX inhibition by indomethacin reduced directed migration into the wound but enhanced early cell spreading and bridge formation. Exogenous PGE2 reversed this effect and increased cell migration into the wound. The differential effects of arachidonate acid metabolites produced by LOX and COX were further confirmed with NIH/3T3 fibroblast cell lines constitutively over- and underexpressing the 5-LOX and COX-2 enzymes. These data suggest that two competing oxidative enzymes in arachidonate metabolism, LOX and COX, differentially regulate sequential aspects of fibroblast wound closure in vitro. involved during the different stages of wound resolution remain unclear. Leukotriene B4 (LTB4), which is produced by the oxidation of arachidonic acid (AA) by 5-lipoxygenase (5-LOX), is known to be an active chemottractant for fibroblasts and monocytes and may regulate fibroblast activities in wound healing (14, 25). Furthermore, inhibitor studies indicate that LOX metabolites regulate reepithelialization in wounded corneas (11). Another oxidative enzyme of AA, cyclooxygenase (COX-1/2), also may be involved in wound healing because it appears to be associated with directed cell motility and new tissue growth in corneal endothelium, airway epithelium, and skin (15, 16, 27). How these two eicosanoids coordinate regulated wound closure, however, remains to be elucidated.

Wound healing in a fibroblast monolayer, in vitro, is similar to fibroblast cell adhesion to an ECM in that discrete transitional stages are observed. In the case of cell-ECM adhesion, sequential spreading and migration are initiated by the cell attachment stage (32), whereas in wound closure, the spreading and migration stages are initiated by wounding of the cell monolayer. Each stage of cell adhesion involves changes in overall morphology and cytoskeletal structure that are regulated inter alia by the oxidative enzymes of the AA cascade: LOX and COX. LOX and COX compete for AA released from membranes by phospholipase A2 (PLA2) to convert it to either leukotriene (LT) or prostaglandin (PG), respectively (1, 6, 33). More specifically, the conversion of AA to LTB4 via 5-LOX initiates a cascade of two messengers leading to actin polymerization and thereby activating cell spreading (4) (Stockton RA, Katsumi A, Dixon DA, Green JA, Roberts LA, and Jacobson BS, unpublished observations). Subsequent migration of spread cells requires redistribution and peripheral bundling of F-actin mediated by phospholipidas A2 (PLA2) to convert it to either leukotriene (LT) or prostaglandin (PG), respectively (1, 6, 33). More specifically, the conversion of AA to LTB4 via 5-LOX initiates a cascade of second messengers leading to actin polymerization and thereby activating cell spreading (4).

IN THE PROCESS OF CUTANEOUS WOUND HEALING, keratinocytes, endothelial cells, fibroblasts, and other cells migrate to the wound from adjacent tissues in response to cytokines and other soluble mediators. Fibroblasts play a major role as primary sources of newly synthesized extracellular matrix (ECM) and connective tissue needed for wound contracture after injury. Fibroblast migration into wound gaps is an initial response to injury and generally precedes reepithelialization and endothelial angiogenesis (see reviews, Refs. 26, 34). These cells proliferate into the wounded area until damaged tissue is replaced by new tissue. Although much is known about the overall process of wound healing, the biochemical signals

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wound healing process as indicated by changes in wound margin cells and overall wound closure revealed phenotypically discernible events. The overall rate of closure of the wound appeared to be biphasic, as determined by measuring both the surface area of cells at wound margins and the area occupied by the entire wound. The use of specific pharmacological inhibitors indicated that LTB4 regulates the initial phase of wound closure by enhancing wound margin cell spreading, whereas prostaglandins regulate subsequent directed intra-wound cell migration. In addition, a novel early postwounding organization of bridge cells acting as foci for later directed migration was noted, occurring at regular intervals perpendicularly to the wound axis, and was also examined with respect to arachidonate regulation. Wound closure was similarly studied with NIH/3T3 fibroblasts constitutively over- or under-expressing the 5-LOX or COX-2 enzymes to verify the results obtained by pharmacological knockout and rescue. The evidence presented supports the possibility that sequential activities of both 5-LOX and COX are essential for wound closure in fibroblasts.

MATERIALS AND METHODS

Cell culture and reagents. NIH/3T3 cells were obtained from American Type Culture Collection (Rockville, MD). Cells were maintained as subconfluent monolayers in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (wt/vol) calf serum (Atlanta Biologicals, Atlanta, GA), 100 μg/ml of dihydrostreptomycin, and 60 U/ml penicillin (Sigma, St. Louis, MO) in a humidified 37°C incubator with 5% CO2. For most assays, cells were serum starved in DMEM with 2% calf serum and 0.5% fatty acid-free bovine serum albumin (BSA) overnight. Tissue culture plates and flasks were purchased from VWR (West Chester, PA). AA861, a specific 5-LOX inhibitor, and indomethacin, a COX inhibitor, were obtained from Biomol (Plymouth Meeting, PA). LTB4 was obtained from Cayman (Ann Arbor, MI), and prostaglandin E2 (PGE2) was obtained from Calbiochem (La Jolla, CA).

5-LOX and COX-2 overexpression and antisense constructs and transfections. NIH/3T3 cell lines either stably overexpressing or having antisense suppression of 5-LOX or COX-2 synthesis were previously described and characterized (Stockton RA, Dixon DA, Green JA, Roberts LA, and Jacobson BS, unpublished observations). In brief, a 2.2-kb fragment of human 5-LOX cDNA inserted in the sense orientation of pEGFP-C1 to promote synthesis of a 5-LOX-green fluorescent protein (GFP) fusion protein (provided by Dr. Colin Funk and described initially in Ref. 3), a 5-LOX antisense construct consisting of a 2.0-kb fragment of 5-LOX cDNA (courtesy of Dr. Jilly Evans) ligated in reverse orientation into pEGFP-C1, and human COX-2 cDNA with deleted 3′ untranslated region (7) cloned into the EcoRI site of pEGFP-C1 in frame in both sense and antisense orientations were transfected into wild-type NIH/3T3 cells with the use of Lipofectamine (GIBCO BRL, Grand Island, NY) according to the manufacturer’s instructions and then selected for genetic resistance. Resulting single-cell clones were isolated and tested for leukotriene or prostaglandin production, generating stable cell lines maintained with 1.0 mg/ml G418. “3T3-SLOX” (5-LOX overexpressing) cells produce fourfold more LTB4 than control cells do, whereas “3T3-XOLS” (5-LOX antisense) cells produce 70% less, “3T3-COX2” (COX-2 overexpressing) cells produce 10-fold more PGE2 than control cells do, whereas “3T3-2XOC” (COX-2 antisense) cells produce 50% less (Stockton RA, et al., unpublished manuscript).

Wound closure assay. Cells were detached from culture vessels with 0.01% trypsin-EDTA, washed with Hank’s balanced salt solution, and resuspended in fresh DMEM with 2% calf serum. Cells were plated on 35-mm polystyrene dishes at 6 × 10^5 cells/plate in DMEM with 2% calf serum overnight to permit formation of monolayers just reaching early confluence by 16–24 h. Cells were washed and incubated for 2 h in DMEM with carrier solvent (control) or in AA861, indomethacin, LTB4, or PGE2 at concentrations indicated in figure legends. After incubation, medium was aspirated and replaced with fresh medium with or without additions, and then prewounding medium aliquots were removed for comparison with postwounding samples at later times. The monolayer was then immediately wounded by being scraped with the corner of a piece of Mylar film (used in copy machines) to produce a wound 300 μm (3–4 cells wide) across the entire length of the 35-mm plate monolayer. Cells were visualized by phase-contrast microscopy (Nikon Diaphot-TMD or Nikon Eclipse TE-300 inverted microscopes) and photographed at times indicated in figure legends with the use of a CCD100 video camera and Scion LG3-01 frame grabber or a Nikon N6000 camera and Kodak TMAX-100 professional film. Images were processed using Scion Image or Adobe Photoshop software.

To measure marginal cell surface area, cell bridges, and gap surface area, we photographed the wound at multiple random sites for each time point. To determine marginal cell surface area, we analyzed 50 cells per time point for each treatment. At each time point, NIH Image J software was used to quantify the cell surface area of those cells exposed to the wound gap. The mean area of treated cells for each time point and each treatment was normalized to the surface area of control cells at time 0 and expressed as a percentage of control cell surface area. Data from all experiments were pooled and subjected to ANOVA.

To evaluate cell bridges, we counted the number of bridges that completely spanned the wound in the entire photographic field (1 mm in length) for each treatment at 2 h postwounding. The result for each treatment was normalized to that for control untreated cells. Data from multiple experiments were then pooled and subjected to ANOVA.

To quantify gap surface area, we measured the gap at each time point for each treatment using NIH Image J analysis software. The results for each time point per treatment were normalized as percentages of control cell surface area at time 0. Data from all experiments were then pooled and subjected to ANOVA. All of the above experiments were graphed, with data points shown as means ± SE.

LTB4 and PGE2 enzyme immunoassays. Cells were seeded as described in Wound closure assay in 35-mm polystyrene plates and grown to early confluence. Cells on triplicate plates for each treatment were wounded after a 2-h incubation in medium containing 100 μM AA861, 75 μM indomethacin, 100 nM LTB4, 100 nM PGE2, or indicated combinations of these additions. Incubation medium was then replaced with fresh medium plus additions immediately before wounding, and aliquots of the fresh medium were removed to serve as indicators of time 0 prewounding comparison control levels of LTB4 or PGE2. Control cell monolayers for each treatment were left unwounded. At indicated times postwounding, cells and medium in plates were lysed with cold enzyme immunoassay buffer (Cayman), in an amount equivalent to the medium volume, on ice. Cells were scraped, and then lysed cells plus medium were incubated on ice for 15 min, ruptured by sonication, and immediately transferred to −70°C. Colorimetric enzyme immunoassay to determine amounts of either LTB4 or PGE2 in the cell-medium lysate was performed according to the manufacturer’s directions. Amounts of both eicosanoids were normalized to percentages of prewounding control levels to indicate relative changes over time.

Scanning electron microscopy. NIH/3T3 fibroblast monolayers were plated on surfaces and wounded as described. The monolayers were then rinsed twice with serum-free DMEM maintained at 37°C. The cells were then fixed with 0.5% glutaraldehyde in serum-free DMEM, also maintained at 37°C for 30 min. The cells were then DMEM rinsed for 5 min each with 0.5% glutaraldehyde and 1% osmium tetroxide for 30 min. The cells were rinsed three times for 10 min each in serum-free DMEM. After postfixation, the cells were dehydrated by immersion in the following concentrations of ethanol
for 2 min each: 20, 30, 40, 50, 60, 80, 90, 96, and 100%. The cells were then dried by critical point drying. Finally, the samples were mounted and coated with gold-palladium and imaged using a JEOL JSM-5400 scanning electron microscope.

Statistical analysis. Data are presented as means ± SE of minimally three separate experiments. Statistical significance was determined using SigmaPlot and SigmaStat software (Jandel Scientific). To determine statistical significance for differences between treatments and controls in kinetic assays, we compared all time point values as a whole for a particular treatment with control values as a whole, unless a particular treatment intersected or merged with control points for a part of the assay. In that case, those distinct sections of the kinetic course were evaluated both overall and as separate parts. P values derived from ANOVA are indicated in figure legends.

RESULTS

The typical sequence of events in wound closure in wild-type NIH/3T3 cells is demonstrated in Fig. 1. During the first 60 min postwounding, the surface area of the cells at the wound margins increases as a result of cell spreading into the wound gap, with filopodial extensions protruding unidirectionally into the empty space (Fig. 1, A and B). By 60–120 min postwounding, after this unidirectional cell spreading, single cells located at regular intervals continue to elongate into the wound at an angle perpendicular to the wound axis (Fig. 1, B and C), until contacting another cell similarly stretching from the opposite side. These initial cell “bridges” become the focal points for later events in closure of the wound. In addition, these bridges appear to have periodicity in spacing along the entire length of the wound (Figs. 1, C and D, and 2C). Once these bridges have formed, cells adjacent to the primary bridge cells begin moving into the wound area both along the bridge and from the peripheral margins between 180 and 300 min postwounding (Fig. 1D). As these bridges thicken, cells in the peripheral areas farther away from the wound are reorganized to align behind the bridge structures and the organized motile cells perpendicular to the wound axis. After moving onto or adjacent to the bridges, cells turn 90° along the axis of the wound and either migrate away from the bridges into unoccupied gap areas to spread or join the bridges to enlarge these structures (Fig. 1E). Cells that have moved into the wound then spread and retain a greater average surface area than cells farther out from the wound until later mitosis plus spreading of new cells completely restores a normal monolayer (Fig. 1F).

Cells that are in the process of division or have recently divided and remain rounded relative to neighboring spread cells are highly refractile in the micrographs and appear as bright spots. A closer view of the cell bridges acting as foci for directed cell migration is shown in Fig. 2A. The filled arrow indicates an established three- to four-cell bridge with other cells moving across the initial bridge toward the wound gap center; the open arrows indicate areas where outlying peripheral cells have reorganized behind the bridges bilaterally to face the wound gap. Interestingly, not every marginal cell that initially protrudes into the wound space attracts an opposite cell to form a bridge, as can be seen directly above the existing bridge in this view. Figure 2B is a scanning electron micrograph of a similar

Fig. 1. Sequence of events during in vitro closure of a 3- to 4-cell width-wounded NIH/3T3 fibroblast monolayer. Monolayers and wounds were created as described in METHODS AND MATERIALS. The time sequence is as follows: 0 min (A), 60 min (B), 120 min (C), 180 min (D), 240 min (E), and 300 min (F). The images are not sequential but are representative. Wound closure can be examined in 3 parts: early spreading (A–C), bridge formation (C), and migration (C–F).
wild-type cells. During the 5-LOX and COX, respectively, over time postwounding in
suring changes in the levels of LTB₄ and PGE₂, metabolites of
wound closure, we performed enzyme activity assays by mea-
shows bridges located at regular intervals at 2 h postwounding.

Fig. 2. Formation of cellular bridges. A: phase-contrast image showing
close-up view of cell-cell bridges and cells elongating into the wound as well
as cell spreading onto the adjacent open wound space from the initial bridge
(filled arrow). Open arrow indicates areas where outlying peripheral cells have
reorganized behind the bridges laterally to face the wound gap. B: scanning
electron microscopy was performed as described in MATERIALS AND METHODS.
Cells are spreading and elongating from either side of the wound to form
cell-cell bridges. Arrow indicates another cell migrating atop the bridge cells
toward the wound gap. C: phase-contrast image showing periodicity of
bridging at 120 min postwounding.

two-cell bridge with the filled arrow showing another cell migrating
atop the bridge cells toward the wound space. Figure 2C
shows bridges located at regular intervals at 2 h postwounding.

To show that LOX and COX are sequentially active in
wound closure, we performed enzyme activity assays by mea-
suring changes in the levels of LTB₄ and PGE₂, metabolites of
5-LOX and COX, respectively, over postwounding in
wild-type cells. During the first 120 min of wound closure, a
transient LTB₄ release correlated with the first marginal cell-
spreading phase of wound closure (Fig. 3A, top). This release
was completely abrogated in the presence of the specific
5-LOX inhibitor AA861 and was both enhanced and sustained longer over time in the presence of the COX inhibitor indo-
methacin. In contrast, a delayed but sustained increase of PGE₂
over the course of wound closure commenced at ~2 h post-
wounding and remained at significantly elevated levels at all
times thereafter (Fig. 3A, bottom). Indomethacin completely
inhibited the PGE₂ release. The results are consistent with a
sequential oxidation of AA released during wounding, first by
5-LOX and then by COX. Interestingly, the LOX inhibitor as
well as exogenous addition of LTB₄ also inhibited the PGE₂
release, which suggests that the metabolite of LOX, LTB₄, may
be sequentially linked to the activation of COX oxidation to
produce PGE₂.

As a control, LOX and COX activity were also assessed
using an unwounded monolayer of wild-type cells treated
exactly as the wounded plates (Fig. 3B). Control unwounded
cell LTB₄ and PGE₂ levels can be assumed to represent basal
levels in the absence of wound stimulation and, in fact, varied
little over the course of the assay. This finding suggests that the
increases and kinetics of synthesis observed for both LTB₄ and
PGE₂ are stimulated in a wound-specific manner.

Figure 4A indicates that during the first 120 min postwound-
ing in wild-type cells, there was a quantitative increase in the
cell surface area of the cells at the edge of the wound; this
unidirectional spreading of the marginal cells increased over
the course of wound closure. This marginal cell spreading can
be further correlated with the kinetics of the LTB₄ increase
shown in Fig. 3. The increase in marginal spread cell surface
area was inhibited by the specific 5-LOX inhibitor AA861; this
spreading inhibition was completely reversed with the addition
of LTB₄, but reversal of inhibition was not seen with coaddi-
tion of AA861 and PGE₂ (Fig. 4A, top). Overcoming AA861
inhibition of LOX by the exogenous addition of LTB₄ with
regard to restoration of cell spreading also confirmed that the
effect of the LOX inhibitor was not due to nonspecific general
cytotoxicity (Fig. 4A, top).

Addition of the COX inhibitor indomethacin slightly in-
creased marginal cell spreading, whereas addition of exoge-
nous PGE₂ to the inhibitor-treated cells reduced marginal cell
spreading (Fig. 4A, middle). Interestingly, the kinetics of LTB₄
synthesis by a monolayer after wounding as shown in Fig. 3A
correspond to the increase in marginal cell surface, as shown in
Fig. 4A. The observation that inhibition of COX by indometh-
acin increased LTB₄ synthesis is consistent with the concept
that LOX and COX compete for the AA released during wound-
ing. In other words, when COX is inhibited with indo-
methacin, more AA can be metabolized to LTB₄ by 5-LOX to
increase the rate of marginal cell spreading (compare Figs. 3A
and 4A, middle). This statement is supported by the observation
that addition of exogenous LTB₄ alone greatly enhanced mar-
ginal cell spreading during the course of wound closure,
indicating that the amount of LTB₄ released after wounding
was in rate-limiting amounts with regard to cell spreading into
the wound (Fig. 4A, bottom). Finally, PGE₂ was antagonistic to
marginal cell surface spreading (Fig. 4A, bottom). This finding
is consistent with the observation that PGE₂ antagonizes cell
spreading during cell adhesion by shifting the cells into the
migration stage. As shown by results discussed below, this is
quite possible.
Additional studies of the role of LOX and COX on wound closure in NIH/3T3 fibroblasts were done with fibroblasts transfected such that they overexpressed or underexpressed either 5-LOX or COX-2. Overexpression of 5-LOX (5LOX in Fig. 4B, top) or underexpression of COX-2 (2XOC in Fig. 4B, bottom) led to an increase in the marginal cell surface area during the course of wound closure, whereas underexpression of 5-LOX (XOL5 in Fig. 4B, top) or overexpression of COX-2 (COX2 in Fig. 4B, bottom) decreased the extent of marginal cell spreading during wound closure. These results confirm and complement the biochemical and pharmacological experiments described above.

After the marginal cell spreading and elongation into the denuded area in wild-type cells, some of the spread, extended cells make contact with a counterpart on the opposite side, eventually forming a perpendicular cell-cell bridge across the wound space (Fig. 2A). The formation of these bridge structures appears to be critical in the shift from directional marginal cell spreading to directional peripheral cell migration. Because the onset of bridging occurs during the initial cell-spread phase of wound gap closure, cells were treated with the 5-LOX inhibitor AA861 to determine whether the role played by leukotrienes in signaling marginal cell spreading also extends to this more selected and limited type of unidirectional spreading. The addition of the 5LOX inhibitor to wild-type cells produced a significant reduction in the frequency of bridge formation to <30% of that in untreated control cells (Fig. 5A, top). Conversely, addition of exogenous LTB4 enhanced the formation of bridges 1.5-fold (Fig. 5A, top). Furthermore, addition of the COX2 inhibitor indomethacin resulted in enhanced bridge formation that corresponds to the increase in LTB4 levels shown in Fig. 3. Addition of exogenous PGE2 also produced a reduction in the number of bridges (Fig. 5A, bottom).

To investigate the possibility that prostaglandins might also influence bridge formation concurrently with the demonstrated leukotriene regulation, we added exogenous PGE2 to cells in conjunction with the 5-LOX inhibitor AA861. The results indicate that a reduction in bridge formation from 5-LOX inhibition cannot be overcome or reversed by stimulating cells with PGE2 (Fig. 5A, top). The reciprocal combination of indomethacin to inhibit COX, plus addition of exogenous

Fig. 3. Changes in leukotriene B4 (LTB4) and prostaglandin E2 (PGE2) levels are specific to wounded cells. A: LTB4 production transiently increased during the first phase of wound healing, peaking at 60 min postwounding and then returning to basal prewounding levels (top); PGE2 production gradually increased during the course of wound healing, but only after 60–120 min postwounding, and remained elevated during the course of wound closure (bottom). Enzyme immunoassay was performed as described in MATERIALS AND METHODS. Concentrations of inhibitors and metabolites used were 100 μM AA861 (5-LOX inhibitor), 200 nM LTB4, 75 μM indomethacin (Indo; COX-1/2 inhibitor), and 200 nM PGE2. The basal values of LTB4 and PGE2 produced for control cells (untreated) at time 0 (prewounding) were 70–90 pg/ml (0.21–0.27 nM) for LTB4 and 150–200 pg/ml (0.43–0.57 nM) for PGE2, depending on the experiment. Data shown represent 2 independent experiments done in triplicate. P values are as follows for LTB4 production: for control vs. Indo, AA861, and LTB4, P 0.001, 0.01, and 0.05, respectively, after 60 min. B: unwounded control cell monolayers were treated such that they overexpressed or underexpressed either 5-LOX or COX-2. Overexpression of 5-LOX (5LOX in Fig. 4B, top) or underexpression of COX-2 (2XOC in Fig. 4B, bottom) led to an increase in the marginal cell surface area during the course of wound closure, whereas underexpression of 5-LOX (XOL5 in Fig. 4B, top) or overexpression of COX-2 (COX2 in Fig. 4B, bottom) decreased the extent of marginal cell spreading during wound closure. These results confirm and complement the biochemical and pharmacological experiments described above.

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LTB₄ produced an increased frequency of bridges relative to wild-type cell untreated wounds (Fig. 5A, bottom). These data, taken together, support the idea that LOX activity and not COX activity is required for bridging.

To further corroborate these findings, we examined the formation of the cell bridges with the stably transfected 5-LOX- and COX-2-over- and -underexpressing fibroblasts at 120 min postwounding. Either 5-LOX overexpression (3T3-5LOX in Fig. 5B, top) or COX-2 underexpression (3T3-2XOC in Fig. 5B, bottom) enhanced bridge formation more than threefold compared with untreated wild-type cells, whereas either COX-2 overexpression (3T3-COX2 in Fig. 5B, bottom) or 5-LOX underexpression (3T3-XOL5 in Fig. 5B, top) decreased the extent of cell bridging to less than 20 and 35%, respectively, of that in control wild-type cells during wound closure.

To determine differences in wound closure mediated by 5-LOX or COX oxidative products between marginal cell spreading as opposed to peripheral cell motility into the gap, we also measured the reduction of wound surface area over time by adding the same enzyme inhibitors and metabolites described above. Although marginal cell spreading was shown to be necessary for wound closure in the previously described assays, most of the total reduction in wound surface area occurred later than 120 min postwounding, which suggests that either directed cell migration (migration of cells resulting from early formation of bridges) or random cell migration (migration of cells without the early formation of bridges) into the gap after earlier marginal cell spreading is responsible for the majority of wound closure. Evaluation of wound surface area in wild-type cells at 300 min postwounding indicates that inhibitors of 5-LOX (AA861) and COX (indomethacin) both slow wound closure, with 5-LOX inhibition resulting in a slower closure overall (Fig. 6A, top and middle). Inhibition of either enzyme in wild-type cells could be reversed by addition of its own respective downstream metabolite but not by addition of PGE₂ to 5-LOX-inhibited cells (Fig. 6A, top) or addition of LTB₄ to COX-inhibited cells (Fig. 6A, middle), although wound closure was partially reversed in the latter case, likely due to increased marginal cell spreading, since motility is inhibited. Interestingly, addition of exogenous PGE₂ alone did not inhibit overall wound closure at 300 min, even though it slowed initial cell spreading and bridge formation (Fig. 6A, bottom).

As expected, 5-LOX overexpression (5LOX in Fig. 6B, top) and COX-2 underexpression (2XOC in Fig. 6B, bottom), effectively increasing LTB₄ or decreasing PGE₂, both enhanced the early phase of wound closure associated with marginal cell spreading. A: surface areas of the marginal wound cells were measured in the presence and absence of AA861 (top), a specific 5-LOX inhibitor; Indo (middle), a COX inhibitor; and exogenous LTB₄ and PGE₂, metabolites of LOX and COX, respectively (bottom). Spreading began immediately after wound was made and plateaued approximately 2 h postwounding. Concentrations of inhibitors and metabolites used were as stated in Fig. 3. Data shown represent 3–5 independent experiments. P values are as follows: for control vs. AA861, P < 0.05 at all time points. For control vs. AA861 + LTB₄, no significant difference at all time points. For control vs. AA861 + PGE₂, P < 0.01 at all time points. For control vs. Indo, P < 0.05 between 0 and 120 min and P < 0.01 after 180 min; no significant difference at 120 and 180 min. For control vs. Indo + LTB₄, P < 0.0015 after 60 min; no significant difference at 30 and 60 min. For control vs. Indo + PGE₂, P < 0.01 between 60 and 300 min; no significant difference at 30, 60, and 300 min. For control vs. LTB₄, P < 0.0015 after 60 min; no significant difference at 30 and 60 min. For control vs PGE₂, P < 0.01 between 0 and 240 min; no significant difference at 240 and 300 min. B: overexpression of 5-LOX or underexpression of COX-2 enhanced wound marginal cell surface area, whereas overexpression of COX-2 or underexpression of 5-LOX inhibited any increases in wound marginal cell surface area. Data shown represent 3 independent experiments. P values are as follows: for control vs. 5-LOX, P < 0.01 at all time points. For control vs. XOL5, P < 0.05 after 120 min; no significant difference at other time points. For control vs. COX-2, P < 0.05 after 180 min; no significant difference at other time points. For control vs. 2XOC, P < 0.001 at all time points.
spreading and bridging but did not accelerate wound closure
over the entire period studied. This effect was also seen with
wild-type cells treated with excess LTB₄, as shown in Fig. 6
A, bottom. 5-LOX underexpression (XOL5 in Fig. 6B, bottom)
conversely inhibited the early (overall rate) stage of wound
closure, but not as much as in wild-type cells treated with the
5-LOX inhibitor AA861, which significantly abrogated wound
closure at 300 min (Fig. 6A, top). COX-2 overexpression
(COX2 in Fig. 6B, bottom), as was the case with the 5-LOX-
derexpressing cells (Fig. 6B, top), inhibited the early stages
of wound closure associated with marginal cell spreading but
eventually was equivalent with that in control cells by 300 min
postwounding (Fig. 6B). The early stage delay shown in these
cells corresponds to the decreased early stage of wound closure
associated with marginal cell spreading as shown in Fig. 4.

DISCUSSION

Previous studies of the signaling effects of AA metabolites
in wound response indicate that there may be variation between
cell types (11, 18, 23), suggesting that either AA regulation of
wound healing is cell type specific or the production of AA
metabolites by one cell type through paracrine signaling acti-
vates or inhibits adhesion of other cell types, thus regulating
the overall process of wound healing. In vivo, in tissue con-
taining multiple cell types, fibroblasts would precede endo-
theilial cells into denuded areas (36) such that the release of
arachidonate metabolites from fibroblasts to coordinate their
own spreading, bridging, and migration would also likely
influence surrounding endothelial, smooth muscle, and neural
cells. Endothelial wound closure in small scrape wounds of
corneal and vascular endothelium have been reported to occur
primarily by enlargement and flattening, or spreading, of pe-
ripheral wound cells adjacent to the wound gap (10, 30). In
larger punch wounds, where cell migration is required to close
the wound, two types of wound-associated movements have
been observed in both epithelium and endothelium. The first
type of motility occurs when cells break contact with the
monolayer, spread, and then migrate into the wound gap. The
second type occurs when cells within the confluent monolayer
spread and migrate as whole connected sheets to fill the wound

Fig. 5. COX inhibition or exogenous LTB₄ enhance bridge formation, whereas 5-LOX inhibition or exogenous PGE₂ inhibit these
structures. A: the number of bridges per millimeter of wound were counted 120 min postwounding after preincubation and
maintenance in cells with either no addition (control) or the addition of 100 μM AA861, 75 μM Indo, 200 nM LTB₄, or 200 nM
PGE₂. Bridges are defined as elongated cells that span the entire width of the wound and contact cells from the other side. AA861
(5-LOX inhibitor) inhibited bridge formation, whereas Indo (COX inhibitor) enhanced bridge formation. In all cases where LTB₄
was added to cells, bridge formation was enhanced. In all cases where PGE₂ was added to cells, bridge formation was inhibited.
Data shown represent 3 independent experiments. Values for control vs. all treatments are significantly different. P values are as
follows: AA861, AA861 + LTB₄, AA861 + PGE₂, and LTB₄, P < 0.01; Indo, P < 0.05; Indo + LTB₄, P < 0.01; Indo + PGE₂
and PGE₂, P < 0.01. B: overexpression of 5-LOX or underexpression of COX-2 enhanced bridge formation whereas overexpres-
sion of COX2 or underexpression of 5-LOX inhibited bridge formation compared with control. Data shown represent 3 independent
experiments. Values for control vs. all treatments are significantly different. P values are as follows: 5LOX, P < 0.0001; XOL5,
P < 0.01; COX2, P < 0.01; 2XOC, P < 0.005.
In the present studies, we observed NIH/3T3 fibroblasts moving singly in a coordinated manner facilitated by cell-cell bridges but not as connected sheets. It is important to study the kinetic and empirical stages of wound healing to better understand both how the process can go awry and how to develop drugs to accelerate wound closure. The goal of these studies was to evaluate whether LOX and COX metabolites might signal the unique directional cell spreading and motility required for wound closure in stable confluent monolayers where cells are already spread, as has been shown to be the case with suspended NIH/3T3 cells plated onto a fibronectin (FN) surface (4–6, 32, 33).

Fig. 6. 5-LOX and COX activities are necessary for complete wound closure. A: inhibition of 5-LOX inhibited kinetics of overall wound closure (top). This inhibition was fully reversible with addition of exogenous LTB₄ but not PGE₂. Inhibition of COX also inhibited overall wound closure. This inhibition was partially reversible with addition of exogenous LTB₄ and fully reversible with PGE₂ (middle). Addition of both exogenous LTB₄ and PGE₂ enhanced overall kinetics of wound closure (bottom). Concentrations of inhibitors and metabolites used were as stated in Fig. 3. Data shown represent 3–5 independent experiments. P values are as follows: values for control vs. all treatments with AA861 are equivalent up to and including 120 min; values at other time points are significantly different as follows: control vs. AA861, P < 0.001; control vs. AA861 + LTB₄, P < 0.05. For control vs. Indo, P < 0.01 after 60 min; no significant difference at other time points. For control vs. Indo + LTB₄, P < 0.05 between 0 and 180 min and after 180 min; no significant difference at 180 min. For control vs. Indo + PGE₂, P < 0.05 between 30 and 180 min; no significant difference at other time points. For control vs. LTB₄, P < 0.01 between 0 and 240 min; no significant difference at 240 min or after. For control vs. PGE₂, P < 0.05 after 120 min; no significant difference at other time points. For control vs. XOL5, P < 0.01 between 60 and 240 min; no significant difference at other time points. For control vs. Indo + XOL5, P < 0.05 between 60 and 300 min; no significant difference at other time points. For control vs. 2XOC, P < 0.01 between 0 and 180 min and P < 0.05 after 180 min; no significant difference at 180 min.

B: overexpression of 5-LOX and underexpression of COX-2 caused increased initial wound closure kinetics, but later wound closure was inhibited. Overexpression of COX2 and underexpression of 5-LOX in cells delayed the rate of wound closure up until 300 min. Data shown represent 3 independent experiments. P values are as follows: for control vs. 5-LOX, P < 0.01 between 30 and 180 min and P < 0.05 after 180 min; no significant difference at 180 min. For control vs. XOL5, P < 0.01 between 0 and 240 min; no significant difference at 240 min or after. For control vs. COX2, P < 0.05 between 60 and 300 min; no significant difference at other time points. For control vs. 2XOC, P < 0.01 between 0 and 180 min and P < 0.05 after 180 min; no significant difference at 180 min.
In previous studies, we (32) showed that the production of LTB4 and PGE2 in these cells during the course of cell spreading and migration is dependent on the production of AA via cytosolic PLA2 activation. Our preliminary work using pan and specific inhibitors of PLA2 also suggested that calcium-dependent secretory PLA2 may be required for wound healing (unpublished observations).

We (32) and others (23) also showed that when cells are stimulated by either mechanical wounding and/or adhesion to the ECM, AA production is also stimulated. We can therefore infer that in this case, wounding, and more specifically, wounded marginal cells, are responsible for the LTB4 and PGE2 production described in these assays, because little activity was observed in the absence of wounding (Fig. 3B). Whether an AA release providing substrate for the various oxidative enzymes is stimulated by mechanical forces from wounding or by exposure of marginal cells to an increased area of available FN surface cannot be determined from these data.

Although marginal cell spreading into a wound gap is unidirectional compared with the spreading of newly plated detached cells, wound marginal cell spreading was similarly shown to be regulated by 5-LOX metabolites (Fig. 4, A and B) and can be correlated to 5-LOX activity as measured by increases in LTB4 levels stimulated rapidly after monolayer wounding. LTB4 increased 2.5-fold over basal levels by 60 min postwounding in untreated wild-type cells, whereas no increases were seen in unwounded monolayers (Fig. 3). This 5-LOX-mediated LTB4 rise in wild-type cells also corresponded to development of periodic cell bridges across the wound gap, which were required for later directed motility (Fig. 5A). Cells constitutively expressing 5-LOX demonstrated a proportional increase in cell bridge formation (Fig. 5B). This finding suggests that initial bridge extensions are a later developing part of the process of marginal cell spreading and that both are regulated by LTB4. Both marginal cell spreading and bridge formation were required for later directed cell motility into wound gaps but not for motility stimulated by prostaglandin release, which seems to occur without much additional spreading (Figs. 4 and 5). Increased spreading and bridge cell formation were also enhanced by indomethacin inhibition of COX, likely due to increased LTB4 production accompanying COX inhibition as shown in Fig. 3. Interestingly, even though 5-LOX overexpression or COX-2 antisense enhanced initial marginal spreading and bridge formation compared with wild-type cells, overall wound closure was inhibited. This is likely due to the observation that those particular cells have impaired motility following the spreading stage of cell adhesion to FN (Stockton RA and Jacobson BS, unpublished observation).

Conversely, COX activity as indicated by PGE2 levels was unchanged in wild-type cells for the first 60–120 min postwounding but then rose continuously over the remaining time course of wound closure (Fig. 3). This increase also corresponded to increased cell migration into the wound gap, as measured by a decrease in wound surface area over time (Fig. 6).

Overall wound closure appeared to be biphasic, in that initial reductions in wound surface area were associated with marginal cell spreading signaled by LTB4 over the first 60–120 min postwounding, whereas subsequent full gap closure was associated with directed motility of reorganized peripheral cells into the wound gap over and adjacent to the established bridges (Figs. 1, 5, and 6). These leukotriene-mediated early bridge structures, to our knowledge, appear to represent a novel and previously unreported stage of wound healing in vitro.

The overall effects of leukotrienes as opposed to prostaglandins in all stages of wound closure can be argued to be largely dominated by the relative ratios of oxidative products over time. Although exogenous PGE2 enhanced overall wound closure in untransfected cells at 5 h postwounding, it inhibited initial LTB4 production and concomitant marginal spreading and bridge formation. This is likely, because LTB4 production in cells treated with PGE2 returns to control levels after 2 h. COX-2 overexpression, like addition of PGE2, caused a delay in the onset of wound healing, presumably because of decreased spreading and bridge formation (Fig. 4 and 5). The effect of COX-2 on overall wound healing can be better observed with the COX-2-overexpressing cells in which gap closure was delayed up until 4 h postwounding. In these cells, no increase in spreading could be observed during the entire period studied; therefore, any movement of the cells occurred by random motility, according to our definition of random motility as that occurring in the absence of any increase in spreading or bridge formation and not specifically wound gap-directed in overall direction. In wild-type cells, exogenous PGE2 enhanced general motility of cells into the wound while reducing initial marginal cell spreading, as stated previously.

Fig. 7. Overall model of eicosanoid signaling in wound closure. 5-LOX activity is stimulated to increase by wounding and signals both spreading of marginal cells and formation of early bridges. As LTB4 then returns to basal levels, PGE2 increases and signals directed migration of peripheral cells through the bridges and from the interbridge space into wound gaps. Coordinated sequential activity of both LOX and COX is required for effective closure.
After 120 min, spreading as well as LTB4 levels catch up to control levels to produce an accelerated rate of wound closure. This is further evidence that both LOX and COX activity are required for optimal closure.

The results seen with the COX-2-overexpressing cells can be compared with untransfected cells treated with both AA861 and PGE2, which also show no increase in spreading during the period studied, indicated by the fact that both have delayed wound closure. Cells treated with both AA861 and PGE2, however, take much longer than the 5-h period shown in this study to close (data not shown). Although we have shown that enhanced PGE2 production inhibits initial LTB4 production, it is likely that 5-LOX activity is not completely inhibited in COX-2 overexpressers but that the mass ratio of products has heavily shifted toward prostaglandin signaling. We cannot rule out, however, other indirect effects that PGE2 might have on LTB4 production such as the inhibition of 5-LOX-activating protein (FLAP) via an IL-10-dependent mechanism as was seen in dendritic cells (12).

Leukotrienes are known to play an important role in the initiation of inflammatory bowel disease (IBD) in rats. Interestingly, IBD is only inhibited when administration of an inhibitor of leukotriene synthesis is begun before the inflammatory stimulus is applied and not after (20). This suggests that leukotrienes may play a role early in the inflammatory process such that inhibition after inflammation has already occurred would be futile. This requirement for predation of wound-associated inhibitors was also observed in corneal endothelial cells treated with indomethacin such that indomethacin added before, but not after, wounding promoted enhanced spreading of the confluent monolayer into the wound (17).

Other recent work showed that inhibition of 5-LOX did not impair healing of 3T6 fibroblast wounds (22); however, in those experiments wound closure was evaluated at more than 5 h postwounding, by which time random migration rather than bridge-directed migration of COX-activated cells would have produced a considerable degree of closure. Our data indicate that 5-LOX inhibition slows the initial stages of gap closure and prevents later directed motility dependent on bridge formation but that it has little effect on random peripheral cell motility. In contrast, cells treated with PGE2 had inhibited LTB4 production and consequential bridge formation, but the later stages of wound closure were not affected (Fig. 6A). Wounds treated with both a 5-LOX inhibitor and exogenous PGE2 showed an initial lag in closure compared with control cell monolayers but did eventually close at later times (data not shown), possibly due to eventual prostaglandin-stimulated random movement into the wound (Figs. 3 and 4). It is also interesting to note that AA861 has a broad inhibitory effect on both LTB4 and PGE2 production. The observation that exogenous LTB4 alone can reverse the inhibitory effects of AA861, however, strongly suggests that 5-LOX activation and COX activation are sequential during wound healing. The overall stages of wound healing and their proposed regulatory sites by LTB4 and PGE2 are schematized in Fig. 7.

Dysregulation of AA signaling leading to sustained prostaglandin production is associated with a variety of hyperproliferative malignancies (8, 29, 35) and diseases of chronic inflammation (13, 24, 28). Clearly, a prostaglandin-stimulated motile cell is a potentially dangerous cell and must be returned to a more quiescent state as soon as possible by being directed to a specific location and induced to spread. The coordinated leukotriene-prostaglandin-mediated process demonstrated in these studies is optimized to stimulate and regulate only that cell motility required for wound closure.

This wound closure regulation by arachidonate pathway switching modulates activities of both branch enzymes, and we hypothesize that in addition to limiting undirected cell motility from prostaglandin effects, this also serves to reduce postwounding localized fibrosis, which may be associated with sustained increases in leukotriene synthases. Elevated leukotriene synthesis is seen systemically in scleroderma (31) and other conditions of systemic fibrosis (19, 21) and specifically in keloid scar formation (2). These data suggest that sequential and coordinated activities of first LOX and then COX form a system of tight regulation of early inflammation-stimulated cell motility, both to optimize wound healing and, possibly, to reduce potentially dangerous increases in fibroblast and epithelial cell random motility associated with increased prostaglandin levels.

**REFERENCES**

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