Endothelial Cell Confluence Regulates Cyclooxygenase-2 and Prostaglandin E₂ Production That Modulate Motility

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Endothelial cells line the vasculature and, after mechanical denudation during invasive procedures or cellular loss from natural causes, migrate to reestablish a confluent monolayer. We find confluent monolayers of human umbilical vein endothelial cells were quiescent and expressed low levels of cyclooxygenase-2, but expressed cyclooxygenase-2 at levels comparable with cytokine-stimulated cells when present in a subconfluent culture. Mechanically wounding endothelial cell monolayers stimulated rapid cyclooxygenase-2 expression that increased with the level of wounding. Cyclooxygenase-2 re-expression occurred throughout the culture, suggesting signaling from cells proximal to the wound to distal cells. Media from wounded monolayers stimulated cyclooxygenase-2 expression in confluent monolayers, which correlated with the level of wounding of the donor monolayer. Wounded monolayers and cells in subconfluent cultures secreted enhanced levels of prostaglandin (PG) E₂ that depended on cyclooxygenase-2 activity, and PGE₂ stimulated cyclooxygenase-2 expression in confluent endothelial cell monolayers. Cells from subconfluent monolayers migrated through filters more readily than those from confluent monolayers, and the cyclooxygenase-2-selective inhibitor NS-398 suppressed migration. Adding PGE₂ to NS-398-treated cells augmented migration. Endothelial cells also migrated into mechanically denuded areas of confluent monolayers, and this too was suppressed by NS-398. We conclude that endothelial cells not in contact with neighboring cells express cyclooxygenase-2 that results in enhanced release of PGE₂, and that this autocrine and paracrine loop enhances endothelial cell migration to cover denuded areas of the endothelium.

Angiogenesis, the formation of new blood vessels from pre-existing blood vessels, is essential for wound repair, tumor growth, and metastasis (1). Endothelial cell migration from the confluent monolayer of endothelial cells of mature vessels into matrix underlying this process. Prostanoids, collectively E₂ (PGE₂),¹ PGF₂α, PGD₂, PGI₂, and thromboxane A₂, are cyclooxygenase products involved in angiogenesis and tumor growth (2, 3). Individual prostanoids are recognized by a family of G protein-coupled receptors whose distribution controls the prostanoid signaling axis (4).

There are two isoforms of cyclooxygenase, cyclooxygenase-1 and cyclooxygenase-2, that have both shared and separate functions (5). Cyclooxygenase-1, found in many tissues, typically is constitutively expressed, although it and not cyclooxygenase-2 is induced in uterine endothelial cells in the third trimester of pregnancy when PGI₂ levels and blood flow increase (6). Cyclooxygenase-2 typically is absent from endothelial cells and white blood cells but accumulates to high levels in endothelial cells over several hours in response to IL-1β (7), lipopolysaccharide (8), phorbol myristate acetate (8), TNFα (9), and oxidized phospholipids (10). Accordingly, cyclooxygenase-2 has numerous transcriptional regulatory elements in its 5'-regulatory region (11, 12) and also is subject to post-transcriptional control (13).

Cyclooxygenase-2 is dramatically induced by growth factors, tumor promoters and mitogens, and is aberrantly expressed in tumors, including those of colon (14), breast, and prostate (15). Cyclooxygenases are the targets of non-steroidal anti-inflammatory drugs (NSAIDs), and NSAIDs decrease cancer risk and suppress tumorigenesis in animal models (15). NSAIDs inhibit endothelial cell spreading, migration, and angiogenesis (16), processes controlled by PGE₂ (17), just as genetic ablation of cyclooxygenase-2 blocks the growth of cyclooxygenase-2-replete tumors by suppressing angiogenesis (3). These gene-targeted animals show that it is cyclooxygenase-2 expression in the host stromal cells, including endothelium, and not by the tumor cells themselves that is critical and suggest that host autocrine and paracrine signaling has a role in tumorigenesis. Tumors express high levels of PGE₂, and pharmacologic suppression of cyclooxygenase-2 activity, and not that of cyclooxygenase-1, depletes PGE₂ and blocks tumorigenesis (18). A similar result was obtained when PGE₂ was depleted with a monoclonal antibody (18), so PGE₂ is one factor controlling angiogenesis and tumor growth.

Endothelial cells migrating during angiogenesis necessarily lack a neighboring cell at the leading edge of the nascent tube. We find that endothelial cells not completely surrounded by neighboring endothelial cells and those not embedded in a confluent monolayer of cells display a characteristic of highly activated cells, cyclooxygenase-2 expression. Re-expression of cyclooxygenase-2, which was down-regulated as cells formed a monolayer, results in enhanced PGE₂ secretion that aids endothelial cell migration to reestablish a confluent monolayer of endothelial cells.

MATERIALS AND METHODS

Reagents—NS-398 was purchased from Biomol (Plymouth Meeting, PA); the monoclonal antibodies against cyclooxygenase-1 and cyclooxy-
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genase-2 were from Cayman Chemical (Ann Arbor, MI); horseradish peroxidase-conjugated goat anti-mouse antibody was from BIO-SOURCE, and PGE₂ ELISA kits were from Assay Designs (Ann Arbor, MI). Calcein AM was the product of Molecular Probes (Eugene, OR), and the cell cycle inhibitors aphidicolin, mimosine, 5-fluorouracil, Ara-C, and nocodazole were from EMD Biosciences (San Diego, CA). Transwell inserts with a black membrane (3 μm pores) were from Discovery Labware. IL-1β, phorbol myristate acetate, TNF, lipopolysaccharide, and all other reagents were from Sigma.

**Cell Culture and Monolayer Wounding**—Human umbilical vein endothelial cells were isolated and cultured as described (19). These cells were allowed to achieve confluence, typically in 3–5 days, and then were serum-starved by culturing for 24 h in media containing 1% human serum before initiating our experiments. Cells were maintained in M199 supplemented with 20% pooled human serum at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The wound repair model used freshly seeded endothelial cells grown to confluence in 6-well tissue culture plates before the cells were mechanically removed from the plate by dragging a 1000-μl pipette tip along the confluent monolayer as the plate rested over a template. Low wounding consisted of seven cell-free lines formed in the monolayer and medium wounding employed 21 parallel wound lines. For the heavily wounded monolayers, the plates were rotated 90° after the first medium wounding, and thirty minutes after wounding, 1000-μl pipette tips were added in triplicate in the wound area. The form square islands of remaining cells. The monolayers were washed after wounding to remove the debris and fed fresh medium that also contained any agonist to be included in the experiments. Images of the wounded cultures were recorded just after medium wounding (t = 0) and then 24 h later for most experiments. Four fields were recorded for each sample, and the assays were repeated 10 or more times.

Endothelial cells from confluent monolayers or individual cells from the same isolation plated at a lower density to preclude monolayer formation were freed from the dish by trypsinization, washed by centrifugation, resuspended in culture medium containing a reduced concentration (1%) of human serum. The recovered cells (1.5–2.5 × 10⁴) were added to the upper chamber of a transwell insert, and the filter inserts were incubated in wells of a 24-well culture plate containing 750 μl of medium. NS-398, when present, was added to both the upper and lower chambers. Basic fibroblast growth factor (10 ng/ml) was added to the lower chamber as a positive control. After 21–22 h, the cells were stained with calcein AM (4 μM for 30 min), and cell migration was quantitated by measuring the fluorescence of the migrated cells in a fluorescent plate reader (Fusion, Hewlett-Packard) using its bottom reading capability. Alternatively, photographs of the migrated cells were recorded by confocal microscopy. Four randomly selected low power fields were chosen, and the number of cells in high-power fields was counted. The migration response was expressed as fold increase over baseline where each condition was assayed in triplicate wells and each experiment was repeated at least twice. Student’s t tests (Graph-Pad Instat) showed all changes were significant (p < 0.05).

**Cell Proliferation**—Inhibition of the cell cycle at various points was accomplished by growing the endothelial cells to confluence in 20% human serum, washing the cells, and then starving them in 1% human serum for 17 h in the presence of agents that interfere with cell cycle progression at distinct stages. Avidin (5 μg/ml) and mimosine (1 mM) block cell cycling during late G₂/S phase; 5-fluorouracil (10 μM) and 18β-arachidinofuranosylcytosine (Ara-C; 1 μM) interfere with deoxyribonucleotide synthesis in S phase; and nocodazole (0.5 μg/ml) blocks microtubule depolymerization required for M phase. The monolayers were then wounded, or not, in the high wounding pattern and incubated with the stated agents for 8 h before cellular material was collected for analysis of cyclooxygenase-2 by Western blotting.

**Immunofluorescence**—Endothelial cells were grown to confluence in 8-well glass chamber slides coated with fibronectin or maintained at a lower density to preclude monolayer formation as before. Multiple wounds were made in each chamber of confluent endothelial cells using a 200-μl pipette tip, and the remaining cells were washed and then fed with endothelial cell medium containing 1% human serum. After the specified time, the medium was flicked out of the wells, and chambers were peeled off. Cells on the slides were fixed in 2% paraformaldehyde followed by permeabilization with 0.5% Triton X-100 for 5 min, and incubated with cyclooxygenase-2 antibodies (1:1000) overnight at 4°C. The next day, the slides were developed with biotinylated goat anti-mouse immunoglobulin (2 μg/ml) for 1 h, followed by Alexa 488-labeled streptavidin for 45 min at room temperature. Propidium iodide (15 μg/ml for 5 min) was used to stain the nuclei before the images were recorded by a confocal laser microscope using a 60× objective (Axiophot, Zeiss, Germany). Images were acquired using a FITC filter set and are representative of at least three independent experiments with similar results. The cyclooxygenase-2 protein expression in cells maintained in a confluent state was not detectable by Western analysis (Fig. 1B). Staining by immunofluorescence shows the enzyme was associated with punctate intracellular structures and particularly stained the nuclear membrane, as anticipated (23). The amount of cyclooxygenase-2 staining by individual cells not organized into a monolayer was equivalent to that found in confluent endothelial cell cultures after treatment with powerful stimuli, such as inflammatory cytokines (TNFα and IL-1β), lipopolysaccharide, or a phorbol ester (Fig. 1B).

**RESULTS**

Cyclooxygenase-2 Protein Production Is Regulated by Cell Contact—Inducible cyclooxygenase-2 has a key role in tumor angiogenesis (15, 20), and the prostaglandin PGE₂ it produces stimulates angiogenesis (21, 22) and endothelial cell adhesion and spreading (17). Endothelial cells primarily exist as a confluent monolayer of quiescent cells (Fig. 1A) but rapidly spread, migrate, and proliferate in response to mechanical denudation as occurs during invasive procedures. We found that confluent monolayers of endothelial cells cultured for 24 h in a reduced amount of serum expressed trace amounts of cyclooxygenase-2 protein by immunocytochemistry (Fig. 1A, lower left). However, cells cultured in numbers insufficient to form a monolayer displayed a sharply increased level of cyclooxygenase-2 expression (Fig. 1A, lower right). Staining shows the enzyme was associated with punctate intracellular structures and particularly stained the nuclear membrane, as anticipated (23). The amount of cyclooxygenase-2 staining by individual cells not organized into a monolayer was equivalent to that found in confluent endothelial cell cultures after treatment with powerful stimuli, such as inflammatory cytokines (TNFα and IL-1β), lipopolysaccharide, or a phorbol ester (Fig. 1B).

Cyclooxygenase-2 protein expression in cells maintained in a confluent state was not detectable by Western analysis (Fig. 2A). In contrast, cells at 40% of the final density of a confluent monolayer expressed this enzyme, and cells seeded even more sparsely to give a density of 20% of confluent cultures contained more cyclooxygenase-2 than this. These samples were normalized for protein content before electrophoresis, which produced equal levels of staining for β-actin, and so this cyclooxygenase-2 staining reflects average cellular content. This enzyme was fully functional because endothelial cells not organized into a confluent monolayer made and released about 17 times more PGE₂ than confluent cultures on a per cell basis when provided with exogenous arachidonate (Fig. 2B). Subconfluent endothelial cells continuously produced twice as much PGE₂ as their confluent counterparts even when exogenous...
Endothelial cell production of PGE₂ from both confluent monolayers and individual cells primarily was a function of cyclooxygenase-2 activity because the non-steroidal anti-inflammatory drug NS-398 that selectively inhibits cyclooxygenase-2 effectively blocked PGE₂ synthesis and release by endothelial cells cultured under either condition (Fig. 2C).

Wounding Endothelial Cell Monolayers Stimulates Cyclooxygenase-2 Expression Proximal and Distal to the Wound—Endothelial cells in a confluent monolayer clearly differ from individual cells before they organize and establish intercellular communications. We tested whether an abrupt disruption of a confluent monolayer, as occurs during invasive clinical procedures, affects cyclooxygenase-2 expression in the same way as seeding the culture at a low density. To do this, we denuded sections of tightly confluent monolayers by dragging a pipette tip along the plate in a pattern to create three levels of wounding (Fig. 3A). We found by Western analysis (Fig. 3B) that cyclooxygenase-2 was present in the cells remaining after

FIG. 1. Subconfluent endothelial cells express cyclooxygenase-2. A, top panels, phase-contrast photographs of confluent (∼200,000 cells/cm²) or subconfluent cultures (∼40,000 cells/cm²) 3 days after plating human umbilical vein endothelial cells. Lower panels, endogenous levels of cyclooxygenase-2 protein in confluent and subconfluent unstimulated endothelial cell cultures were imaged by immunocytochemistry as described under “Materials and Methods.” Alexa-488 green fluorescence, cyclooxygenase-2; red fluorescence, propidium iodide counter-stained nuclei. B, endothelial cells unable to form monolayers expressed cyclooxygenase-2 at levels equivalent to agonist-activated monolayers of endothelial cells. Endothelial cells were plated at a density that allows rapid monolayer formation or at lower density not compatible with monolayer formation. Confluent cultures were stimulated with the stated agonist (5 ng/ml IL-1β, 50 ng/ml TNFα, 100 ng/ml Escherichia coli lipopolysaccharide (LPS), or 0.5 μM phorbol myristate acetate (PMA), or not, for 4 h) before the cells were fixed and stained for cyclooxygenase-2 protein expression and nuclear DNA as above. Subconfluent cells were not exposed to an exogenous agonist.
wounding the monolayer and that the level of protein expression increased with the number of lines drawn through the culture. The amount of cyclooxygenase-2 accumulated by wounded cultures was nearly that reached following phorbol ester stimulation. Cyclooxygenase-1 did not change by wounding the monolayer, although phorbol myristate acetate stimulation modestly enhanced the cellular content of this isoform.

We visualized cyclooxygenase-2 expression by wounded monolayers to determine whether cells adjacent to the wound line, which are the cells directly affected by the wounding procedure, were the only cells to express cyclooxygenase-2. We found that each cell adjacent to the wound expressed this enzyme, but many cells distal to the wound also expressed immunoreactive cyclooxygenase-2 (Fig. 3C). Enhanced expression of cyclooxygenase-2 was detected just 2 h after wounding the monolayer, with a further enhancement 4–8 h after wounding. In Fig. 3C, the lower two panels are composite images that show the cells remaining between two parallel wound lines that are the black, acellular regions at the extreme right, and left portions of the image marked by a white dotted line.

Endothelial Cells Release PGE₂ after Wounding, Which Stimulates Cyclooxygenase-2 Expression—Confluent cultures of endothelial cells make and release PGE₂, and wounding the monolayer increased this secretion (Fig. 4A, upper panel). The amount of PGE₂ released from the cells remaining in the mechanically disturbed monolayer varied with the extent of wounding, and the most heavily scored monolayers released...
The most PGE$_2$. We pretreated the monolayers with NS-398 to inhibit cyclooxygenase-2 activity, and we found that nearly all of the PGE$_2$ released from wounded monolayers came from this enzyme (Fig. 4A, lower panel). The positive control for cyclooxygenase-2 expression, phorbol myristoyl acetate, also stimulated PGE$_2$ release from intact monolayers that was also abolished by NS-398 pretreatment.

PGE$_2$ stimulates cyclooxygenase-2 expression in some (24), but not all (12), contact-inhibited, and so we questioned whether PGE$_2$ acted on endothelial cells to induce the rate-limiting enzyme for its synthesis, cyclooxygenase-2. We found that 10 nM PGE$_2$, the lowest concentration we tested, effectively stimulated cyclooxygenase-2 expression and that stimulated cyclooxygenase-2 accumulation was maximal at 33 nM (Fig. 4B). This observation, coupled with enhanced secretion of PGE$_2$ by wounded monolayers, suggested that the supernatants from wounded monolayers should increase cyclooxygenase-2 expression in confluent endothelial cell cultures. We extensively wounded endothelial cell cultures as before, collected the media overlaying these cells 4 h after wounding, and then incubated new, confluent cultures of endothelial cells containing little cyclooxygenase-2 with this media for 4 h. We found that media from wounded cultures, but not that from unmanipulated monolayers, stimulated cyclooxygenase-2 protein expression in target cells maintained as a confluent monolayer (Fig. 4C). The level of this cyclooxygenase-2 expression increased with the level of wounding of the donor culture, just as the amount of secreted PGE$_2$ increased with wounding density (Fig. 4A). We also found that medium from subconfluent endothelial cell cultures induced cyclooxygenase-2 expression in confluent monolayers to a greater extent than media from confluent cultures (Fig. 4C).

Inhibition of Cyclooxygenase-2 Suppresses Endothelial Cell Migration—Disruption of the integrity of an endothelial barrier causes adjacent cells to fill in the denuded areas by spreading, migration, and proliferation (16). We established that endothelial cells migrated into the areas denuded by the soft pipette tip, which did not score the plate and create a barrier to migration, over time (Fig. 5A). In contrast to the clean edge just after wounding, individual cells had broken away from the monolayer adjacent to the wound and entered the denuded area 24 h after the wounding. This created a disorganized edge of the wound with a few individual cells migrating individually into the denuded zone. Primarily, however, closure of the wound resulted from the entry of groups of adjacent cells that remained in contact with one another. We found extensive closure of the wound by 34 h, with little movement prior to 6 h (not shown) after wounding the monolayer (Fig. 5A). Endothelial cell migration into the denuded region depended on cyclooxygenase-2 activity because NS-398 suppressed cell entry into the acellular area and wound closure (Fig. 5B). The wound edge after NS-398 treatment remained uniform, although there was a modest decrease in the distance between the remaining endothelial cells.

Progression through the Cell Cycle Does Not Control Cyclooxygenase-2 Expression after Monolayer Wounding—Endo-

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**Fig. 4.** Wounding stimulates cyclooxygenase-2 expression and stimulates PGE$_2$ release, and PGE$_2$ is an agonist for cyclooxygenase-2 expression in confluent endothelial cells. A, endothelial cells were grown to confluence in 6-well plates, and the monolayer was wounded in patterns that produced three levels of injury, treated or not with 30 μM NS-398, washed and incubated with fresh media containing 1% pooled human serum, and no exogenous arachidonate, for 8 h. PGE$_2$ released over this time was quantitated by ELISA as in Fig. 2. Ctl, control; Med, medium. B, PGE$_2$ stimulates cyclooxygenase-2 (COX-2) protein expression. Confluent monolayers of endothelial cells were incubated with PGE$_2$ at the stated concentrations for 4 h before cellular protein was collected for electrophoresis and immunoblotting with anti-cyclooxygenase-2 or β-actin antibodies. C, media from wounded monolayers, or subconfluent endothelial cells, contains an agonist for cyclooxygenase-2 expression. Endothelial cell monolayers were extensively wounded, washed, and given fresh media with 1% human serum. Alternatively, medium overlaying subconfluent cells was changed at this time to the reduced serum medium. After 4 h, medium from these cultures was transferred to fresh, undisturbed confluent endothelial cell monolayers that had just been serum-deprived by overnight incubation in media containing 1% human serum. These target monolayers were incubated for 4 h with media from wounded or subconfluent cells before the assay was stopped, and material collected for protein analysis and electrophoresis before cyclooxygenase-2, cyclooxygenase-1, and β-actin was determined by immunoblotting as before.
Endothelial cell monolayers are growth-arrested, and either release from an enforced cell cycle arrest or the addition of the growth factors in serum stimulates cyclooxygenase-2 expression in serum-starved fibroblasts (25). We tested the role of progression through the cell cycle on cyclooxygenase-2 expression in endothelial cells after wounding by including agents that interfere with deoxynucleotide synthesis (Ara-C and 5-fluorouracil) in S phase, that introduce a block at the G1/S boundary (aphidicolin and mimosine), and M phase (nocodazole) were present during this period at the concentrations stated under "Materials and Methods." The monolayers were then wounded, or not, in the high wounding pattern and incubated in the continued presence of the stated agents for 8 h before cellular material was collected for analysis of cyclooxygenase-2 (COX-2) expression by Western blotting. SDS-polyacrylamide gels contained 35 μg of cellular protein per well.

We found that endothelial cell motility was suppressed by about half after treating the cells with NS-398 (Fig. 7B). The effect of NS-398 was statistically significant at the 30 μM we used in other experiments, but we were unable to further reduce endothelial cell motility with higher concentrations of this cyclooxygenase-2 inhibitor. We conclude that cyclooxygenase products modulate motility but are not essential for cell migration. We inhibited cyclooxygenase-2 activity in cells isolated from subconfluent cultures, which again suppressed a portion of their motility, and then added PGE2 back to the isolated cells. We found that the addition of PGE2 partially restored cell motility in cells treated with NS-398 (Fig. 7C). The increase in migration is a chemokinetic enhancement of cell motility rather than direct chemotaxis by a gradient of PGE2 because PGE2 was added to both the upper and lower well and because a gradient would not be maintained over the 22 h of the experiment.

**DISCUSSION**

We find that individual endothelial cells not organized into a tightly confluent monolayer of cells express cyclooxygenase-2. Expression of this regulatory enzyme then diminishes as the cells form the intercellular contacts made possible by closely opposed cells in confluent monolayers. Cyclooxygenase-2 expression and PGE2 production were rapidly reestablished after an abrupt loss of neighboring cells when we mechanically wounded cultures of quiescent endothelial cell monolayers. Reactivation of cyclooxygenase-2 expression by wounding leads to increased synthesis and release of PGE2 to the surrounding media because the selective inhibitor NS-398 completely suppressed enhanced PGE2 secretion. Juxtacrine signaling, i.e., signaling by a neighboring cell, and paracrine and autocrine signaling by PGE2 are previously unknown mechanisms used to control expression of cyclooxygenase-2. This mode of regulation is relevant to angiogenesis before perfusion of the new vessel can occur, and is one that will have a major effect on the apparent background expression of cyclooxygenase-2 in cultured endothelial cells.

Denudation of vascular endothelium is a consequence of invasive clinical procedures from stent placement to grafting to venipuncture. This constitutes an inflammatory signal for the endothelium as shown by the synthesis of cyclooxygenase-2 and PGE2. Endothelial cells remaining after mechanical denudation responded to the extent of monolayer disruption with a graded accumulation of PGE2 and cyclooxygenase-2. Immunocytochemistry showed cyclooxygenase-2 protein expression by...
both cells adjacent and distal to the wound line, which suggests cells distal to the mechanical wound received information regarding the loss of cellular contacts from the cells immediately adjacent to the wound edge. Disrupting an endothelial cell monolayer with a fine scratch results in a wave of Ca$^{2+}$ excitation from the injured/surviving cells adjacent to the wound to distal cells that is critical for motility (26) and proliferation (27). In addition to this, we find a positive feed-forward loop involving the prostanoid PGE$_2$ after mechanical disruption of endothelial cell monolayers that aids recovery of monolayer integrity. PGE$_2$ stimulates endothelial cell cyclooxygenase-2 expression at low nanomolar levels, as recently found for cyclooxygenase 2 accumulation in pulmonary artery smooth muscle cells after bradykinin stimulation (28). PGE$_2$ accumulated to levels ($\sim$6 nM) sufficient to stimulate cyclooxygenase-2 expression after wounding confluent monolayers even in the absence of an exogenous source of arachidonate. We cannot directly support the autocrine/paracrine role of PGE$_2$ in stim-

![Image of Figure 7]
ulating cyclooxygenase-2 in our systems because inhibitors of cyclooxygenase-2 catalytic activity, including NS-398, themselves induce cyclooxygenase protein expression (12).

The amount of PGE₂ made in vivo may be enhanced by exogenous sources of arachidonate such as high density lipoprotein (29), but blood flow washing over the endothelium will likely restrict PGE₂ induction of cyclooxygenase-2 expression to those cells that synthesized the PGE₂. The PGE₂ feedback signaling loop, however, may extend to paracrine signaling in some compartments that are closed. For example, cyclooxygenase-2 accumulates in the endothelium throughout the central nervous system during acute peripheral inflammation (30) or burn injury (31). This increases PGE₂ levels in cerebrospinal fluid, to 3 and 0.3 nM respectively, and associates with hyperalgesia in these models. PGE₂ may also accumulate during angiogenesis where the endothelial cells at the leading edge are not part of an organized monolayer, and any PGE₂ released before perfusion of the new microvessel is established will be confined to that area.

Endothelial cells express cyclooxygenase-2 in response to diverse soluble agonists ranging from serum (32) to endotoxin (8) to cytokines (7, 9) to lipid agonists of peroxisome proliferator-activated receptors (12, 33). Cyclooxygenase-2 expression has not been characterized as a response to environmental stimuli such as intercellular contacts, although prostaglandin production from arachidonate by endothelial cells is modulated by the number of population doublings (34), and cell density has been shown to affect the amount of PGE₂ made in response to IL-1 stimulation of an osteoblast-like cell line (35). Cyclooxygenase-2 is also induced in a fibroblastic cell line after addition of serum to cells synchronized by complete serum starvation (25), but wounds endothelial cell monolayers does not induce cyclooxygenase-2 in this way. Growth factors were not added back to endothelial cell cultures after wounding, and inhibition of the cell cycle at various points did not block the increase in cyclooxygenase-2 induced by disrupting the integrity of the monolayer. Previous work (36, 37) shows that incorporation of [³H]thymidine into endothelial cells adjacent to a wound in a monolayer is a late event that occurs well after our experiments ended.

Cells adjacent to the mechanical wound migrate into the area by releasing at least some of their contacts with adjacent cells in the undisturbed portion of the monolayer to migrate as individual cells. Thus, we found a few individual cells in the denuded area 24 h after injuring the monolayer, but we also found a disorganized monolayer edge that extended into the wound space. We can ascribe a role for cyclooxygenase-2 in the migration of endothelial cells into the wound because NS-398 suppressed migration. However, we found it impossible to quantitate the rate of wound edge migration or cellular coverage of the denuded area over time because of the large heterogeneity in the organization of the monolayer edge. Instead, we recovered cells from both confluent and pre-confluent cultures, and we assayed their migration through 3-µm filters to find cells obtained from the pre-confluent condition migrated faster than their counterparts isolated from confluent monolayers. We also found that NS-398 suppressed migration, and so the recovered cells mimicked their counterparts in wounded cultures of endothelial cells. We also found that adding PGE₂ back to the NS-398-inhibited cells at least partially overcame the reduction in cell migration caused by the loss of cyclooxygenase-2 activity.

Cyclooxygenase-2 activity of host stromal tissue, e.g. the vasculature and supporting structures, has a profound effect on tumorigenesis (15). Cyclooxygenase-2 products act on tumor cells (3) and underpin the angiogenic response of host vascular tissue induced by tumor cells (38, 39). Thus, blocking the synthesis of cyclooxygenase-2-derived PGE₂ with inhibitors (18), genetically deleting cyclooxygenase-2 (3), or sequestering PGE₂ with an antibody suppresses tumor growth (18). Similarly, cyclooxygenase-2 has a critical role in the angiogenesis induced by inflammatory cytokines (22) and inflammatory insults (40). The production of PGE₂ also underlies the angiogenic effects of vascular endothelial cell growth factor and basic fibroblast growth factor (16, 41). Accordingly, application of PGE₂ into the connective tissue of rat femoral vessels causes intense vascular sprouting (42). PGE₂ functions as an autocrine and paracrine signal to modulate endothelial cell monolayer formation, and the regulatory enzyme cyclooxygenase-2 is controlled by the integrity of the monolayer.

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