Endothelial Cell Confluence Regulates Cyclooxygenase-2 and PGE$_2$

Production that Modulate Motility

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Running title: Endothelial Cell PGE$_2$ autocrine signaling
Summary

Endothelial cells line the vasculature, and, after mechanical denudation during invasive procedures or cellular loss from natural causes, endothelial cells migrate to reestablish a confluent monolayer. We find confluent monolayers of human umbilical endothelial cells are quiescent and expressed low levels of cyclooxygenase-2, but express cyclooxygenase-2 at levels comparable to cytokine-stimulated cells when present in a sub-confluent 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 sub-confluent cultures secrete enhanced levels of PGE$_2$ that depended on cyclooxygenase-2 activity, and PGE$_2$ stimulated cyclooxygenase-2 expression in confluent endothelial cell monolayers. Cells from sub-confluent monolayers migrated through filters more readily than those from confluent monolayers, and the cyclooxygenase-2 selective inhibitor NS-398 suppressed migration. Adding PGE$_2$ 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 endothelial cells not in contact with neighboring cells express cyclooxygenase-2 that results in enhanced release of PGE$_2$, and that this autocrine and paracrine loop enhances endothelial cell migration to cover denuded areas of endothelium.
Introduction

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 underlies this process. Prostanoids, which includes prostaglandin E$_2$ (PGE$_2$), PGF$_{2\alpha}$, PGD$_2$, PGI$_2$ and thromboxane A$_2$ (TXA$_2$), are cyclooxygenase products that are involved in angiogenesis and contribute to 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 PGI$_2$ 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 IL1β (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 PGE$_2$ (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 stromal cells, including endothelium, and not by the tumor cells themselves that is critical, and suggests that autocrine signaling in host stromal cells has a role in tumorigenesis. Tumors express high levels of PGE$_2$ and pharmacologic suppression of cyclooxygenase-2 activity, and not that of cyclooxygenase-1, depletes the PGE$_2$ and blocks tumorigenesis (18). A similar result obtained when PGE$_2$ was depleted with a monoclonal antibody (18), so PGE$_2$ 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 tubule. 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 of cells, results in enhanced PGE$_2$ secretion that aids endothelial cell migration to reestablish a confluent monolayer of endothelial cells.
Material and Methods

Reagents—NS-398 was purchased from Biomol (Plymouth Meeting, PA), the monoclonal antibodies against cyclooxygenase-1 and cyclooxygenase-2 from Cayman Chemical (Ann Arbor, MI), horseradish peroxidase conjugated goat anti-mouse antibody was from Biosource (Camarillo, CA), and PGE$_2$ 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 nacodozol were from EMD Biosciences (San Diego, CA). Transwell inserts with a black membrane (3 µm pore) were Discovery Labware obtained from BD Biosciences (San Jose, CA). IL1β, PMA, 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 three to five days, and then 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% CO$_2$. The wound repair model used freshly isolated endothelial cells grown to confluence in 6 well tissue culture plates, before 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, medium wounding employed 21 parallel wound lines, while for heavily wounded monolayers the plates were rotated 90° after the first medium wounding and then a further 21 parallel lines were etched in the monolayer to form square islands of remaining cells. The monolayers were washed after
wounding to remove the debris, and fed fresh medium that also contained any agonists to be included in the experiment. 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 cell migration**—Migration of endothelial cells was determined with a modified Boyden chamber assay. Briefly, polycarbonate filter wells (3 µ pores, BD Bioscience) were coated with fibronectin (10 µg/ml) for 1 h at room temperature and washed once with PBS. 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, the cells washed by low speed centrifugation, and then resuspended in culture medium containing a reduced concentration (1%) of human serum. The recovered cells (1.5 to 2.5 x 10⁴) were added to the upper chamber of a transwell insert and the filter inserts incubated in wells of a 24-well culture plate containing 750 µl of medium. NS398, 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 µg/ml for 30 min) and cell migration 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 (10x) fields were chosen and the number of cells in each field were 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 t-tests (GraphPad 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 starving them in 1% human serum for 17 h in the presence of the agents that interfere with cell cycle progression at distinct stages. Aphidicolin (5 µg/ml) blocks during G2 phase, mimosine (1 mM) blocks cell cycling during late G1/S phase, 5-fluorouracil (10 µM) and 1β-D-arabinofuranosylcytosine (Ara-C; 1 µM) interfere with deoxynucleotide synthesis in S phase, and nacodazole 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 wound lines were made through in each chamber of confluent endothelial cells using a 200 µl pipet tip, the remaining cells were washed and then fed with endothelial cells 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 4% paraformaldehyde, 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 (1.5 mg/ml for 5 min) was used to stain the nuclei before the images were recorded by confocal microscopy.
Immunoblot analysis—Cells were washed twice with PBS, and then with iced cell lysis buffer (20 mM Tris/HCl, 16 mM CHAPS, 0.5 mM DTT, 1 mM EDTA, 1 mM benzamidine hydrochloride, 1μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor). A cell scraper removed the cellular material, the lysates were kept on ice for 30 min, and then centrifuging at 4 °C for 5 min at 10,000 x g. Protein content of the supernatants was quantitated using a BCA protein assay (Pierce). Thirty micrograms of each sample was mixed with sample buffer and resolved in a 10% acrylamide gel by electrophoresis. Proteins in the gel were transferred to PVDF membranes and the resulting membranes were blocked overnight at 4 °C with 5 % dried milk, and then incubated with antigen specific antibodies (cyclooxygenase-2, Cayman Chemical Co; β-actin, ICN Biomedicals, Aurora, OH) and further reacted with appropriate secondary antibody. The horseradish peroxidase of the secondary antibody was detected by chemiluminescence (ECL, Amersham) according to manufacturer’s directions.

PGE\textsubscript{2} ELISA—Endothelial cells were grown to confluence, or their cognate subconfluent cultures from the same cord, in six well plates and extensively wounded with a 1000 µl pipette tip as described above. The residual cells were washed with serum free endothelial cell medium and fed fresh endothelial cell growth medium containing 1% human serum. After 8 hours, this medium was collected and PGE\textsubscript{2} was quantitated by a sandwich ELISA according to the manufacturer’s protocols. Some cultures were supplemented with arachidonic acid by washing the culture with HBSS and then adding 20 μM arachidonic acid in HBSS containing 0.1 % human serum for 30 min before this media was collected for PGE\textsubscript{2} ELISA. The data in these experiments were normalized by cell number.
Results

Cyclooxygenase-2 and PGE$_2$ production are regulated by cell contact. Inducible cyclooxygenase–2 has a key role in tumor angiogenesis (15,20) and the prostaglandin PGE$_2$ 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 (Fig. 1A) 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—inflammatory cytokines (TNF$\alpha$ and IL1-$\beta$), 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 $\beta$-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 PGE$_2$ than confluent cultures on a per cell basis when provided with exogenous arachidonate (Fig. 2B). Sub-confluent endothelial cells continuously produced twice as much PGE$_2$ as their confluent counterparts even when exogenous arachidonate was not provided to overcome the limiting level of endogenous arachidonate (Fig. 2C). Endothelial cell production of PGE$_2$ 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 PGE$_2$ 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 pipet 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 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 PMA 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 that in addition 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 to 8 h after wounding. The lower two panels of the figure are composite images that show the cells remaining between two wound lines, with the wounds being the black acellular area at the ends of the picture marked by the introduction of a white dotted line. These images show the majority of the green fluorescence representing cyclooxygenase-2, and indeed the brightest cells, was found well away from the wound edge, so cyclooxygenase-2 expression occurs in cells not mechanically affected by the wounding or by loss of neighboring cells.

*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*). 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 PGE₂. We pretreated the monolayers with NS-398 to inhibit cyclooxygenase-2 activity, and found that nearly all of the PGE₂ released from wounded monolayers came from this enzyme (Fig. 4A *lower*). The positive control for cyclooxygenase-2 expression, phorbol myristoyl acetate, also stimulated PGE₂ 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 cells and so we questioned whether PGE$_2$ acted on endothelial cells to induce the rate limiting enzyme for its synthesis, cyclooxygenase-2. We found (Fig. 4B) 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. 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 (Fig. 4C) that medium from sub-confluent endothelial cell cultures induced cyclooxygenase-2 expression in confluent monolayers to a greater extent than media from confluent cultures.

*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 (Fig. 5A) that endothelial cells migrated into the areas denuded by the soft pipet tip, which did not score the plate and create a barrier to migration, over time. 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 (Fig. 5A) extensive closure of the wound by 34 h, with little movement prior to 6 h (not shown) after wounding the monolayer. 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._ 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, 5-fluorouricil) in S phase, that introduce a block at the G\(_1\)/S boundary (aphidicolin, mimosine), or block microtubule cycling during the M phase (nacodazole). We found (Fig. 6) that wounding increases cyclooxygenase-2 protein in the presence of any of these inhibitors, and so wounding was unlike serum stimulation and was not dependent on cell replication.

_Endothelial cell motility is enhanced by cyclooxygenase-2 activity and PGE\(_2\)._ We quantified endothelial cell motility using a modified Boyden chamber by imaging and counting calcein-labeled cells. We found (Fig. 7A) that endothelial cells isolated from confluent cultures
migrated through the filter in the absence of an added agonist, and that the addition of the chemoattractant β-FGF increased the number of migrating cells. Endothelial cells isolated from sub-confluent cultures, however, were inherently more motile than their counterparts isolated from a confluent monolayer of cells. In fact, cells from a sub-confluent culture were as motile as cells from a confluent culture after being stimulated with β–FGF.

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 sub-confluent cultures, which again suppressed a portion of their motility, and then added PGE₂ back to the isolated cells. We found (Fig. 7C) that the addition of PGE₂ partially restored cell motility in cells treated with NS-398. The increase in migration is a chemokinetic enhancement of cell motility rather than direct chemoattraction 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. A parallel regulation of cyclooxygenase-2 expression and PGE\(_2\) production occurs subsequent to the abrupt loss of neighboring cells caused by mechanically wounding cultures of quiescent endothelial cell monolayers. Reactivation of cyclooxygenase-2 expression by wounding leads to increased synthesis and release of PGE\(_2\) to the surrounding media because the selective inhibitor NS-398 completely suppressed enhanced PGE\(_2\) secretion. Juxtacrine signaling, that is signaling by a neighboring cell, and paracrine and autocrine signaling by PGE\(_2\) is a previously unknown mechanism 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 endothelium as shown by the synthesis of cyclooxygenase-2 and PGE\(_2\). Endothelial cells remaining after mechanical denudation responded to the extent of monolayer disruption with a graded accumulation of PGE\(_2\) and cyclooxygenase-2. Immunocytochemistry showed cyclooxygenase-2 protein expression by both cells adjacent and those 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"+-excitation from the injured cells/surviving cells adjacent to the wound to cell 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 PGE₂ after mechanical disruption of endothelial cell monolayers that aids recovery of monolayer integrity. PGE₂ stimulates endothelial cell cyclooxygenase-2 expression at low nM levels, as recently found for cyclooxygenase 2 accumulation in pulmonary artery smooth muscle cells after bradykinin stimulation (28). PGE₂ accumulated to levels (~6 nM), sufficient to stimulate cyclooxygenase-2 expression after wounding confluent monolayers even in the absence of an exogenous source of arachidonate. We can not directly support the autocrine/paracrine role of PGE₂ in stimulating 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 HDL (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 feed forward signaling loop, however, may extend to paracine signaling in some compartments that are closed. For example, cyclooxygenase-2 accumulates in endothelium throughout the central nervous system during acute peripheral inflammation (30) or burn injury (31). This increases PGE₂ levels in cerebrospinal fluid, to 3 nM 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 \( \text{PGE}_2 \) 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 re-addition of serum to cells synchronized by complete serum starvation (25), but wounding endothelial cell monolayers does not induce cyclooxygenases-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 \([^3\text{H}]\text{thymidine}\) 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. We found it impossible, however, 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 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 found that here too 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 overcome 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 vasculature 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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Figure Legends

Figure 1. Sub-confluent endothelial cells express cyclooxygenase-2. (a). Top panels: Phase-contrast photographs of confluent (~200,000 cells/cm²) or subconfluent cultures (~40,000 cells/cm²) three days after plating human umbilical vein endothelial cells. Lower Panels: Endogenous levels of cyclooxygenase-2 protein in confluent and sub-confluent unstimulated endothelial cell cultures were imaged by immunocytochemistry as described in “Material 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 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 E. 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. Sub-confluent cells were not exposed to an exogenous agonist.

Figure 2. Endothelial cells down regulate cyclooxygenase-2 expression as they organize into confluent monolayers. (a) Western blot of cyclooxygenase-2 as a function of seeding density. Endothelial cells were plated at three densities, grown until the most densely plated culture became a tightly confluent monolayer before the cells were harvested for western analysis of cyclooxygenase-2 expression as described in “Material and Methods.” (b) Total PGE₂ synthetic capacity of confluent and pre-confluent endothelial cell cultures. The amount of PGE₂ made and released to the media over 30 min in the presence of 20 μM arachidonic acid.
acid was determined by ELISA as described in “Material and Methods.” (p< 0.001) (c) Cycloxygenase-2 accounts for the majority of PGE$_2$ released from either sub-confluent or confluent endothelial cells. Endothelial cells were pretreated, or not, in the absence of an exogenous source of arachidonate with 30 µM NS-398, and then maintained in this amount of cyclooxygenase-2 inhibitor over the subsequent 8 h.

Figure 3. Cyclooxygenase-2 and PGE$_2$ production are stimulated by wounding endothelial cell monolayers. (a) Patterns of monolayer wounding. Endothelial cells were grown to confluence in 6-well plates, and then portions of the monolayer were removed by scraping a pipet tip over the plate to create 7 (low wounding), 21 (medium wounding) or 42 (high wounding) cell free lines. (b) Wounding confluent endothelial cell monolayers induced cyclooxygenase-2 re-expression that correlates with the level of wounding. The 6-well plates were washed after wounding, the remaining cells fed with media containing 1% pooled human serum and incubated for 4 h before the cells were lysed and material was recovered for protein assay and electrophoresis. Equal amounts of protein were loaded on a 10% SDS-PAGE gel, the proteins resolved by electrophoresis, transferred to PVDF membranes, and immunoblotted with antibodies to cyclooxygenase-1, cyclooxygenase-2, or β-actin. (c) Spatial expression of cyclooxygenase-2 after wounding. Confluent monolayers of endothelial cells were grown to confluence on glass chamber slides that had been coated with fibronectin before multiple wound lines in a high wound pattern were created with a 200 µl pipette tip as above, or not, and then fixed at the stated times. Cyclooxygenase-2 protein expression was detected by immunocytochemistry and cell nuclei counterstained with propidium iodide as before. The lower two elongated panels are composite images capturing all the cells 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.

**Figure 4. Wounding stimulates cyclooxygenase-2 expression, stimulates PGE\(_2\) release, and PGE\(_2\) is an agonist for cyclooxygenase-2 expression in confluent endothelial cells.** Endothelial cells were grown to confluence in 6-well plates, the monolayer wounded in patterns that produce 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 Figure 2.

(b) PGE\(_2\) stimulates cyclooxygenase-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 \(\beta\)-actin antibodies. (c) Media from wounded monolayers, or sub-confluent 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, media overlaying sub-confluent cells was changed at this time to the reduced serum medium. After 4 h media 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 sub-confluent cells before the assay was stopped, material collected for protein analysis and electrophoresis, and cyclooxygenase-2, cyclooxygenase-1, and \(\beta\)-actin was determined by immunoblotting as before.
Figure 5. Closure of monolayer wounds is blocked by NS-398. (a) Endothelial cell monolayers were wounded with a pipet tip as above, and allowed to recover for the stated times. (b) Endothelial cell monolayers were imaged just after wounding with a pipet tip or maintained in the continuous presence of 100 µm NS-398 for 24 h before imaging.

Figure 6. Endothelial cell monolayers need not transverse the cell cycle to induce cyclooxygenase-2 after monolayer wounding. Endothelial cells were grown to confluence in 20% human serum, washed and serum deprived for 17 h in media containing just 1% human serum. Agents that interfere with cell cycle progression during S phase (5-fluorouracil, Ara-C), during late G1/S phase (aphidicolin, mimosine) and M phase (nacadazol) were present during this period at the at the concentrations stated in “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 expression by western blotting SDS-PAGE gels containing 35 µg of cellular protein per well.

Figure 7. PGE2 from cyclooxygenase-2 enhances endothelial cell migration. (a) Endothelial cells isolated from sub-confluent cultures migrate more slowly than those from sub-confluent cultures. Endothelial cells recovered from either confluent or sub-confluent cultures were added to the upper chamber of a transwell over a filter with 3 µm pores. After 22 h, the insert was removed, the cells stained with Calcein-AM and cells that had migrated through the filter were imaged by confocal microscopy and the number of cells in four random fields low power (10x) were enumerated (bottom). The positive control was 10 ng/ml β-fibroblast growth factor (βFGF). (b) NS-398 inhibits some, but not all, spontaneous
endothelial cell migration. Endothelial cells were recovered from sub-confluent cultures, added to the upper well of a transwell, and then NS-398 was added to the upper and lower migration chambers at the stated concentrations. Cells that had migrated through the filter after 22 h were imaged after staining with the fluorescent dye calcein AM as in panel a. (c) PGE\textsubscript{2} partially overcomes NS-398 inhibition of migration. Endothelial cells were isolated and treated with 100 \( \mu \text{M} \) NS-398 as above, but some wells additionally contained 5 nM PGE\textsubscript{2} in both the upper and lower wells. Migration was quantitated as in panel a.
References

Figure 3
H. Jiang, et al.
051404cs
Figure 4
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060104
Figure 6
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090304.ai
Figure 7
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Fold Change

NS398 (mM)

0 0.2 0.4 0.6 0.8 1.0

Fold Change

Vehicle NS398 + PGE2

Conf 30 50 100

NS398 Veh + PGE2

30 50 100 µM NS398

Non-confluent

βFGF (10ng/ml)

Confluent + βFGF (10ng/ml)

Confluent

Non-confluent

NS398

NS398 + PGE2

Vehicle

50 µm

50 µm

50 µm

50 µm