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Therapeutic levels of aspirin and salicylate directly inhibit a model of angiogenesis through a Cox-independent mechanism

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ABSTRACT **A range of antineoplastic properties is attributed to aspirin, thought to be due to inhibition of cyclooxygenase (Cox) enzymes in tumor cells. One important outcome is that by reducing angiogenic factor secretion by cancer cells, aspirin also inhibits angiogenesis, thereby restricting tumor growth. However, aspirin may also have direct effects on endothelial cells to regulate angiogenesis. Our aim was to quantitate these effects and determine whether they occurred through inhibiting Cox enzymes. The effects of aspirin, salicylate (the natural deacetylated form of aspirin), and the selective Cox inhibitors SC560 and Celecoxib on endothelial cell proliferation, viability, and angiogenesis were compared. Therapeutic aspirin concentrations (0.5 mM) had no detectable effect on endothelial cell viability or proliferation but caused a striking reduction in tubule formation in a three-dimensional collagen angiogenesis assay. This was also seen with equimolar concentrations of salicylate, while selective Cox inhibitors did not inhibit angiogenesis in this assay either alone or in combination. Furthermore, high doses of aspirin or salicylate (5 mM), well above therapeutic plasma concentrations, lead to endothelial cell apoptosis. We conclude that aspirin, at therapeutic concentrations, directly inhibits angiogenesis via a Cox-independent mechanism, which may significantly contribute to its neoplastic protective effects. —Borthwick, G. M., Johnson, A. S., Partington, M., Burn, J., Wilson, R., Arthur, H. M. Therapeutic levels of aspirin and salicylate directly inhibit a model of angiogenesis through a Cox-independent mechanism. *FASEB J.* 20, 2009–2016 (2006)**

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There is great interest in aspirin as a chemopreventive agent in colorectal cancer, as epidemiological data show it can reduce bowel cancer risk by as much as 50% (1). The mechanisms involved are complex, but accumulating evidence suggests that the protective effects of aspirin are partly due to its anti-angiogenic properties (2–6). If this is the case, then progression of small neoplastic lesions to more highly vascularized tumors is more likely to be affected by aspirin. Consistent with this hypothesis, recent randomized, placebo-controlled trials of aspirin in patients with prior adenomas showed equivocal effects on the frequency of small polyp formation but significant protective effects on the incidence of advanced adenomas (7–9). As targeting angiogenesis is showing great promise as an anticancer treatment in combination with standard chemotherapies, it is timely to pursue the anti-angiogenic properties of aspirin (10).

The protective effect of aspirin is often attributed to its ability to inhibit the cyclooxygenase enzymes Cox-1 and Cox-2 that are responsible for the synthesis of prostaglandins and to regulate many homeostatic functions as well as pain and inflammation (11, 12). Cox-1 is constitutively expressed in most cell types, whereas Cox-2 is generally present at low levels and is strongly induced in response to inflammatory stimuli (13–15). Recently, Cox-3, an isozyme of Cox-1, has been identified, which might be the therapeutic target of paracetamol and responsible for pain and fever (16).

Although aspirin has protective properties, its regular use carries with it an increased risk of severe gastrointestinal bleeding (17). In an effort to avoid these side effects, selective inhibitors that specifically target the inducible Cox-2 isoform were developed. There was particular interest in these inhibitors in the cancer field, because Cox-2 expression is associated with tumor progression (18, 19). Also, high Cox-2 expression in colorectal adenomas and colorectal carcinoma is associated with a high microvascular density and poor prognosis (20–23). Furthermore, Cox-2-selective inhibitors reduce polyp burden in animal models

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of intestinal neoplasia and, more importantly, in patients with familial adenomatous polyposis (24–26). Celecoxib is a selective Cox-2 inhibitor, and early work showed it had effective anti-inflammatory and analgesic properties with no gastric bleeding side effects (27). More recently, however, clinical trials of Celecoxib for colorectal adenoma prevention have highlighted a significant cardiovascular risk (28).

At present, the roles of Cox-1 and Cox-2 enzymes in tumor progression are still unclear. Their roles are complex as they function in both the tumor cells and the peri-tumor stromal environment. A key stromal response that regulates tumor progression is the angiogenic response, which is required to generate a sufficient blood supply for a solid tumor to grow beyond 2–3 mm^3 in size (29). Tumor cells secrete growth factors such as vascular endothelial growth factor (VEGF) that stimulate vascular cells nearby to form new blood vessels. A number of studies have shown that Cox-2 expression by tumor or stromal cells stimulates angiogenesis at the earliest stage of tumor development via induction of VEGF synthesis (2–5). Cox-2 inhibitors appear to modulate angiogenesis indirectly by reducing expression of angiogenic factors, such as VEGF in the tumor environment (6). However, any direct effects of non steroidal anti-inflammatory drugs (NSAIDs) on endothelial cells remain unclear. These are important to establish, because they should be more predictable than the responses of highly variable mutating tumor cells. This study focuses on the effect of aspirin on angiogenesis, and an *in vitro* approach was used to examine the direct effect of aspirin on endothelial cells.

MATERIALS AND METHODS

Drugs

Aspirin, salicylate (Sigma), Celecoxib (SC-58635, Pfizer), and SC-560 (Cayman) were dissolved in DMSO, and DMSO alone was used as a carrier control. Aspirin is rapidly converted to salicylate in water and was therefore replaced every 24 h by replacing the culture medium with fresh medium containing aspirin. Drugs were added after cells became adherent, normally 6 h after seeding. All the experiments below were performed in triplicate on at least three separate occasions.

Cell Culture

The immortalised endothelial cell line HMEC-1 was a kind gift from the Centre of Disease Control (Atlanta, GA) and was grown on plasticware precoated with 0.1% gelatin (30). Endothelial cell culture medium was MCDB131 medium (Invitrogen) supplemented with 10% FCS, L-glutamine (4 mM), hydrocortisone (1 μg/ml), and epidermal growth factor (10ng/ml).

Reverse transcriptase-polymerase chain reaction

Expression of Cox-1, Cox-2, and β-actin in HMEC1 cells was determined by extracting RNA using Trizol (following manufacturer's instructions) and performing rtPCR with reverse transcriptase (RT; Superscript II) and the following primers: Cox-1, forward primer 5′ggagtctcttgctccggttc; Cox-1 reverse primer, 5′aggtggcattgacaaactcc; Cox-2 forward primer, 5′caccctttcaaattcatgaccag; Cox-2 reverse primer, 5′caaatgattcatagggcttcag; β-actin forward primer, 5′tccacgaaactaccttcaac; β-actin reverse primer, 5′tttaggatggcaagggac. All primer pairs were designed to bracket at least one intron to allow positive recognition of polymerase chain reaction (PCR) products derived from cDNA (as opposed to genomic) templates on the basis of product size.

Cell proliferation and cell viability assays

The proliferation rate of endothelial cells was measured by seeding cells at 5×10^4 cells/well in 12-well plates and performing hemocytometric counting of adherent cells after incubation with different drugs concentrations for 96 h. 3-(4,5-dimethyl-2-thizolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assays were used to measure cell viability (31). This colorimetric assay is based on the cleavage of tetrazolium salts to form insoluble purple formazan in metabolically active cells. Cells were seeded at 5×10^3 cells/well in 96-well plates and cultured for 48 h in the indicated drug concentration before 10 μl MTT (5 mg/ml) was added to each well, and they were incubated for 5 h at 37°C. Cell media were removed, and formazan was solubilized in 100 μl DMSO before absorbance was measured at 570 nm.

Terminal dUTP nick-end-labeling assay

Apoptosis was assessed using a terminal dUTP nick-end-labeling (TUNEL) kit (Roche Diagnostics). Cells were seeded onto chamber slides (LAB-TEK II) and treated with the indicated drug concentrations for 48 h. The TUNEL assay was performed as directed by the manufacturer. Essentially cells were fixed in 4% paraformaldehyde, washed in PBS, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. Cells were washed well in PBS before addition of 50 μl TUNEL reaction mixture containing FITC conjugated dUTP to each well and incubated for 1 h at 37°C in the dark. Slides were washed thoroughly in PBS and mounted with Vectashield aqueous mounting medium for fluorescence with 4′,6′-diam idino-2-phenylidole (Vector Laboratories). Apoptotic cells were counted in three fields of view in each chamber at ×100 magnification.

Angiogenesis assays

Both Matrigel and three-dimensional (3D) collagen assays were used; 250 μl Matrigel (BD Biosciences) were added to prechilled wells on 12-well culture plates and allowed to set at 37°C for 30 min before seeding with cells. Cells were cultured for 24 h with various drug concentrations, and the effects of each treatment were recorded using digital photography at ×50 magnification.

For the 3D collagen angiogenesis assay, type I collagen (Sigma) was reconstituted in 0.1 M acetic acid, neutralized with sodium hydroxide, diluted in PBS and MCDB 131 medium, and used to coat 12 well plates at a final concentration of 5 mg/ml, with each well seeded with 1×10^5 cells (32). Endothelial cell medium containing ascorbic acid (50 μg/ml, Sigma), PMA (80 nmol/l), and the fibroblast growth factor (40 ng/ml) and VEGF (40 ng/ml; Peprotech) was added to each well. Tubule formation was quantified after 7 days using digital images of three different fields of view per well at ×80 magnification, and the total number of tubule branch points within each field was manually counted to quantify angiogenesis (33, 34).

Statistical analysis

Data were analyzed where relevant by unpaired Student's *t* tests and are mean ± SE. Prism 4.0 software (GraphPad) was used for statistical analyses.

RESULTS

Aspirin significantly inhibits endothelial cell proliferation and viability

The human endothelial cell line HMEC1 was used for these experiments, as it is a well-characterized microvascular cell line and represents the endothelial cell type that undergoes tumor angiogenesis *in vivo* (30). It has three important practical advantages for this study: it does not show the rapid progression to senescence shown by primary microvascular cells, it is not subject to batch variability due to donor heterogeneity, and it responds rapidly to angiogenic signals. To determine the suitability of this cell line for selective Cox inhibition studies, expression of both Cox isoforms, Cox-1 and Cox-2, was first confirmed by rtPCR (**Fig. 1*A***).

During angiogenesis, endothelial cells proliferate to form new blood vessels. To investigate the effect of aspirin on endothelial cell proliferation, cell numbers were counted after 96 h of culture in the presence of a range of aspirin concentrations (0, 0.5, 2 and 5 mM). *In vivo*, typical therapeutic levels range from 0.1–2 mM, while 5 mM is toxic (35). We found that aspirin inhibits endothelial cell proliferation in a dose-dependent manner at 2 and 5 mM, while little effect is seen at 0.5 mM compared with controls (Fig. 1*B*). In parallel, an MTT assay was used to determine the effect of aspirin on cell viability (31). Figure 1*B* shows that there was no detectable effect of 0.5 mM aspirin, whereas 2 and 5 mM aspirin caused a significant decrease in cell viability after 48 h. Because of the time scale of the MTT assay (48 h), it is possible that the apparent loss of viability may partly reflect the reduced levels of cell proliferation seen earlier. In addition, both outcomes may be due to increased levels of apoptosis triggered by aspirin. To investigate this, a TUNEL assay was performed. Figure 1*D* clearly shows that 5 mM aspirin triggers an extensive apoptotic response, but almost no apoptosis was detected in cells exposed to 0.5 mM aspirin.

Therapeutic levels of aspirin inhibit angiogenesis

The effect of aspirin on the ability of endothelial cells to form new vessels was initially assessed using a Matrigel assay. When endothelial cells are seeded onto Matrigel, they cease to proliferate and differentiate to form capillary-like tubules within 24 h (36). Aspirin inhibited angiogenesis in this assay in a dose dependent manner (**Fig. 2*A***). Compared with controls, tubes were more fragile and short-lived at 0.5 mM aspirin and few tubes formed at 2 and 5 mM aspirin. However, Matrigel suffers from the disadvantage that it triggers very transient tube formation that does not require new gene expression and is only two dimensional. In contrast, angiogenesis in a 3D collagen matrix requires gene expression and endothelial cells form stable capillary like tubes with lumens, making this a more physiological angiogenesis model (32, 37, 38). For these reasons, we used the 3D collagen system as our preferred angiogenesis model for quantitation. In this assay, aspirin significantly reduced levels of angiogenesis at all doses used (Fig. 2*B,C*). At the toxic aspirin dose (5 mM), this was largely due to extensive loss of endothelial cells (Fig. 2*B*) and was consistent with the high levels of apoptosis seen in Fig. 1*C*. We were particularly interested to note that the lowest aspirin concentration (0.5 mM) had a large and significant decrease in angiogenesis (60% reduction); yet, this dose had not generated any detectable decrease in cell viability or proliferation (Fig. 1*A,B*).

Inhibitors of Cox-1 and Cox-2 do not inhibit angiogenesis at selective doses

Both cyclooxygenase enzymes, Cox-1 and Cox-2, are inhibited by 0.5 mM aspirin; therefore, it may exert its inhibitory effects on angiogenesis through either of these enzymes. We therefore assayed the effects of selective inhibitors to determine whether inhibition of Cox-1 or Cox-2 or both caused the observed inhibition of angiogenesis. The specific Cox-1 inhibitor SC-560 (39) had no effect on cell proliferation or cell viability (data not shown) or on angiogenesis at selective doses of 50 nM and 1 μM (**Fig. 3*A***). However, 50 μM SC560, a dose 5,000 times the IC50 for Cox-1, did lead to a small decrease in branch formation (Fig. 3*A*). Selective doses of Celecoxib, a specific Cox-2 inhibitor, had no effect on cell proliferation or cell viability (data not shown). Similarly, Celecoxib had no detectable effect on angiogenesis even at 5 μM, 50 times higher than the selective dose (Fig. 3*B*). At 400 times the selective dose (40 μM), Celecoxib caused an 85% decrease in branch formation (Fig. 3*B*), but the targets of Celecoxib at such high doses are not known. To test for a possible combined effect of Cox-1 and Cox-2 inhibition, selective doses of Celecoxib and SC-560 were added simultaneously. There was still no detectable effect on angiogenesis (Fig. 3*B*), supporting the conclusion that aspirin inhibited angiogenesis through a Cox-independent pathway.

Salicylate inhibits angiogenesis

Aspirin is rapidly hydrolyzed to salicylate in contact with water, and it is possible that aspirin acts through salicylate to produce the observed effects in this study. Therefore, the effect of salicylate alone was determined. In the MTT assay, low concentrations of salicylate (1 and 0.5 mM) had no detectable effect on cell viability (**Fig. 4*A***). However, 2 and 5 mM caused a significant reduction in viability in a similar way to aspirin (see Fig. 1*B*). With the use of TUNEL assays to

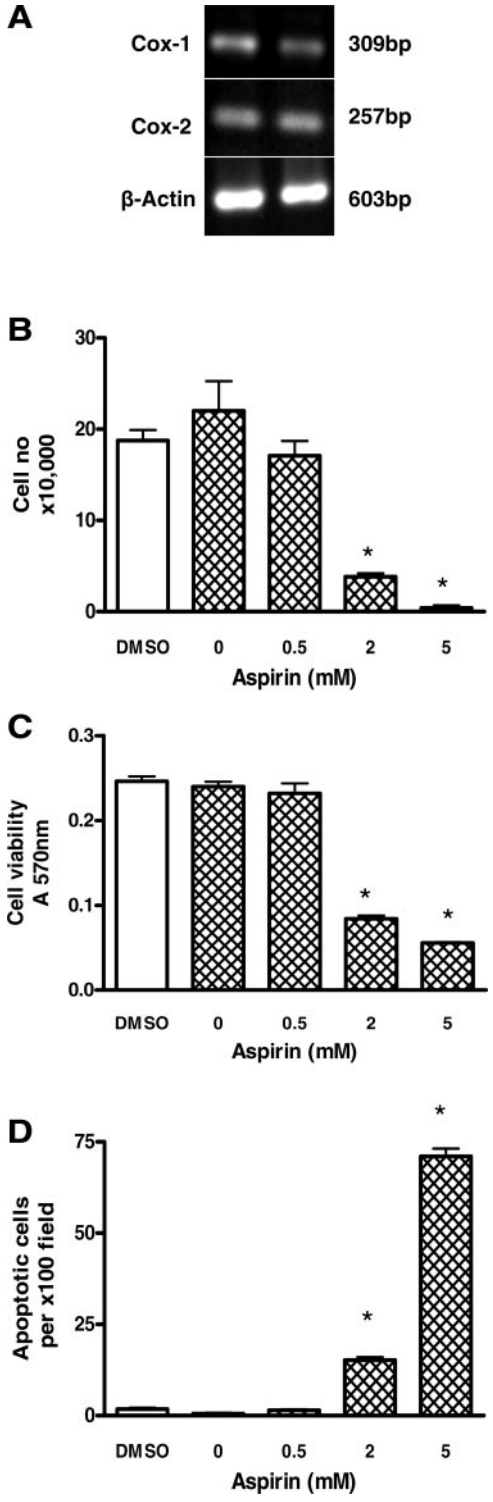

Figure 1. Aspirin inhibits endothelial cell proliferation and reduces cell viability. rtPCR showing expression of Cox-1 and Cox-2 in HMEC cells in 2 independent experiments. PCR products sizes are indicated, and β-actin product was used as a template control (*A*). Treatment with 2 and 5 mM aspirin leads to significant reduction in HMEC cell numbers after 96 h, but there is no change at 0.5 mM aspirin compared with carrier control (DMSO; *B*). Cell viability in the MTT assay is significantly decreased at 2 and 5 mM aspirin after 48 h, but there is no detectable change at 0.5 mM aspirin compared with carrier control (*C*). Apoptotic cells, detected by TUNEL staining, were significantly increased after 48 h treatment with 2 and 5 mM aspirin, but there was no increase at 0.5 mM aspirin compared with carrier control (*D*). Data, expressed as mean ± SE, were analyzed using Student's *t* test; *statistical significance, at $P < 0.0001$, compared with controls. Each figure represents 3 independent experiments.

investigate apoptosis, high levels of apoptosis are seen when cells are treated with 5 mM salicylate, again mirroring the effects seen with aspirin (Fig. 4*B*). Finally, salicylate had almost identical effects to aspirin in the 3D collagen angiogenesis assay (Fig. 4*C*). The complete abolition of angiogenesis in cells exposed to 5 mM salicylate may have been due to the high frequency of apoptosis at this dose. In contrast, a low dose (0.5 mM) of salicylate caused a 68% decrease in angiogenesis without detectable loss of cell viability, mirroring the effects seen with equimolar concentrations of aspirin (compare with Fig. 2*C*).

DISCUSSION

We have shown that high levels of aspirin or salicylate (5 mM) cause apoptosis of microvascular endothelial cells. This could be due to a number of possible mechanisms. Salicylate is known to activate the p38 MAP kinase stress pathway and inhibit the ERK survival pathway, and either response would lead to increased levels of apoptosis (40, 41). In addition, apoptosis may result from activation of the NF-κB pathway. Although aspirin and salicylate are generally considered to inhibit this pathway (42), more recent studies have shown that aspirin can activate NF-κB signaling and stimulate apoptosis in colorectal cancer cell lines (43). We have preliminary data showing loss of Iκ-Bα in endothelial cells treated with 5 mM aspirin for 24 h (not shown) suggesting that aspirin induced activation of NF-κB signaling may play a part in the apoptotic response. *In vivo*, when aspirin is ingested, the stomach is exposed to particularly high concentrations, and even two standard 300 mg aspirin tablets (3.3 mM) may result in stomach fluid concentrations of up to 30 mM. These high concentrations of aspirin are known to cause ulceration of the gastrointestinal epithelium (44). Our data suggest that these locally toxic aspirin concentrations will also lead to endothelial cell apoptosis, which would increase the likelihood of gastric bleeding and contribute to poor healing of ulcerated epithelium.

Aspirin concentrations approximating to systemic pharmacological concentrations (0.5 and 2 mM) did not lead to notable levels of apoptosis. However, there was significant reduction in endothelial cell viability and proliferation at 2 mM aspirin, corresponding to a high therapeutic plasma concentration. At 0.5 mM aspirin, equivalent to a low therapeutic plasma concentration, we did not detect any significant decrease in endothelial cell proliferation nor loss of cell viability. Yet, this dose leads to a significant decrease in the

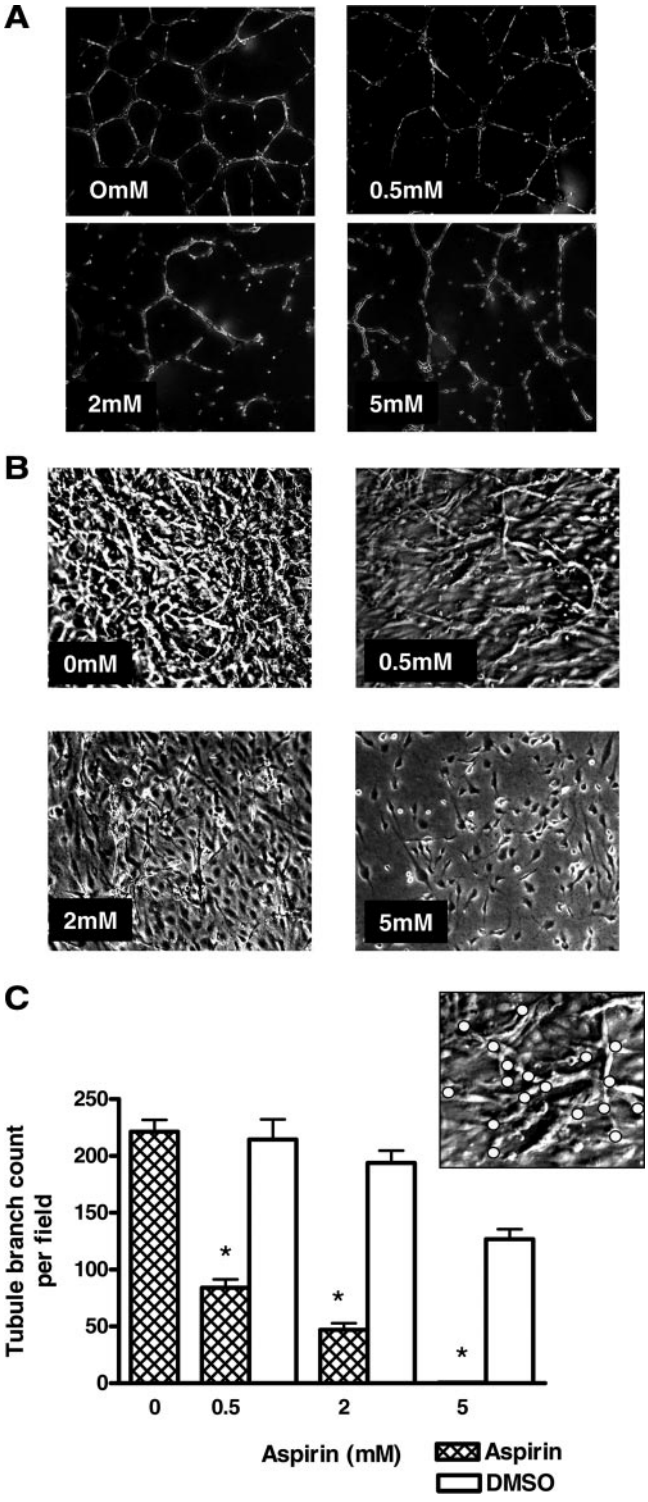

Figure 2. Aspirin inhibits angiogenesis. HMECs were treated with a range of aspirin doses in Matrigel (*A*) and 3D collagen (*B*). Transient tube formation in Matrigel and stable tube formation in 3D collagen were recorded photographically. Quantitation of effect of aspirin on angiogenesis in 3D collagen at day 7 was performed by counting branch points (insert) and 3 fields of view were analyzed per replicate well. *C)* A significant decrease ($P<0.0001$) in angiogenesis was observed at 0.5, 2, and 5 mM aspirin. Data, expressed as mean ± SE, were analyzed using Student's *t* test; *statistical significance, at $P < 0.0001$, compared with controls. Each figure represents 3 independent experiments.

ability of cells to form tubules in the 3D collagen angiogenesis assay. This suggests that low therapeutic doses of aspirin significantly inhibit the ability of endothelial cells to undergo the remodeling involved during angiogenesis. Reduced angiogenesis may be an important feature of gastric injury and reduced healing but will also be vitally important in restricting cancer growth.

Interestingly, we did not detect reduced angiogenesis after treatment with the specific inhibitors of Cox-1 or Cox-2 at selective doses. Although Cox-2 is known to be important in regulating angiogenesis, its reported mode of action on endothelial cells is an indirect one. Essentially it induces high levels of VEGF in cancer or peritumor stromal cells that stimulate angiogenesis (2–5, 45–48). Inhibition of Cox-2 reduces VEGF production, which leads to decreased endothelial cell proliferation, increased apoptosis, and decreased angiogenesis (49, 50). The latter outcomes may be due to losing the powerful prosurvival and antiapoptotic signals generated by VEGF (51, 52). Thus, current evi-

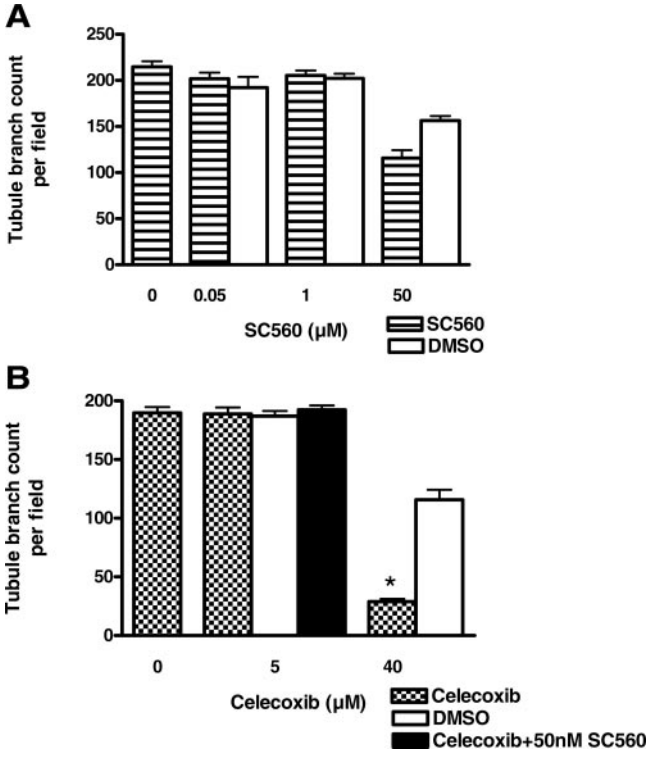

Figure 3. Angiogenesis is not inhibited by selective doses of Cox inhibitors. Angiogenesis in 3D collagen was quantitated at day 7 of treatment with various inhibitors in same way as for Fig. 2. The selective Cox-1 inhibitor, SC560, did not affect angiogenesis at selective concentrations (50 and 1 μM), but there was an inhibitory effect of 50 μM SC560, 5000× selective dose (*A*). Celecoxib did not inhibit angiogenesis at selective dose of 5 μM, but did have an inhibitory effect at 40 μM, 400× greater than selective dose (*B*). When selective concentrations of SC560 and celecoxib were combined, no decrease in angiogenesis was observed (*B*). Data, expressed as mean ± SE, were analyzed using Student's *t* test; *statistical significance at $P < 0.0001$, compared with controls. Each figure represents 3 independent experiments.

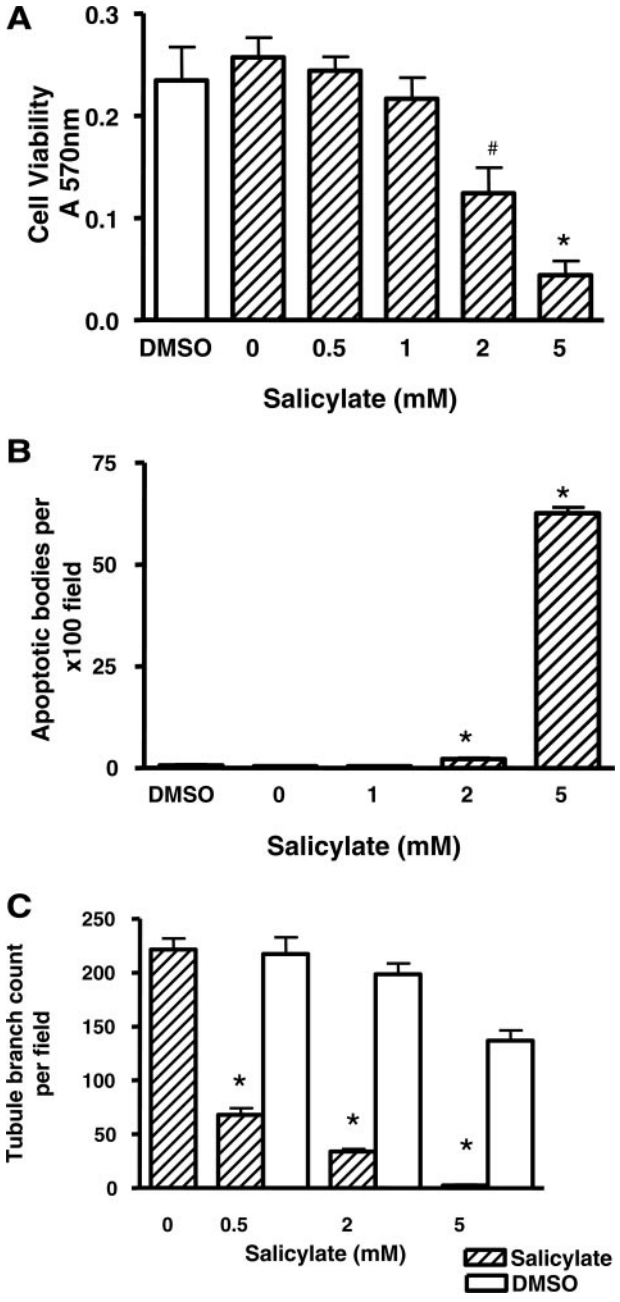

Figure 4. Salicylate reduces cell viability and inhibits angiogenesis. Cell viability in the MTT assay is significantly decreased at 2 and 5 mM salicylate after 48 h, but there is no detectable change at 0.5 mM salicylate compared with carrier control (*A*). Apoptotic cells, detected by TUNEL staining, were significantly increased following 48 h treatment with 2 and 5 mM salicylate, but there was no increase at 0.5 mM salicylate compared with carrier control (*B*). Salicylate treatment caused a significant decrease in angiogenesis in the 3D collagen assay at 0.5, 2, and 5 mM, mirroring effects of aspirin at equimolar concentrations (*C*). Data, expressed as mean ± SE, were analyzed using Student's *t* test. *Statistical significance at $P < 0.0001$ and #statistical significance at $P < 0.02$, compared with controls. Each figure represents 3 independent experiments.

dence suggests that Cox-2 promotes angiogenesis indirectly through a cancer-stromal interaction via VEGF, and, consistent with this evidence, VEGF synthesis in colon cancer cells is inhibited by Cox-2 inhibitors (53). This is in agreement with early *in vitro* work that showed that in the absence of growth factors, a selective Cox-2 inhibitor (NS-398) can inhibit tube formation in Matrigel angiogenesis assays (54). However, in the presence of the exogenous growth factors VEGF and fibroblast growth factor, we observed that Cox-dependent inhibition is lost and additional inhibitory effects of aspirin on angiogenesis become apparent. We would argue that the Cox-independent responses to low dose aspirin would also be important *in vivo* for inhibiting angiogenesis in cancer, particularly when growth factor secretion by tumor cells is strongly stimulated in the hypoxic environment of a growing tumor.

Inhibition of Cox-1 with selective doses of SC560 had no detectable effect on cell viability, proliferation, or angiogenesis in our assays. In contrast, it has been reported that depletion of Cox-1 in HUVECS using antisense oligonucleotides caused a reduction in angiogenesis, suggesting that Cox-1 may directly regulate angiogenesis of endothelial cells (48). A possible reason for the discrepancy here is that different endothelial cell types have different prostanoid profiles. PGF2a and PGE2 are the major metabolites of microvascular endothelial cells such as HMEC1, which we used in this study, whereas PGI2 is the major metabolite in large blood vessels (55). Thus, Cox-1 may have roles in HUVECs that are not required in microvascular cells. The Cox-1-independent angiogenesis of microvascular cells reported here may be more relevant in the context of tumor angiogenesis than umbilical vein endothelial cells. When Celecoxib and SC560 treatments were combined to inhibit both Cox-1 and Cox-2, again there was no detectable effect on endothelial cell viability or angiogenesis at selective doses, suggesting that aspirin inhibits angiogenesis of microvascular cells by a Cox-independent mechanism.

Aspirin inactivates Cox-1 and Cox-2 enzymes by acetylating them, but aspirin is rapidly hydrolyzed to salicylate in water and salicylate does not efficiently inhibit cyclo-oxygenase activity (56). As salicylate inhibited angiogenesis equally potently to aspirin in our assays, this is consistent with the idea that aspirin and salicylate inhibit angiogenesis by directly targeting endothelial cells through a Cox-independent pathway. There are a number of possible alternative pathways that could be responsible and that are altered in the presence of either aspirin or salicylate (57). These include pathways regulating ERK kinase, cyclin E, and cdc2 (41). There is also evidence that these pathways are important for angiogenesis (58), but further work is required to dissect which pathway is responsible for the inhibitory effect of aspirin and salicylate on angiogenesis and how this contributes to the neoplastic protective effect of NSAIDs in cancer prophylactic treatments. Nonetheless, it is clear that low therapeutic doses of aspirin have a direct inhibitory effect on the ability of endothelial cells to undergo angiogenesis and that this may con–

tribute to the antineoplastic affects claimed for aspirin in a wide range of tissue types. FJ

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