[CANCER RESEARCH 59, 4574–4577, September 15, 1999]

Advances in Brief

Thromboxane A_2 Is a Mediator of Cyclooxygenase-2-dependent Endothelial Migration and Angiogenesis[1]

Thomas O. Daniel,[2] Hua Liu, Jason D. Morrow, Brenda C. Crews, and Lawrence J. Marnett

Departments of Medicine, Cell Biology, Biochemistry, and Pharmacology, Divisions of Nephrology and Hypertension and Clinical Pharmacology, The Vanderbilt Center for Vascular Biology, Vanderbilt Cancer Center, Vanderbilt University, Nashville, Tennessee 37232

Abstract

Cyclooxygenase-2 (COX-2) inhibitors reduce angiogenic responses to a variety of stimuli, suggesting that products of COX-2 may mediate critical steps. Here, we show that thromboxane A_2 (TXA_2) is one of several eicosanoid products generated by activated human microvascular endothelial cells. Selective COX-2 antagonists inhibit TXA_2 production, endothelial migration, and fibroblast growth factor-induced corneal angiogenesis. Endothelial migration and corneal angiogenesis are similarly inhibited by a TXA_2 receptor antagonist, SQ29548. A TXA_2 agonist, U46619, reconstitutes both migration and angiogenesis responses under COX-2-inhibited conditions. These findings identify TXA_2 as a COX-2 product that functions as a critical intermediary of angiogenesis.

Introduction

Recent evidence suggests that COX-2[3] metabolic products contribute to neovascularization and may support vasculature-dependent solid tumor growth and metastasis. Selective COX-2 inhibitors are antiangiogenic (1), and COX-2-null mice are substantially protected in a genetic model of human familial adenomatous polyposis (2). Forced COX-2 overexpression enhances the metastatic potential of CaCo-2 colon carcinoma cells through processes that are sensitive to COX-2 inhibitors (3). Coculture of endothelial cells with tumor cells promotes COX-2-dependent endothelial motility and assembly into capillary-like structures (4), an effect that is attributed to tumor cell release of angiogenic peptides and nitric oxide. Alternatively, eicosanoids synthesized by endothelial COX-2 may contribute to this effect.

COX-2 expression or function is induced in cultured endothelial cells in response to phorbol esters (5, 6), basic FGF (7), hypoxia (8), cyclic strain (9), thrombin, interleukin 1α (10), or interleukin 1β (11). Hypoxia (12) or lipopolysaccharide administration (13) induce microvascular endothelial COX-2 expression *in situ*. Moreover, COX-2 inhibitors have been shown to decrease urinary excretion of prostacyclin, a major product of vascular endothelium in human subjects (14). These findings motivated our efforts to identify a COX-2 product or products that are capable of functioning as intermediaries of angiogenesis.

Received 5/21/99; accepted 8/2/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by USPHS Awards RO1 DK38517 and P50 DK39261(to T. O. D.), CA47479 (to L. J. M.), and DK48831, GM42056, DK26657, and GM15431 (to J. D. M.) as well as a by a center grant from the National Cancer Institute (Grant CA68485) . J. D. M. is the recipient of a Burroughs-Wellcome Fund Clinical Scientist Award in Translational Research. The T. J. Martell Foundation provided support critical to this work.

[2] To whom requests for reprints should be addressed, at Division of Nephrology, MCN S3223, Vanderbilt University Medical Center, Nashville, TN 37232-2372. Phone: (615) 343-8496; Fax: (615) 343-7156; E-mail: tom.daniel@mcmail.vanderbilt.edu.

[3] The abbreviations used are: COX-2, cyclooxygenase-2; FGF, fibroblast growth factor; bFGF, basic FGF; TXA_2, thromboxane A_2; PMA, phorbol myristate acetate.

Materials and Methods

Eicosanoids and Quantitation. All of the eicosanoids and eicosanoid agonists were purchased from Cayman Chemical, (Ann Arbor, MI). Eicosanoids were quantified by gas chromatographic negative ion chemical ionization mass spectrometric assays using the precise and accurate stable isotope dilution technique (15).

Endothelial Migration. Confluent human renal microvascular endothelial cells were grown to confluency and serum-depleted in medium containing 1% (w/v) bovine albumin for 18 h prior to assay (16). Triplicate circular "wounds" (600–900 μm in diameter) were generated in confluent endothelial monolayers within a single well, using a rotating silicon-tipped drill bit mounted on a drill press, to avoid scoring subjacent surfaces. Medium was supplemented at the time of wounding with test agents at concentrations indicated in the figures. Residual fractional wound areas were measured using a Bioquant (Nashville, TN) software package calibrated to a Nikon Diaphot microscope. Mean fractional residual areas of three wounds, calculated at each of two or three time points (see Fig. 1*b*), were used to derive linear regressions, reflecting migration rates (expressed as percentage closure per h $\pm$ 95% confidence intervals).

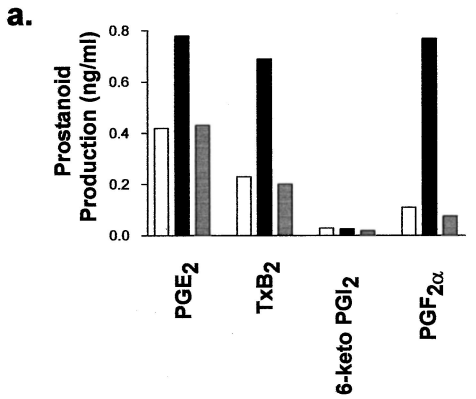
Mouse Corneal Angiogenesis Assay. Hydron pellets incorporating sucralfate with vehicle alone, basic FGF (a kind gift from Scios, Inc.), or bFGF in combination with other agents indicated in the figure legends were surgically implanted into corneal stromal micropockets, created 1 mm medial to the lateral corneal limbus of C57BL mice (7–10 weeks old), as described previously (17). On day 5, corneas were photographed at an incipient angle of 35–50° from the polar axis in the meridian containing the pellet, using a Zeiss slit lamp. Images were digitalized and processed by subtractive color filters (Adobe Photoshop Version 4.0), as displayed (see Figs. 3 and 4). Images were analyzed using Bioquant image analysis software to determine the fraction of the two-dimensional total corneal image that was vascularized and the fraction of pixels within that area (regional density) or within the corneal perimeter (total density) that exceeded a threshold matching visible capillaries.

The dose of VU08 used (5 mg/kg) was selected based on anti-inflammatory responses in a carrageenan foot pad assay and on effects of this dose to suppress TXA_2 production by endogenous COX activity in prostate tumor tissue issue (85% inhibition at 3 days daily i.p. injection). Concentrations of SQ29548 were selected based on a dose-response experiment optimizing for inhibition of FGF-induced angiogenesis (data not shown).

Results and Discussion

Fig. 1*a* provides the profile of eicosanoids produced by cultured microvascular endothelial cells under COX-2-induced conditions, following stimulation by PMA. Prostaglandin E_2, TXA_2 (measured indirectly as its thromboxane B_2 metabolite), and prostaglandin F-2α were the dominant PMA-induced products, and induced endothelial production of each was blocked by coincident exposure to a COX-2-selective inhibitor. Notably, basal endothelial capacity to produce these metabolites was maintained in the presence of COX-2 inhibition, consistent with production by constitutive endothelial COX-1.

To assess functional consequences of endothelial COX-2 inhibition, we evaluated endothelial motility. The rates at which endothelial cell migration closed replicate circular wounds in confluent monolayers were determined by quantitating residual wound areas in digital images that were captured at multiple points during a 12-h time



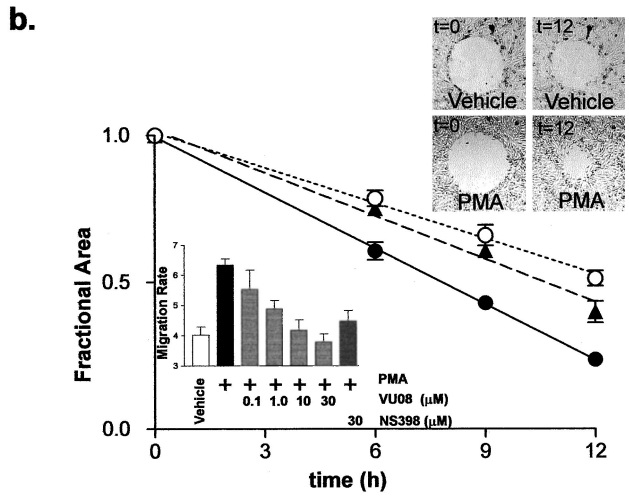


Fig. 1. *a*, COX-2-selective antagonist, VU08, inhibits PMA-induced eicosanoid production. Cultured human renal microvascular endothelial cells were exposed to vehicle (□), PMA (■; 20 ng/ml), or PMA supplemented with a COX-2 selective antagonist, VU08 (▨; 10 μM; IC_{50} against purified COX-2 was 50 nM; IC_{50} against purified COX-1 was 66 μM), for 8 h. Arachidonic acid (10 μM) was added, medium was collected after a 60-min incubation, and prostanoid products were quantified (15). *b*, COX-2-selective inhibitor, VU08, inhibits PMA-induced endothelial migration. PMA-induced endothelial migration was assayed using a video capture/image analysis system (Bioquant, Nashville, TN) to follow the rate at which endothelial migration covered triplicate 600–900-μm-diameter circular wounds created in a confluent monolayer ("Materials and Methods"). At the time of wound initiation, medium was supplemented with vehicle (○), PMA (20 ng/ml; ●) or PMA with the COX-2 inhibitor, VU08 (▲), at the concentrations indicated. *Top inset*, example of the residual wound areas remaining after 12 h incubation in cells treated with vehicle (*top*) or PMA (*bottom*). *Data points*, means of residual wound areas expressed as fractions of the original wound (*Fractional Area*) at 6, 9, and 12 h; *bars*, SE. *Columns*, migration rates, modeled by linear regression ($r^2 > 0.97$ for each condition) and expressed as the percentage of the original wound area covered per hour; *bars*, 95% confidence limits (*bottom inset*). Two different COX-2 inhibitors, NS398 and VU08, inhibited the PMA induced migration rate. The effective IC_{50} was ~1 μM for VU08.

course. PMA reproducibly stimulated the rate of endothelial migration over that of untreated cells (Fig. 1*b*), and the PMA-induced migration was blocked by two different COX-2-selective inhibitors, NS398 and VU08.

The effect of COX-2 inhibition to reduce endothelial production of eicosanoids, coupled with its effect to inhibit endothelial migration, led us to ask whether supplementation with specific eicosanoids would reconstitute PMA-induced migration in the presence of COX-2 inhibition. Shown in Fig. 2*a*, the TXA_2 mimetic, U46619, reconstituted a near full migratory response to PMA under COX-2-inhibited conditions. Because endothelial cells produce TXA_2 (Ref. 18; Fig. 1*a*) and express functional TXA_2 receptors (19), we evaluated further the effects of U46619 and the thromboxane receptor antagonist, SQ29548.

Shown in Fig. 2*b*, U46619 reconstituted PMA induced migration responses in the presence of COX-2 inhibition, with an ED_{50} in the range of 0.1–1.0 μM, consistent with its affinity for thromboxane receptors (20). Importantly, Fig. 2*c* shows the TXA_2 receptor antagonist, SQ29548, blocked PMA-induced endothelial migration, with an IC_{50} of ~0.1 μM, also consistent with its reported affinity for thromboxane receptors (20). U46619 alone did not stimulate migration rates above basal levels in the absence of PMA (data not shown), suggesting TXA_2 participates as a requisite but permissive contributor to induced migration.

To test the hypothesis that TXA_2 may be a mediator of angiogenic responses promoted by COX-2, we evaluated effects of systemic COX-2 inhibition upon corneal angiogenesis in a mouse pellet implantation model. Systemic administration of a selective COX-2 in-

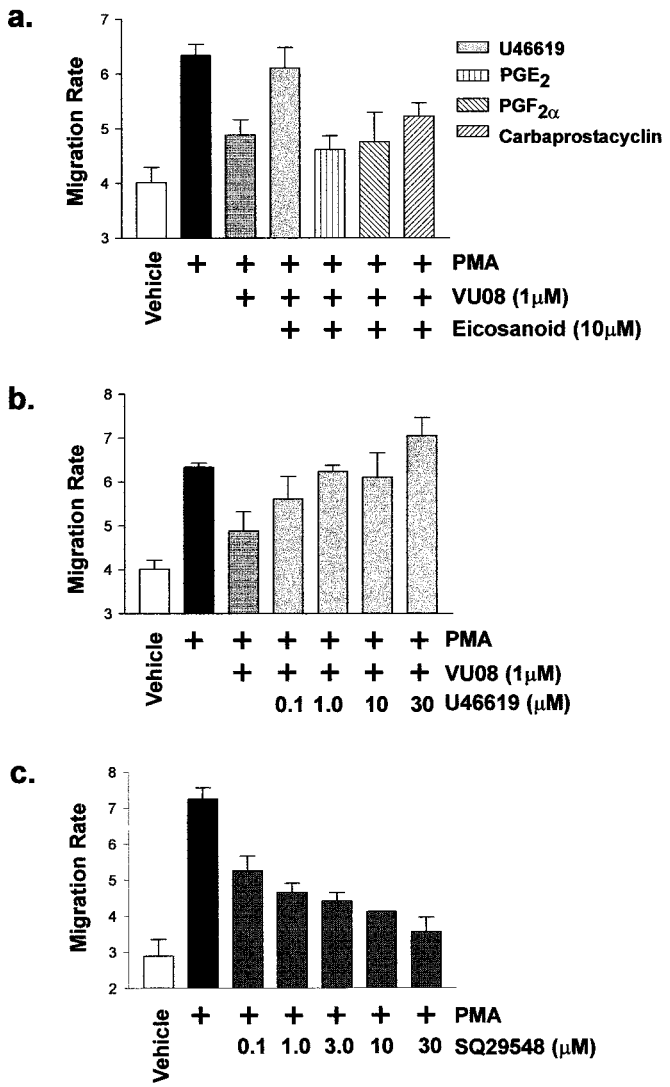


Fig. 2. *a*, TXA_2 agonist, U46619, reconstituted PMA-induced endothelial migration during COX-2 inhibition. Endothelial migration rates (percentage reduction in residual area/h were evaluated over 12 h following addition of PMA (20 ng/ml); a selective COX-2 inhibitor, VU08 (1 μM); and specific eicosanoids in combinations and at concentrations indicated. Values represent linear regression of the slope ($-\Delta$ area/time) ± 95% confidence intervals. Differences in migration rate in the absence or presence of U46619 (*third and fourth bars from the left*) is significant ($P < 0.05$). *b*, dose response of TXA_2 agonist, U46619, to reconstitute PMA-induced migration under COX-2-inhibited (VU08, 1 μM) conditions. Endothelial migration rates were evaluated as above (ED_{50} of ~0.1 μM). *c*, TXA_2 receptor antagonist, SQ29548, blocks PMA-induced endothelial migration. Endothelial migration rates were evaluated for endothelial cells exposed to vehicle, PMA (20 ng/ml) or PMA supplemented with SQ29548 at concentrations indicated. *a–c*: *columns*, migration rates; *bars*, 95% confidence intervals.

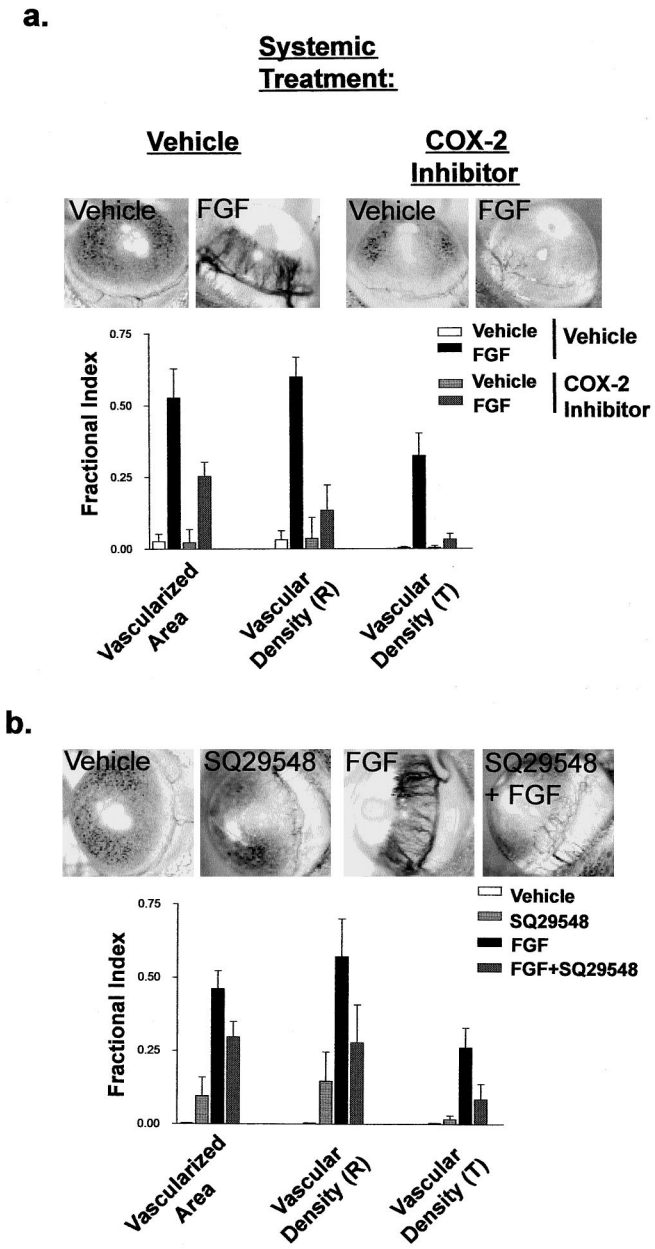


Fig. 3. COX-2 inhibitor, VU08, and TXA$_2$ receptor antagonist, SQ29548, attenuate FGF-induced corneal angiogenesis. *a*, hydron/sucralfate pellets impregnated with vehicle or basic FGF (3 pmol) were placed, as described previously (17), in the corneal stroma of mice treated systemically by daily i.p. injection of vehicle ($n = 4$) or COX-2 inhibitor, VU08 (5 mg/kg; $n = 8$), beginning 1 day prior to implantation. Corneal angiogenic responses were photographed on day 5, and digitized images were quantified ("Materials and Methods"). COX-2 inhibition markedly attenuated (significant at $P < 0.05$) FGF-induced corneal angiogenesis, expressed as the fractional vascularized area, the regional vascular density (*R*), or the total vascular density (*T*). *b*, hydron/sucralfate pellets impregnated with vehicle, SQ29548 (1.5 nmol, $n = 9$), bFGF (3pmol, $n = 9$), or SQ29548 combined with bFGF ($n = 9$), were placed in the corneal stroma and evaluated on day 5, as above. SQ29548 attenuated FGF-induced angiogenesis by each parameter (significant at $P < 0.05$).

hibitor imposed a marked inhibitory effect on the angiogenic response to basic FGF in the corneal model, reducing the area vascularized by 52% and the density of vascularity within that area by 80% (Fig. 3*a*). This provided strong evidence that a COX-2 metabolite participates in angiogenic responses to FGF.

Consistent with a role for TXA$_2$, local administration of the TXA$_2$ receptor antagonist, SQ29548, in the corneal pellet inhibited FGF-stimulated angiogenesis, reducing the vascularized area by 40% and the vascular density within that vascularized area by 51% (Fig. 3*b*). Although vascular flow in the ocular circulation is sensitive to TXA$_2$ mimetics (21), the response is vasoconstrictive, and the TXA$_2$ receptor antagonist should promote vasodilatation and capillary filling rather than the attenuation we observed. Thus, TXA$_2$ appears to function as an *in vivo* mediator of FGF-stimulated angiogenesis.

The action of the TXA$_2$ agonist U46619 to reconstitute endothelial migration under COX-2-inhibited conditions (Fig. 2*a*) suggested the possibility of reconstituting corneal angiogenic responses to bFGF in the setting of systemic COX-2 inhibition. Shown in Fig. 4, locally administered U46619 showed a striking capacity to repair the attenuated angiogenic response seen in the setting of COX-2 inhibition, returning the vascularized area to 80% and the vascular density within that area to 95% of levels achieved with FGF in the absence of COX-2 inhibition. U46619 alone was not angiogenic, and it did not amplify on the bFGF response in animals with intact COX-2 function (vehicle).

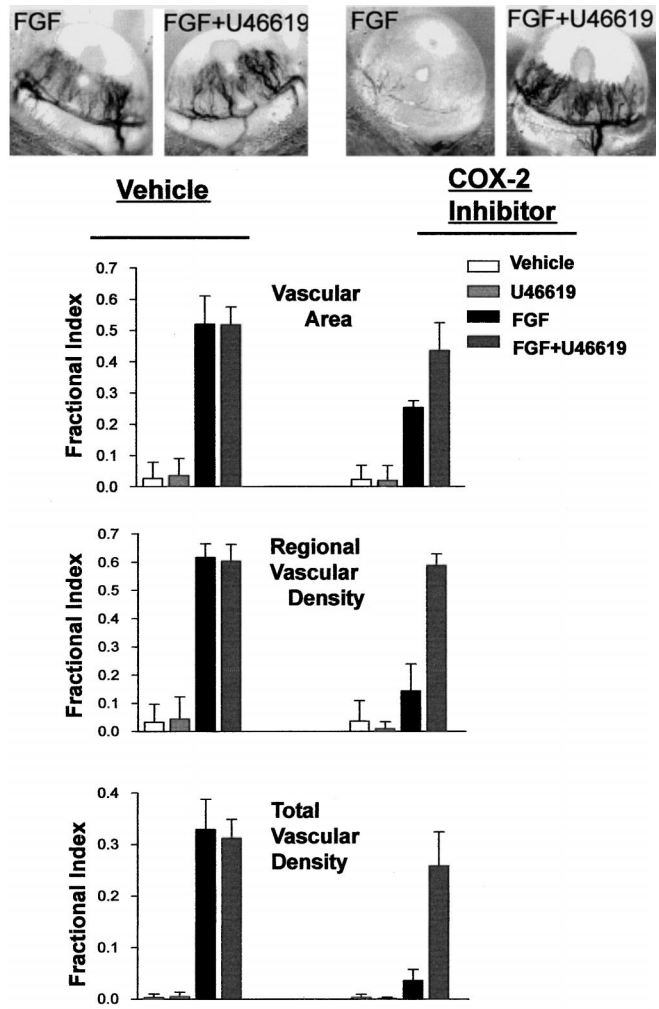


Fig. 4. TXA$_2$ agonist, U46619, reconstitutes the COX-2 dependent FGF-induced angiogenic responses *in vivo*. Hydron/sucralfate pellets impregnated with vehicle ($n = 4$), U46619 (1.7 nmol, $n = 6$), bFGF (3pmol, $n = 6$) or bFGF supplemented with U46619 (1.7 nmol, $n = 6$) were placed in corneal pockets of animals treated daily by i.p. injection with either vehicle (*left*), or VU08 (5 mg/kg; *right*), beginning 1 day prior to implantation. Above, representative images are displayed from animals receiving FGF alone, or with the TXA$_2$ receptor agonist, under COX-2 active (*Vehicle*) or COX-2 inhibited (*COX-2 Inhibitor*) conditions. Summarized data include fractional indices of the vascularized area (*top*) and regional vascular density within the vascularized area (*bottom panels*). *Columns*, means; *bars*, SE. U46619 addition to FGF pellet in VU08 treated mice (*right*) promotes an increase in regional vascular density and total vascular density (significant at $P < 0.05$).

These findings provide *in vivo* validation of a critical role for TXA_2 in neovascularization responses. Extrapolation from the endothelial migration responses *in vitro* (Fig. 2*a*) suggests that a critical threshold level of TXA_2 is required to support angiogenesis in this system, one that is not met under non-COX-2-induced conditions. Although COX-2 induction may lead to endothelial production of TXA_2, other cellular sources may be relevant in the context of neovascularization in specific tissue circumstances.

Platelets generate TXA_2 from endogenous COX-1-derived substrate prostaglandin H_2 and can convert endothelial-derived prostaglandin H_2 to TXA_2 (22). We speculate that TXA_2 from either source may support angiogenesis adjacent to microthrombi in tumors and other vascular sites. Indeed, reported effects of thromboxane synthase inhibitors and thromboxane receptor antagonists to inhibit metastatic behavior of tumor cells in mouse models were attributed to interruption of adhesive platelet interactions with tumor cells (23). Our findings suggest that TXA_2 axis antagonists may, alternatively, act primarily to inhibit endothelial responses to angiogenic peptides that are required for tumor vascularization and metastasis. TXA_2 axis antagonists may retain antiangiogenic activity under circumstances in which COX-2 inhibition is ineffective in eliminating TXA_2 production that is dependent upon COX-1-derived substrate.

Although TXA_2 receptor null mice show no overt developmental vascularization defects or disorders of pregnancy, gestation, or delivery (24), other critical mediators of neovascularization in mature animals, such as $\alpha_v\beta_3$, are not required for developmental vascularization to proceed (25). The requirement for thromboxane receptors to mediate COX-2 induced responses provides a focal point for intervention and a rationale for application of thromboxane receptor and synthase antagonists to potentiate therapeutic efficacy of COX-2 inhibitors.

Acknowledgments

Our thanks go to Ray Harris and Lee Limbird for critical reading of this manuscript.

References

1. Majima, M., Isono, M., Ikeda, Y., Hayashi, I., Hatanaka, K., Harada, Y., Katsumata, O., Yamashina, S., Katori, M., and Yamamoto, S. Significant roles of inducible cyclooxygenase (COX)-2 in angiogenesis in rat sponge implants. Jpn. J. Pharmacol., *75:* 105–114, 1997.
2. Oshima, M., Dinchuk, J. E., Kargman, S. L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J. M., Evans, J. F., and Taketo, M. M. Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell, *87:* 803–809, 1996.
3. Tsujii, M., Kawano, S., and DuBois, R. N. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. Proc. Natl. Acad. Sci. USA, *94:* 3336–3340, 1997.
4. Tsujii, M., and DuBois, R. N. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. Cell, *83:* 493–501, 1995.
5. Hla, T., and Neilson, K. Human cyclooxygenase-2 cDNA. Proc. Natl. Acad. Sci. USA, *89:* 7384–7388, 1992.
6. Creminon, C., Habib, A., Maclouf, J., Pradelles, P., Grassi, J., and Frobert, Y. Differential measurement of constitutive (COX-1) and inducible (COX-2) cyclooxygenase expression in human umbilical vein endothelial cells using specific immunometric enzyme immunoassays. Biochim. Biophys. Acta, *1254:* 341–348, 1995.
7. Kage, K., Fujita, N., Oh-hara, T., Ogata, E., Fujita, T., and Tsuruo, T. Basic fibroblast growth factor induces cyclooxygenase-2 expression in endothelial cells derived from bone. Biochem. Biophys. Res. Commun., *254:* 259–263, 1999.
8. Schmedtje, J. F. J., Ji, Y. S., Liu, W. L., DuBois, R. N., and Runge, M. S. Hypoxia induces cyclooxygenase-2 via the NF-κB p65 transcription factor in human vascular endothelial cells. J. Biol. Chem., *272:* 601–608, 1997.
9. Kito, H., Yokoyama, C., Inoue, H., Tanabe, T., Nakajima, N., and Sumpio, B. E. Cyclooxygenase expression in bovine aortic endothelial cells exposed to cyclic strain. Endothelium, *6:* 107–112, 1998.
10. Karim, S., Habib, A., Levy-Toledano, S., and Maclouf, J. Cyclooxygenase-1 and -2 of endothelial cells utilize exogenous or endogenous arachidonic acid for transcellular production of thromboxane. J. Biol. Chem., *271:* 12042–12048, 1996.
11. Blanco, A., Habib, A., Levy-Toledano, S., and Maclouf, J. Involvement of tyrosine kinases in the induction of cyclo-oxygenase-2 in human endothelial cells. Biochem. J., *312:* 419–423, 1995.
12. Busija, D. W., Thore, C., Beasley, T., and Bari, F. Induction of cyclooxygenase-2 following anoxic stress in piglet cerebral arteries. Microcirculation, *3:* 379–386, 1996.
13. Matsumura, K., Cao, C., Ozaki, M., Morii, H., Nakadate, K., and Watanabe, Y. Brain endothelial cells express cyclooxygenase-2 during lipopolysaccharide-induced fever: light and electron microscopic immunocytochemical studies. J. Neurosci., *18:* 6279–6289, 1998.
14. McAdam, B. F., Catella-Lawson, F., Mardini, I. A., Kapoor, S., Lawson, J. A., and FitzGerald, G. A. Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: the human pharmacology of a selective inhibitor of COX-2. Proc. Natl. Acad. Sci. USA, *96:* 272–277, 1999.
15. Coffey, R. J., Hawkey, C. J., Damstrup, L., Graves-Deal, R., Daniel, V. C., Dempsey, P. J., Chinery, R., Kirkland, S. C., DuBois, R. N., Jetton, T. L., and Morrow, J. D. Epidermal growth factor receptor activation induces nuclear targeting of cyclooxygenase-2, basolateral release of prostaglandins, and mitogenesis in polarizing colon cancer cells. Proc. Natl. Acad. Sci. USA, *94:* 657–662, 1997.
16. Stein, E., Lane, A. A., Cerretti, D. P., Schoecklmann, H. O., Schroff, A. D., Van Etten, R. L., and Daniel, T. O. Eph receptors discriminate specific ligand oligomers to determine alternative signaling complexes, attachment, and assembly responses. Genes Dev., *12:* 667–678, 1998.
17. Kenyon, B. M., Voest, E. E., Chen, C. C., Flynn, E., Folkman, J., and D'Amato, R. J. A model of angiogenesis in the mouse cornea. Invest. Ophthalmol. Vis. Sci., *37:* 1625–1632, 1996.
18. Bustos, M., Coffman, T. M., Saadi, S., and Platt, J. L. Modulation of eicosanoid metabolism in endothelial cells in a xenograft model. Role of cyclooxygenase-2. J. Clin. Invest., *100:* 1150–1158, 1997.
19. Ishizuka, T., Kawakami, M., Hidaka, T., Matsuki, Y., Takamizawa, M., Suzuki, K., Kurita, A., and Nakamura, H. Stimulation with thromboxane A2 (TXA2) receptor agonist enhances ICAM- 1, VCAM-1 or ELAM-1 expression by human vascular endothelial cells. Clin. Exp. Immunol., *112:* 464–470, 1998.
20. Armstrong, R. A., and Wilson, N. H. Aspects of the thromboxane receptor system. Gen. Pharmacol., *26:* 463–472, 1995.
21. Krauss, A. H., Woodward, D. F., Burk, R. M., Gac, T. S., Gibson, L. L., Protzman, C. E., Abbass, F., Marshall, K., and Senior, J. Pharmacological evidence for thromboxane receptor heterogeneity: implications for the eye. J. Ocular Pharmacol. Ther., *13:* 303–312, 1997.
22. Camacho, M., Lopez-Belmonte, J., and Vila, L. Rate of vasoconstrictor prostanoids released by endothelial cells depends on cyclooxygenase-2 expression and prostaglandin I synthase activity. Circ. Res., *83:* 353–365, 1998.
23. Honn, K. V. Inhibition of tumor cell metastasis by modulation of the vascular prostacyclin/thromboxane A2 system. Clin. Exp. Metastasis, *1:* 103–114, 1983.
24. Thomas, D. W., Mannon, R. B., Mannon, P. J., Latour, A., Oliver, J. A., Hoffman, M., Smithies, O., Koller, B. H., and Coffman, T. M. Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A2. J. Clin. Invest., *102:* 1994–2001, 1998.
25. Bader, B. L., Rayburn, H., Crowley, D., and Hynes, R. O. Extensive vasculogenesis, angiogenesis, and organogenesis precede lethality in mice lacking all αv integrins. Cell, *95:* 507–519, 1998.

Thromboxane A_2 Is a Mediator of Cyclooxygenase-2-dependent Endothelial Migration and Angiogenesis

Thomas O. Daniel, Hua Liu, Jason D. Morrow, et al.

Cancer Res 1999;59:4574-4577.