The cyclooxygenase (Cox) enzyme catalyzes the rate-limiting oxidative and peroxidative enzymatic steps in the biosynthesis of prostanoids. Both Cox-1 and -2 genes encode the two isoenzymes that carry out similar enzymatic steps. Enhanced Cox activity is associated with proliferative diseases such as colon cancer. To determine if a cause and effect relationship exists between Cox isoenzyme overexpression and tumorigenesis, the human Cox-1 and Cox-2 isoenzymes were transfected into ECV immortalized endothelial cells. Although numerous clones of Cox-1 expressing cells were obtained, Cox-2 overexpression resulted in growth disadvantage and increased cell death. In contrast, Cox-1 overexpressing cells expressed high levels of the functional Cox-1 polypeptide in the endoplasmic reticulum and the nucleus. In vitro proliferation of these cells was reduced compared with vector-transfected ECV cells. Cox-1 overexpression also enhanced the tumor necrosis factor-α-induced apoptosis of ECV cells 2-fold. In contrast to the in vitro behavior, ECV-Cox-1 cells proliferated aggressively and formed tumors in athymic “nude” mice, whereas the vector-transfected counterparts did not. The growth of Cox-1-induced tumors was not inhibited by indomethacin, suggesting a nonprostanoid function of Cox-1. ECV-Cox-1-derived tumors were angiosarcoma-like and contained numerous host-derived neovessels. These data suggest that Cox-1 overexpression in immortalized ECV endothelial cells results in nuclear localization of the polypeptide and tumorigenesis.
enzymes in tumorigenesis, however, is poorly understood. Overexpression of the Cox-2 enzyme in a gastrointestinal epithelial cell line is associated with the inhibition of apoptosis (19) and altered cell cycle kinetics (20). Because cells resistant to apoptosis may be prone to mutagenic events, inhibition of apoptosis is a potential mechanism for Cox-induced tumorigenesis. However, overexpression of Cox isoenzymes in other cell types promotes rather than inhibits apoptosis (see "Results and Discussion"). Exaggerated and dysregulated angiogenesis may be another mechanism via which Cox isoenzyme overexpression may regulate tumorigenesis. Dysregulated angiogenesis is a major hallmark of tumors and is thought to be necessary for the successful growth of many if not all solid tumors (21). PGE2, a major prostanoid produced by a variety of tumors (15), induces the production of angiogenic growth factors such as vascular endothelial cell growth factor (22) and induces angiogenesis in vivo (23). In this report, we investigated the relationship between the overexpression of the Cox-1 isoenzyme and tumorigenesis.

**MATERIALS AND METHODS**

**Cell Culture, Transfection, and Selection—**ECV304 cells (24) (CRL1994) were obtained from American Type Culture Collection (Rockville, MD) and were grown in medium 199 containing 10% fetal bovine serum (Hyclone) and antibiotic/antimycotic mixture (Life Technologies, Inc.). Cells were transfected with an expression vector pCDNA-I-Neo (Invitrogen) containing the 1.8-kilobase open reading frame of human Cox-1 or Cox-2 cDNAs (25). The characterization of Cox-1 and -2 cDNAs and expression of respective polypeptides have been described previously (25, 26). Transfection was achieved by a liposome-mediated method using Lipofectamine (Life Technologies, Inc.). After transfection, cells were split and selected in growth medium containing 1 mg/ml of G418 (Life Technologies, Inc.). Expression of transgenes was assessed (see below) in pools of transfected cells (ECV-neo and ECV-Cox-1) after 3 weeks of selection. Two independently
derived pools of transfected cells (for both Cox-1 and neo) were used in the studies described below.

**Cell Growth and Apoptosis in Vitro**—For cell growth assays, ECV-Cox-1 and ECV-neo cells (10^4 cells/well) were plated in six-well tissue culture plates containing normal growth medium plus G418. Cell numbers were determined by trypsinization and counting the cells in a Coulter counter. To induce apoptosis, subconfluent ECV cells were treated for 16 h with 100 ng/ml of TNF-α (R & D Systems) and 10 µg/ml of cyclo-heximide (Sigma). This treatment was shown previously to induce apoptosis of endothelial cells (27). After 16 h, apoptotic bodies were determined by staining with propidium iodide and counting under a fluorescent microscope.

Northern and Western Blot Analyses—Total RNA from ECV-neo or ECV-Cox-1 cells were isolated by guanidinium isothiocyanate/phenol procedure (28). RNA was separated by 1% agarose gel electrophoresis and transferred onto a nylon membrane, and Northern blot analysis was conducted using the radiolabeled Cox-1 probe as described previously (29). Western blot analysis was conducted using the polyclonal Cox-1 antiserum as described before (12).

**TLC-Autoradiography Procedures**—ECV cells were superfused with 12.5 µM [14C]arachidonic acid (NEN Life Science Products) in plain medium 199 for 15 min at 37 °C. Medium was then acidified and extracted, and TLC analysis of prostanoids was conducted using the solvent system L as described previously (25). Prostanoids were identified by comparison of Rf values of authentic standards (Cayman Chemical). The TLC plates were then autoradiographed.

**Nude Mouse Studies**—Equal numbers (10^5) of ECV-neo and ECV-Cox-1 cells were trypsinized, resuspended in 200 µl of sterile phosphate-buffered saline, and injected intradermally into athymic nu/nu "nude" mice. Tumor volume was measured at indicated time points (30). Upon completion of the experiments, mice were inspected for the appearance of metastases in major organs by gross as well as by histological procedures (see below).

**Immunohistochemistry**—ECV-Cox-1 cells were grown on glass coverslips and fixed in 10% formalin for 24 h and 70% ethanol for at least 24 h. Cells were then subjected to immunostaining with 1:100 dilution of anti-Cox-1 antibody. This antiserum was determined to be selective for Cox-1 by Western blot procedures of expressed human Cox-1 and -2 polypeptides in Cos-7 cells (8, 10). For immunohistochemistry, antibody staining was visualized using a second antibody coupled to peroxidase (ABC kit, Vector Laboratories). Tumor tissues were dissected, fixed as above, and immunostained with anti-Cox-1 antibody (12) and anti-von Willebrand factor antibody (DAKO Inc.).

**RESULTS AND DISCUSSION**

**Stable Transfection of Cox-1 and -2 cDNAs into ECV Cells**—The ECV cell line is derived from spontaneous immortalization of human umbilical vein endothelial cells (HUVEC) (24). Although these cells retain several endothelial cell markers, such as induction of E-selectin and the formation of capillary-like tubular structures (24, 29, 31), the growth properties of these cells are significantly different from that of the parental HUVEC. For example, ECV cells proliferate maximally in serum containing medium alone, whereas HUVEC require growth factors such as fibroblast growth factor for survival (24, 27, 29). In addition, ECV cells are immortalized and do not exhibit the phenomenon of in vitro senescence, whereas HUVEC do (24, 27, 31). ECV cells do not express either Cox-1 or -2 genes under basal conditions (29). However, stimulation with phorbol myristic acetate or interleukin-1 results in the dramatic up-regulation of Cox-2 expression and the inhibition of cell growth (29). In contrast, no Cox-1 expression was detected.

Cox-1 and -2 cDNAs in the expression vector pCDNANeo were transfected into ECV cells, and G418-resistant cells were selected. Although numerous G418-resistant colonies (approximately 50 colonies/100-mm dish) were obtained from the Cox-1-transfected ECV cells, very few were obtained from Cox-2 transfection. When assayed for the expression of the transgene by Northern blot analysis, several slow growing ECV colonies expressed low but detectable levels of the Cox-2 transcript. These cells, however, exhibited poor survival in serum-containing medium, even in the presence of Cox inhibitors (10 µM indomethacin or NS-398). Numerous apoptotic cells were observed in these colonies. After several weeks of culture, the expression of Cox-2 was lost and rapid growth of these cells resumed. These studies suggested that Cox-2 overexpression in ECV cells conferred a growth disadvantage. In contrast to intestinal epithelial cells (19), Cox-2 overexpression may induce apoptosis in endothelial cells. These colonies were not further studied. In contrast, robust expression of Cox-1 cDNA was observed in stably transfected ECV cells, and the transfectants were further characterized.

These cells and the vector-transfected counterparts were grown in medium containing 1 mg/ml of G418. The expression of the transfected gene was assessed by Northern and Western blot analyses. As shown in Fig. 1a, high levels of the polypeptide and the mRNA for Cox-1 was detected in ECV-Cox-1 cells, whereas the vector-transfected ECV-neo cells did not express detectable levels of Cox-1 mRNA and protein. The functionality of the transfected Cox-1 cDNA was assessed by measuring the production of prostanoids after incubating the cells with [14C]arachidonic acid. Prostanoids were extracted from the me-
**Fig. 4.**

*a*, histological analysis of ECV-Neo and ECV-Cox-1-induced tumors. The site of injection was dissected at 50 days, fixed, and stained with hematoxylin and eosin (panels A and C) or immunostained with anti-Cox-1 antibody (panels B and D) as described. ECV-Neo injection site contained only few injected cells (arrow in panel A) surrounded by a fibroblastic tumor capsule. The fibroblast-like cells in the capsule that express low levels of Cox-1 are presumably of mouse origin. ECV-Cox-1-induced tumors (panels C and D) contains numerous proliferating, malignant ECV cells (large arrow in panels C and D) surrounded by vessel-like structures (small arrow in panels C and D). The nuclear structure of the vessels is flat and normal, suggesting that they are host-derived. The magnification of the photomicrographs are as follows: panels A and B, 60×; panels C and D, 120×.

*b*, angiogenesis in Cox-1-induced tumors. Gross photograph of the ECV-Cox-1-induced tumor at day 50 after injection (panel A). Note the redness of the tumor, suggesting high vascularity. Histological analysis of the tumor after hematoxylin and eosin staining (30×).
dium, analyzed by thin layer chromatography, and visualized by autoradiography as described (25). As shown in Fig. 1a, significant production of 6-keto-PGF$_{1\alpha}$, a stable metabolite of prostacyclin and PGF$_{2\alpha}$ and PGE$_{2\alpha}$ as well as several unidentified arachidonic acid metabolites were observed. Vector-transfected ECV-neo cells did not metabolize exogenous [${}^{14}$C]arachidonic acid, which is consistent with the nondetectable expression of the Cox-1 and -2 isoenzymes in these cells. These data suggest that high level expression of the transfected Cox-1 gene was achieved in ECV cells.

Subcellular Localization of the Transfected Cox-1 Polypeptide—The Cox-1 and -2 polypeptides were shown to be localized in different subcellular compartments in NIH 3T3 cells, transfected Cos cells, and endothelial cells (7). Although the Cox-1 isoenzyme was localized primarily in the ER, the Cox-2 polypeptide was observed in both ER and nuclear compartments (1, 2, 7). Because products of the Cox isoenzymes may have functions extracellularly (8) as well as intracellularly (9), we characterized the localization of the overexpressed Cox-1 polypeptide in stably transfected ECV cells. Immunohistochemical analysis with the polyclonal antisera against Cox-1 on ECV-neo cells did not indicate appreciable staining, consistent with nondetectable expression of Cox-1 in these cells. However, high expression of Cox-1 immunoreactivity was seen in cobblestone-shaped ECV-Cox-1 cells (Fig. 1b). Strong immunoreactivity was observed in the nuclear membrane, within the nucleus and the ER. Confocal immunofluorescence microscopy confirmed these results (data not shown). These data suggest that overexpression of Cox-1 in ECV cells resulted in the localization in the ER and the nucleus. The nuclear localization of Cox-1 could be due to high intracellular concentration of this polypeptide, which is achieved by overexpression. Alternatively, it could be due to a characteristic unique to the immortalized nature of the ECV endothelial cells.

In Vitro Growth and Apoptosis of ECV-neo and ECV-Cox-1 Cells—To determine if elevated Cox-1 expression in ECV cells resulted in the aberrant regulation of growth or apoptosis, we measured the growth rate of ECV-Cox-1 cells and compared it with that of ECV-neo cells. As shown in Fig. 2a, both ECV-Cox-1 and ECV-neo cells proliferated; however, the growth of ECV-Cox-1 cells was reduced (approximately 45%) at day 7. The doubling times of the ECV-Cox-1 and ECV-neo cells were 23.32 and 23.28 h, respectively. The growth rates of both cell lines were not modulated significantly by varying the serum concentration. These data suggest that Cox-1 overexpression resulted in modest inhibition of in vitro growth rate of ECV cells.

The effect of Cox-1 overexpression on the apoptosis of ECV cells was assessed next. Treatment of ECV cells with TNF-α and cycloheximide potently induced apoptosis within 16 h. The rate of apoptosis of ECV-Cox-1 cells was enhanced approximately 2-fold compared with the ECV-neo cells (Fig. 2b). These data suggest that Cox-1 overexpression enhances the TNF-induced apoptosis of ECV cells in vitro. Enhanced apoptosis in ECV-Cox-1 cells perhaps was the reason why the growth rates of these cells were blunted in vitro (Fig. 2a). Interestingly, inhibition of prostaglandin synthesis with 2 μm indomethacin did not block enhanced apoptosis in ECV-Cox-1 cells, suggesting that the ability of Cox-1 to promote apoptosis is dissociable from its enzymatic property of prostaglandin synthesis. Thus, overexpression of Cox-1 in ECV cells does not dramatically alter the in vitro phenotype of ECV cells. For example, the morphology of Cox-1 transfected cells in vitro is indistinguishable from ECV-neo cells in that both cell lines undergo contact inhibition, fail to grow under anchorage-independent conditions, and exhibit a cobble-stone morphology typical of endothelial cells. However, reduction in growth rate and enhanced response to TNF-α-induced apoptosis was observed.

In Vivo Tumorigenesis of ECV-Cox-1 Cells—To test the tumorigenic potential of Cox-1, ECV-neo and ECV-Cox-1 cells were injected subcutaneously into the athymic nude mice. Although ECV cells are immortalized, they are not transformed (24). ECV-neo cells behaved similarly and did not grow as tumors at the site of injection or at distant sites. In contrast, ECV-Cox-1 cells grew aggressively as tumors (Fig. 3a). Large tumors that are frequently red on the surface grew at the site of injection; however, distant metastases were not observed. These data suggest that Cox-1 overexpression induces tumorigenic transformation of ECV cells in vivo. To determine if secretion of prostanoids by the transfected Cox-1 gene is involved in tumorigenesis, mice were administered indomethacin in drinking water (14 μg/ml) for the duration of the experiment. Because mice drink approximately 3–4 ml of water per day, this translates to a dose of 1–1.4 mg/kg/day, which is effective in blocking prostanoïd synthesis under clinical situations (33). As shown in Fig. 3b, indomethacin treatment did not inhibit Cox-1-induced tumorigenesis. These data suggest that the ability of Cox-1 to induce tumorigenesis is independent of prostanoïd biosynthesis.

The morphology of the tumor as well as expression of the transfected Cox-1 polypeptide was analyzed by histopathological procedures. As shown in Fig. 4a, the site of injection of ECV-neo cells (panels A and B) indicates the presence of a few ECV cells that are intermingled with mononuclear cells and host-derived fibroblastic capsule engulfing the injected cells. Many of the ECV-neo cells appear necrotic, and mitotic bodies were not observed. In contrast, ECV-Cox-1 cells appear highly malignant (Fig. 4a, panels C and D). The mass of the tumor is composed of ECV cells and vessel-like structures. The nuclear morphology of ECV cells appears highly abnormal, whereas the vessel-like structures contain flat nuclei and thus may be host-derived. Numerous mitotic and apoptotic bodies were observed in the ECV-Cox-1 tumors, suggesting a high turnover. In addition, various degrees of differentiation of ECV-Cox-1 cells were observed; some were highly mitotic and undifferentiated, whereas some appeared more differentiated with an adenomatous phenotype. The ECV-Cox-1 cells in the tumors still express the transfected Cox-1 gene, albeit in a heterogenous manner. As determined by immunohistochemical procedures (Fig. 4a, panel D). Such a pattern of heterogenous yet exaggerated expression in tumor cells resembles the pattern of Cox-2 expression in human colorectal cancer (12), a tumor of epithelial origin.

The histology of the ECV-Cox-1 tumors with respect to the vasculature was characterized next. As shown in Fig. 4b, tumors derived by ECV-Cox-1 cells are highly angiogenic, as evident from the redness of the tumor. The tumor is composed of inflammatory and angiogenic periphery and a solid mass of...
rapidly proliferating ECV-Cox-1 cells (Fig. 4b). A necrotic center in the center of the tumor can be frequently seen (Fig. 4b). The vessels in the tumor appear to be host-derived because their nuclear architecture is normal and flat, in contrast to the proliferative ECV-Cox-1 cells that possess abnormally shaped nuclei. The vessel-like structures, especially those at the periphery of the tumor are positive for von Willebrand factor and Tom Maciag for support and encouragement.

indomethacin treatment did not reverse the enhanced apoptotic down-regulate angiogenic suppressors (32). However, because in vivo place in ECV cells that would express angiogenic factors and/or expression of Cox-1 may allow a nuclear signaling event to take up-regulate vascular endothelial growth factor expression and

The vessels in the tumor appear to be host-derived because their nuclear architecture is normal and flat, in contrast to the periphery of the tumor can be frequently seen (Fig. 4b).

In conclusion, we have shown that high level expression of Cox-1 is responsible for the enhanced angiogenesis and tumorigenesis. Indeed, PGE2, which is produced by the Cox-1 pathway is a potent inducer of angiogenesis (23). PGE2 is known to induce rapid proliferation of the tumor. In addition, ECV-Cox-1 cells induced enhanced angiogenesis of the host and thus allowed rapid proliferation of the tumor. In addition, this system may be useful to further dissect nonprostanoid-mediated function of Cox-1. Further studies are needed to address such mechanisms.

In conclusion, we have shown that high level expression of Cox-1 in ECV cells, which normally do not express either Cox-1 or -2 isoenzymes, results in nuclear and ER localization of functional Cox-1 polypeptide. Cox-1 overexpression induced tumorigenesis and exaggerated angiogenesis in vivo. Such mechanisms may be important in Cox-induced tumorigenesis in vivo. In addition, this system may be used to further dissect the mechanisms involved.

Acknowledgments—We thank Carolyn Hue for expert technical assistance and Tom Maciag for support and encouragement.