Selective Cyclooxygenase (COX)-1 or COX-2 Inhibitors Control Metastatic Disease in a Murine Model of Breast Cancer

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ABSTRACT

Using a highly metastatic mammary tumor cell line that expresses both cyclooxygenase (COX) isoforms, we now show that oral administration of either a selective COX-2 inhibitor (celecoxib) or a selective COX-1 inhibitor (SC560) to mice with established tumors results in significant inhibition of tumor growth. Administration of the dual inhibitor, indomethacin, leads to even better growth control. Metastatic capacity is also reduced by treatment of tumor-bearing mice with either COX-1 or COX-2 selective inhibitors. Pretreatment of tumor cells with COX inhibitors also reduces metastatic success, indicating that tumor cells may be a direct target of action by COX inhibitors. Growth of a second cell line, which does not express COX-2 in vivo, is also reduced by celecoxib, implicating both COX-dependent and COX-independent mechanisms.

INTRODUCTION

The inducible isoform of COX, COX-2, is commonly overexpressed in solid tumors, suggesting that this enzyme may contribute to malignant behavior (1, 2). Epidemiological studies as well as early clinical trials also suggest that administration of either dual COX-1/COX-2 or selective COX-2 inhibitors may reduce the risk of cancer development (3). Preclinical studies also indicate that COX inhibition may be useful in models of chemoprevention (4). More limited studies suggest that COX inhibitors may have potential in the treatment of established disease (5). Although these early results are encouraging, most of these studies have been carried out in models of solid tumors in which metastatic disease does not occur. We have now examined the efficacy of COX-2 inhibition in a model of metastatic breast cancer. Much less attention has been paid to a possible role for the COX-1 isoform in cancer. Murine mammary tumors express both COX-1 and COX-2 isoforms, and we have also determined the effect of treatment with a selective COX-1 inhibitor on tumor behavior.

MATERIALS AND METHODS

Cell Lines and Tumors. Murine mammary tumor cell line 410 was derived from a spontaneously arising tumor of a BALB/cByJ mouse. This line is tumorigenic but rarely metastasizes. Line 410.4 was derived from a metastatic lesion that occurred in a mouse bearing a s.c. implant of line 410. Both cell lines are maintained in DMEM supplemented with 10% FCS (Gemini Bio-Products, Inc., Calabasas, CA), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.1 mM nonessential amino acids. For growth studies in vivo, 5–50 × 10^3 viable cells were injected s.c. into syngeneic BALB/cByJ female mice (Jackson Laboratories, Bar Harbor, ME). When tumors become palpable, tumor diameters were determined twice weekly by caliper, and tumor size was expressed as the mean of the longest and the perpendicular tumor diameters. To determine the degree of metastatic disease, mice bearing s.c. tumor implants were sacrificed, on an individual basis, when tumors achieved an average diameter of 18 mm; lungs were removed, and surface lung tumor colonies were counted under a dissecting microscope.

COX Inhibitor Treatments. The selective COX-2 inhibitor, celecoxib, and the selective COX-1 inhibitor, SC560, were a generous gift of Pharmacia (St. Louis, MO). The dual COX inhibitor indomethacin was purchased from Sigma Chemical Co. (St. Louis, MO); the selective COX-2 inhibitor NS398 was from Cayman Chemical Co. (Ann Arbor, MI). All drugs were dissolved in a solution of methylocellose (0.5%) and Tween 20 (0.025%) and administered by oral gavage twice/day to achieve a dose of 5 mg/kg/day (celecoxib and SC560) or 1 mg/kg/day (indomethacin). Drugs or vehicle were administered beginning on the day of tumor transplantation or 1 week later, when 410.4 tumors become palpable. Treatment of mice bearing the slower growing tumor 410 was initiated on either day 0 or day 14. To determine the direct effect of COX inhibitors on tumor cells, in the absence of host effects, line 410.4 tumor cells were cultured in the presence of SC560 (0.01 μM), celecoxib (0.1 μM), NS398 (1.0 μM), indomethacin (1.0 μM), ethanol (vehicle control for indomethacin), or DMSO (control for SC560, celecoxib, and NS398). Forty-eight h after drug addition, cells were washed, and 3 or 5 × 10^3 viable tumor cells were injected into the lateral tail vein of syngeneic mice. No further drug treatments were carried out. On day 20, mice were sacrificed, and pulmonary metastases were quantitated.

RESULTS AND DISCUSSION

COX-2 is commonly overexpressed in both rodent and human tumors, suggesting that this isoform is an important determinant of tumor behavior (1, 2). Less attention has been focused on the COX-1 protein, which is also commonly detected in tumors. We have shown previously that the highly metastatic murine mammary tumor line 410.4 expresses both the COX-1 and COX-2 isoforms in culture and in tumors arising from transplantation to syngeneic hosts (6). We have also shown that a dual COX-1/COX-2 inhibitor, indomethacin, has potent antitumor activity in a murine model of metastatic breast cancer (7). Those studies could not distinguish a role for COX-1 versus COX-2 inhibition in the therapeutic effect. Using COX inhibitors that are selective for either COX-1 (SC560) or COX-2 isoforms (celecoxib), we have now examined tumor inhibition and compared the efficacy of these selective drugs to indomethacin.

We transplanted 5 × 10^3 of line 410.4 tumor cells s.c. to syngeneic BALB/cByJ mice. Drugs were administered by oral gavage beginning either on the day of tumor transplantation or at day 7, when all tumors are palpable. Drug treatments were continued on a daily basis until day 28. Fig. 1 shows that all these drugs resulted in statistically significant inhibition of tumor size in comparison with vehicle-treated control animals. We confirmed our previous study showing that indomethacin, initiated on day 0, results in the most marked tumor inhibition of 410.4. In comparison to the dual inhibitor, the selective COX-2 inhibitor celecoxib led to somewhat less tumor inhibition than indomethacin; however, tumors in these mice were still significantly smaller than in vehicle-treated animals. Treatment with either indomethacin or celecoxib was still effective, even if treatment commenced in mice with established (day 7) tumors. Interestingly, treatment with the selective COX-1 inhibitor, SC560, also resulted in significant tumor growth inhibition comparable with that achieved with the COX-2 inhibitor. Therapeutic activity of a selective COX-1 inhibitor has not been described in vivo previously.

Because 410.4 tumors express both COX isoforms, it was possible...
that both enzymes contribute to the behavior of these tumors. This would be consistent with our finding that inhibition of either single isoform does not lead to the same degree of tumor inhibition as is achieved with the dual inhibitor, indomethacin. To address this question more directly, we determined the effect of selective inhibitors on a tumor that expresses only one isoform in vivo. For these studies, we used tumor cell line 410. We have shown that although this tumor line can express both COX isoforms in culture, only the COX-1 protein and mRNA is detected by the time these tumors are palpable when transplanted to syngeneic mice (6). Thus, COX-2 expression appears to be actively down-regulated in these tumors in vivo. If tumor COX protein is an important therapeutic target of these drugs, one would predict that SC560, but not celecoxib, would inhibit 410 tumors. We transplanted $5 \times 10^6$ cells of line 410 to syngeneic mice, and drugs were administered by gavage. This tumor grew progressively in 75% of mice treated with vehicle alone (Fig. 2) and in 33% of indomethacin-treated mice. Because these tumors do not express COX-2 in vivo, either in the stroma or in malignant cells (6) at a time when tumors are palpable, it was interesting that the selective COX-2 inhibitor, celecoxib, also led to significant tumor inhibition (17% of tumors grew progressively). There are two possible interpretations of this result: either a COX-2 inhibitor has therapeutic activity versus tumors that express little or no COX-2 protein, or celecoxib acts on a COX-2-positive cell that is eliminated early after transplantation. Treatment with SC560 had a modest effect on tumor incidence (60% progressive growth).

When tumor line 410.4 is transplanted to the subcutis of mice, tumor cells metastasize spontaneously to the lungs. When the tumor-bearing animals shown in Fig. 1 became moribund, mice were sacrificed on an individual basis, and pulmonary tumor colonies were examined. Similar to s.c. tumor growth, spontaneous metastases were reduced by all three COX inhibitors (Fig. 3). The effect of COX inhibitors on metastatic disease has rarely been examined, and this is the first report showing antimitastatic activity of a selective COX-1 inhibitor.

Few studies have examined COX levels in relation to metastatic potential. COX-2 overexpression in gastric carcinoma was positively correlated with lymphatic invasion (8). A comparison of COX-2 expression in lung adenocarcinomas revealed higher COX-2 in metastatic cells in the lymph node than in the primary tumor (9). We have also reported that metastatic potential is positively correlated with both COX-2 protein and enzymatic (prostaglandin synthesis) activity (6). In contrast, other studies have reported that although COX-2 expression is positively correlated with size and local invasion of colorectal carcinomas, no relationship with distant metastasis was detected (10). Thus, the precise relationship of COX-2 expression to metastatic potential has not been firmly established.

Studies described to this point could not determine whether the COX inhibitors are acting primarily on the host or directly on the tumor cells. Others have demonstrated COX-2 expression in the tumor stroma (5, 11) and that host COX-2 can affect tumor behavior (12). Our previous studies have shown that, in these mammary tumors, COX-2 expression is observed primarily in the malignant epithelial cells, with occasional weaker COX-2 staining observed in the stroma (6). To determine whether tumor COX-2 was a target in the current model, we carried out studies in which the tumor cells were treated with COX inhibitors in vitro, before i.v. administration, and determined the effect on experimental metastasis. This model permits the
examination of later steps in the metastatic process and specifically targets the tumor cell, rather than the host. For these studies, line 410.4 tumor cells were cultured in the presence of SC560 (0.01 μM), celecoxib (0.1 μM), the additional COX-2 inhibitor NS398 (1.0 μM), indomethacin (1.0 μM), ethanol (vehicle control for indomethacin), or DMSO (control for SC560, celecoxib, and NS398). Forty-eight hours after drug addition, cells were washed, and 3 or 5 × 10³ viable tumor cells were injected into the lateral tail vein of syngeneic mice. No further drug treatments were carried out. On day 20, mice were sacrificed, and pulmonary metastases were quantitated. Table 1 shows two independent experiments in which treatment with any of the four COX inhibitors resulted in a dramatic reduction (50–89%) in the numbers of lung tumor colonies. Thus, these studies confirm data obtained using the spontaneous model showing that either COX-1 or COX-2 inhibitors have potent antimetastatic activity. Furthermore, because the host was never exposed to Cox inhibitors, these data provide strong evidence that the tumor cell can be a direct target of Cox inhibitors.

These studies confirm data from other laboratories showing that dual or COX-2-selective inhibitors can have potent antitumor activity (5). We provide new evidence that these drugs may be more potent inhibitors of metastatic disease than of established primary (s.c.) tumors. Furthermore, we show for the first time that selective COX-1 inhibitors may also have important therapeutic activities. Indomethacin was superior to either selective COX-1 or COX-2 inhibitors in controlling growth of s.c. 410.4 tumors, suggesting that targeting of both enzymes would be more therapeutic. In the clinical setting, however, this therapeutic gain might be limited by the significant toxicities associated with inhibition of both isoforms. The greater efficacy of a dual inhibitor could be related to the expression of both isoforms in epithelial cells of these tumors. Additionally, COX-1 activities expressed by tumor-infiltrating endothelial cells may contribute to tumor behavior. For example, COX-1 expression regulates angiogenesis in endothelial cells (13). Induced overexpression of COX-1 in endothelial cells leads to malignant transformation (14). Additional evidence that COX-1 can contribute to cancer development comes from studies of colon polyp formation in Min mice (15). Disruption of either the COX-1 or COX-2 genes reduces the incidence of polyp formation, suggesting that both COX gene products play a role in cancer development. Thus, the ability of the COX-1 inhibitor SC560 to limit tumor growth may be by an indirect effect on tumor angiogenesis. Studies by Masferrer et al. (5) support an antiangiogenic mechanism for celecoxib in a corneal angiogenesis assay, but those studies could not identify an antiangiogenic activity for SC560. Thus, the mechanism by which SC560 inhibits tumor growth and metastasis in the current model will require further study.

Identification of the mechanism by which COX-2 inhibitors act is also confounded by the finding that tumors derived from transplantation of line 410 cells are also inhibited by a COX-2 targeting drug, although these tumors express little or no COX-2 protein. These data are consistent with studies by others showing that NSAIDs can inhibit the growth of COX-null cells in vitro (16). Alternatively, COX-2 could be expressed very early after transplantation of 410 cells, and celecoxib could act on this population. Thus, our studies add to the growing body of literature that indicates that these drugs may affect tumor growth by COX-dependent as well as COX-independent pathways.

Our studies showing that pretreatment of tumor cells with COX inhibitors markedly inhibits experimental metastasis supports a mechanism that involves direct effects of these drugs on tumor cells. We have shown previously that although these cells express both isoforms in vitro, >90% of the prostaglandin synthesis is inhibited by a COX-2 inhibitor (NS398) alone. Thus, the ability of SC560 to inhibit metastasis may not be related principally to the inhibition of COX enzyme activity. Likewise, sulindac sulfone, which lacks the prostaglandin synthetase inhibitory activity of the parent compound, sulindac sulfone, still inhibits mammary carcinogenesis (17).

Other activities have been attributed to COX inhibitors including induction of apoptosis and antiproliferative actions. In other studies, we have examined the effects of COX inhibitors on behavior of tumor cells in vitro. Those data indicate that these same drugs can limit growth of cultured mammary tumor cells, induce cell cycle arrest, and increase intracellular ceramide levels (6). Unlike many cells examined, these mammary tumor cells do not undergo apoptosis in response to COX inhibitors. The relevance of those findings to the current study are unclear, however, because adverse effects on cell behavior were observed only at drug concentrations in the 10–100 μM range, much higher than the concentrations required in the current study to observe changes in metastatic potential (Table 1). Clearly, more studies are needed to discern the mechanisms responsible for the antitumor and antimetastatic effects of these drugs.

Breast and other tumors often have increased levels of prostaglandins, particularly PGE, but the pathophysiological role of this metabolic activity was uncertain (18). Bennett et al. (19, 20), authors of two early retrospective studies, reported high levels of PGE-like material present in breast tumors that had metastasized to bone and found that postsurgical survival time was shorter in women with high PGE tumors. More recently, epidemiological studies have indicated that chronic use of nonsteroidal anti-inflammatory drugs reduces the incidence of many cancer types including breast cancer (21, 22). Examination of human tumors other than breast has frequently revealed high level expression of the COX-2 protein. Examination of human breast tumors has been limited; two studies reported overexpression of COX-2 (23, 24); the other study rarely detected COX-2 protein but commonly observed COX-1 (25). Thus, unlike colon cancer where COX-2 is clearly dominant, the relative importance of these two isoforms has not been established in breast cancer. Even if the COX-1 isofrom is not overexpressed, it may still be an important target of therapy. Taken together, these data indicate that COX inhibitors have potent activity in a model of highly aggressive breast cancer. Both COX-1- and COX-2-selective drugs have efficacy. Several mechanisms may be important, including both COX-dependent and COX-independent activities.

### Table 1 Effect of COX inhibitor pretreatment on experimental metastasis of Line 410.4

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lung Metastases Exp.</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>P</th>
<th>Lung Metastases</th>
<th>Exp.</th>
<th>Exp. 2</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>63 ± 12</td>
<td>217 ± 27</td>
<td></td>
<td>0.002</td>
<td>108 ± 17 (50%)</td>
<td>0.001</td>
<td></td>
<td></td>
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<tr>
<td>Indomethacin (1 μM)</td>
<td>23 ± 4 (37%)</td>
<td>108 ± 17 (50%)</td>
<td>0.002</td>
<td></td>
<td>96 ± 14 (33%)</td>
<td>0.001</td>
<td></td>
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<tr>
<td>DMSO</td>
<td>93 ± 34</td>
<td>287 ± 23</td>
<td></td>
<td></td>
<td>96 ± 14 (33%)</td>
<td>0.001</td>
<td></td>
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</tr>
<tr>
<td>Celecoxib (0.1 μM)</td>
<td>10 ± 2 (11%)</td>
<td>96 ± 14 (33%)</td>
<td>0.001</td>
<td></td>
<td>96 ± 14 (33%)</td>
<td>0.001</td>
<td></td>
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<tr>
<td>NS398 (1 μM)</td>
<td>10 ± 4 (11%)</td>
<td>96 ± 14 (33%)</td>
<td>0.001</td>
<td></td>
<td>96 ± 14 (33%)</td>
<td>0.001</td>
<td></td>
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<tr>
<td>SC560 (0.01 μM)</td>
<td>18 ± 13 (19%)</td>
<td>31 ± 5 (11%)</td>
<td>0.001</td>
<td></td>
<td>31 ± 5 (11%)</td>
<td>0.001</td>
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</table>

* Exp., experiment.

* Mean ± SE lung metastases is expressed as a percentage of control (ethanol or DMSO) value. P values are calculated for lung metastases in COX inhibitor-treated versus vehicle-treated cells.

### REFERENCES

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