In Vivo Effects of Indomethacin on the Growth of Murine Mammary Tumors

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ABSTRACT

We have examined the effect of the prostaglandin synthesis inhibitor indomethacin on the s.c. growth of three murine mammary tumors that are heterogeneous with regard to immunogenicity, metastatic ability, in situ prostaglandin levels, and many other characteristics. Continuous p.o. administration of indomethacin, beginning on the day of tumor transplantation, led to complete regression of the poorly metastatic, low-prostaglandin E (PGE), highly immunogenic Tumor 410 in 11 of 12 animals, whereas 83% of ethyl alcohol-treated controls developed progressively growing tumors. The high-PGE, highly metastatic, poorly immunogenic Tumor 4501 was partially inhibited by p.o. indomethacin, resulting in an increased survival time for tumor-bearing mice (59 days versus 53 days for controls). Progressive growth of the high-PGE, highly metastatic, poorly immunogenic Tumor 4526 was seen in 25% of indomethacin-treated mice compared to progressive growth in 80% of control mice.

In contrast, when these tumor cells were cultured in vitro in the presence of indomethacin, slight stimulation of cell division was seen, suggesting that indomethacin-mediated growth inhibition in vivo is not due to direct inhibitory effects of indomethacin on tumor cells.

INTRODUCTION

Human and experimental tumors are rich sources of prostaglandins and other cyclooxygenase products of arachidonic metabolism (14). Although many studies have shown that administration of prostaglandin synthesis inhibitors such as indomethacin results in inhibition of tumor growth, other studies have shown that prostaglandins themselves inhibit tumor growth (reviewed in Ref. 12). Most studies have used the Moloney sarcoma virus-induced tumors or other fibrosarcomas (19, 22). Our particular interest is in the role of prostaglandins in the growth and metastasis of breast cancer. Our prospective studies of human breast tumor-associated prostaglandins show that high levels of PGE\textsuperscript{2} and PGF are associated with some factors contributing to a poor prognosis, such as lack of histological differentiation in tumor specimens, but are also seen in estrogen receptor-positive tumors that generally have a better prognosis (10). Several studies of human breast cancer have suggested that high prostaglandin levels are associated with clinically aggressive tumors (1, 2, 20, 21).

In the present study, we have used an animal model consisting of a well-characterized system of closely related mouse mammary lesions that possess a wide range of phenotypes for tumorigenicity, immunogenicity, metastatic ability, and many other properties, to examine the biological role of prostaglandins in breast cancer. We have shown previously that high PGE levels in these tumors are correlated positively with metastatic potential (6). In addition, both tumor cells and tumor-associated macrophages synthesize prostaglandins (7, 17). The purpose of this study is to examine the effects of a cyclooxygenase inhibitor on a heterogeneous series of mammary tumors. These studies aim to clarify the role of cyclooxygenase products in the s.c. growth of these transplantable tumors, as well as to provide the groundwork for studies in progress on the role of such products in the metastatic dissemination of these tumors. We report here the results of studies of the primary (s.c.) growth of these tumors.

MATERIALS AND METHODS

Mice. Male and female BALB/c mice, 4 to 6 weeks old, were purchased from the Cancer Research Laboratory (University of California, Berkeley, CA) or from Charles River Breeding Laboratories. Mice were allowed to acclimate for at least 1 week before use. Mice were maintained on standard laboratory chow.

Tumor Cell Lines. The derivation of the mammary tumors used here has been described previously (17, 18). Tumor line 410 was derived from a spontaneously arising mammary tumor of a BALB/cF3H mouse. It is rarely metastatic. Line 410.4 was isolated from a lung metastasis from the same parent tumor growing s.c. Lines 4501 and 4526 are cloned subpopulations of the uncloned 410.4 line. Lines 4501 and 4526 metastasize spontaneously from s.c. implants at a frequency of >80%. All lines were maintained in Waymouth’s medium supplemented with 7% horse serum, 7% newborn bovine serum, 1% fetal bovine serum, 2 mm glutamine, penicillin (100 units/ml), and streptomycin (100 \mu g/ml) and were buffered with NaHCO\textsubscript{3}. Tumor cells were free of Mycoplasma, pneumonia, reovirus type 3, Sendai, encephalomyelitis, K. polyoma, minute, mouse adenovirus, mouse hepatitis, lymphocytic choriomeningitis, and ectromelia viruses (Microbiological Associates, Bethesda, MD).

Subconfluent cell cultures were treated with 0.25% trypsin-EDTA, washed, counted, and resuspended to appropriate concentrations in serum-free Waymouth’s medium prior to s.c. injection into the inguinal region of syngeneic mice.

Cell Replication. For determination of cell proliferation rates, 5 x 10\textsuperscript{4} viable cells were plated in 60-mm plastic Petri dishes (Corning Glass Works, Corning, NY) in 4 ml of Waymouth’s supplemented medium. At 24-hr intervals, dishes were randomly selected, cells were removed by trypsinization, and cell number was determined. To determine the effect of the prostaglandin synthesis inhibitor indomethacin on cell replication, cells were cultured in the presence of indomethacin (Sigma Chemical Co., St. Louis, MO) or in ethanol control, and cell number was determined. Indomethacin was dissolved in absolute ethanol, filter sterilized, and diluted in Hank’s balanced salt solution to the appropriate concentrations. Ethanol vehicle was added to control cultures to achieve a final concentration of 0.01%, the highest concentration used in cultures containing indomethacin. Indomethacin or ethanol was added daily to cell cultures without additional change of medium.

Indomethacin. Immediately after tumor transplantation, mice were...
transferred to cages containing water bottles with 1% absolute ethanol (vehicle) or indomethacin, dissolved in ethanol, and diluted in water to achieve a final concentration of 7 μg/ml. Water was changed twice weekly, and mice were maintained on standard laboratory chow. Twice weekly, mice were weighed, and 2 tumor diameters were determined by vernier caliper measurement. Drug treatment and observation were continued until animals reached a moribund state, at which time they were sacrificed by cervical dislocation.

Prostaglandin Assays. Isolation and measurement of tumor-associated prostaglandins have been described previously (10). Nonneoplastic tumors (lesser than 10 mm in diameter) were removed and immediately frozen to −70°C in neutral buffer. On the day of assay, tumors were thawed, weighed, and homogenized in phosphate-buffered saline using a Willems Polytron (Brinkman Instruments, Inc.). ³H-labeled PGE₂ was added to tumor homogenates to determine recovery efficiency. The entire homogenate was extracted with three 4-ml volumes of ethyl acetate. The 3 extracts were pooled and evaporated to dryness under a stream of nitrogen. The residue was resuspended in a solution of benzene:ethyl acetate:methanol (60:40:2) in a volume of 1.0 ml. This solution was separated into PGA, PGE, and PGF by silicic acid chromatography using 3 solvent mixtures [benzene:ethyl acetate (60:40) and benzene:ethyl acetate:methanol (60:40:2 or 60:40:20)]. These solvent mixtures were added in volumes of 8, 16, and 8 ml, respectively. Chromatography was carried out in glass minicolumns (16 x 1 cm; New England Nuclear, Boston, MA) containing a mixture of silicic acid and Hyflo Super Cell (10:1, w/w) supported by a glass fiber disc. Recovery of radioactive PGE₂ was estimated as 86 ± 3.3% (S.E.). The fractions were collected and evaporated to dryness, and the levels of PGE₂ were determined by radioimmunoassay. PGE₂ was converted to PGB₂ by boiling at pH 12.5 and was measured with a commercially prepared anti-PGB₂ antibody (Clinical Assays, Cambridge, MA). The antibody used to measure PGE₂ cross-reacts with PGE₁ (17%).

Statistical Methods. Contingency table analyses were used to test for association between treatment type and categorical outcomes such as tumor incidence. The usual χ² test was used to test for statistical significance of the tables. Breslow's modification of Gehan's generalized Wilcoxon test (3) was used to test for differences in survival time between treatment groups.

RESULTS

We have shown previously that mouse mammary tumors are heterogeneous in their levels of PGE₂ and PGF₂α in situ (6). Experiments designed to determine if prostaglandins play a biological role in mammary tumor growth were carried out by administering the prostaglandin synthesis inhibitor indomethacin to tumor-bearing animals. Tumor cells growing logarithmically in vitro were trypsinized, washed, counted, and injected into the s.c. tissue of BALB/c mice in the inguinal area. At this time, mice were switched to drinking water containing indomethacin (7 μg/ml) or ethanol vehicle (1%). Mice were weighed and examined biweekly, and tumors were measured in 2 diameters by vernier caliper. Drug administration and observation were continued until animals reached a moribund condition, at which time they were sacrificed.

As shown in Chart 1, administration of indomethacin to mice transplanted with 9 × 10⁵ cells of the low-PGE, poorly metastatic, highly immunogenic Tumor 410 resulted in the regression of tumors in 11 of 12 animals, with one animal developing a progressive, fatal tumor. In contrast, 10 of 12 control animals transplanted on the same day developed progressively growing tumors. At Day 353 posttransplantation, all surviving animals were sacrificed, necropsied, and found to be tumor free.

When mice given injections of 1 × 10⁶ cells of the high-PGE, highly metastatic, poorly immunogenic Tumor 4501 were given indomethacin, the inhibition of tumor growth was less dramatic (Chart 2). Seventeen of 23 control animals (74%) developed progressive tumors, whereas 18 of 27 (67%) indomethacin-treated animals developed progressive tumors. While this difference is not statistically significant, the mean survival time of tumor-bearing mice that died was 89 ± 9 days for indomethacin and 53 ± 7 days for control mice. The Breslow test for survival time differences between the 2 groups was highly significant (p < 0.005).

Chart 3 shows the mean body weight of Tumor 4501-bearing mice receiving ethanol or indomethacin in their drinking water. As can be seen, indomethacin ingested at a concentration of 7 μg/ml does not lead to body weight loss and, in fact, these animals continued to gain weight at a time when untreated control animals were becoming moribund as a result of tumor burden.

When 3.5 × 10⁵ cells of another high-PGE, highly metastatic line, 4526, were transplanted to mice, 16 of 20 untreated mice (80%) developed progressively growing tumors with no regression, whereas, of the 23 mice receiving oral indomethacin, 15 developed palpable lesions, 9 of which regressed and 6 (26%) of which led to progressively growing tumors (Chart 4). This difference in progressive tumor incidence was highly significant.
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\((p < 0.0005)\). Mean survival time for animals with progressing
tumors that died was not greatly affected by drug treatment \((111
days ± 20 for indomethacin-treated mice \textit{versus} 91 days ± 11
for control mice)\).

Table 1 shows the effects of indomethacin treatment on levels
of \(\text{PGE}_2\) \textit{in situ}. Mice received tumor cells transplanted s.c. and
were switched to ethanol or indomethacin-containing water.
Tumors were removed when they had achieved an average
diameter of 15 mm and were frozen and assayed subsequently
for \(\text{PGE}\). Analysis of the effects of indomethacin on \textit{in situ} \(\text{PGE}\)
levels in Tumor 410 was not possible, due to the complete
inhibition of tumor growth. Levels of \(\text{PGE}\) are shown for Tumor
410.4, the parent tumor of 4501, and Tumor 4526. This experi-
mnt establishes that indomethacin is able to inhibit cyclooxy-
genase activity within the tumor mass.

Chart 5 shows that culturing tumor cells in the presence of
indomethacin did not result in inhibition of tumor cell division,
except at the highest concentration used \((1 \times 10^{-6} \text{ M})\). At all
other concentrations, some stimulation of cell replication was
seen when cell number was compared to cultures containing
ethanol vehicle only. All increases in cell number greater than
25% were statistically significantly greater than those of the
control cultures \((p < 0.05)\).

\begin{table}
\centering
\begin{tabular}{|c|c|}
\hline
\textbf{Line 410.4} & \textbf{Line 4526} \\
\hline
\text{Control} & 374 ± 49* \\
\text{Indomethacin} & 171 ± 62 \\
\hline
\end{tabular}
\caption{Effect of indomethacin on \textit{in situ} \(\text{PGE}\) levels}
\end{table}

\textbf{DISCUSSION}

It is well established that many experimental and human
tumors synthesize high levels of prostaglandins both \textit{in vivo} and
\textit{in vitro} \((14)\). Early studies by Straussner and Humes \((22)\) and
Plescia \textit{et al.} \((19)\) showed that indomethacin and aspirin could
inhibit tumor growth and increase survival times, and these
studies have been confirmed in other animal tumor systems by
ourselves and other investigators \((9, 12)\). These studies were
carried out using either Moloney sarcoma virus-induced lesions
\((22)\), which spontaneously regress at a high rate, or single,
nonmetastasizing fibrosarcomas \((9, 19)\). Interestingly, growth of
the B-16 melanoma, which does metastasize, is accelerated in
indomethacin-treated mice \((5)\).

The advantage of the present system is that it enables us to
study the role of prostaglandins in the growth of heterogeneous
mammary tumors of common origin that differ in many biological
properties. We have reported previously that these lesions, as
well as other related mammary lesions, such as proneoplastic
hyperplastic alveolar nodules, mammary tumors of low tumori-
genicity, and highly metastatic tumors, are heterogeneous in
their \textit{in situ} prostaglandin levels \((6)\). Those studies showed that

\begin{figure}
\centering
\includegraphics{chart3.png}
\caption{Effect of indomethacin on body weight. Mean body weight of Tumor 4501-bearing mice. \textbullet, control (vehicle-treated) mice; \textcircled{O}, drug-treated (indomethacin) mice. Bars, S.E.}
\end{figure}

\begin{figure}
\centering
\includegraphics{chart4.png}
\caption{Indomethacin-mediated growth inhibition of Tumor 4526. Mean tumor diameter in control (vehicle-treated) mice (\textbullet) and indomethacin-treated mice (\textcircled{O}) is shown. Numbers in parentheses, number of surviving mice with palpable tumors, used to calculate mean tumor diameter; bars, S.E. MST, mean survival time.}
\end{figure}

\begin{figure}
\centering
\includegraphics{chart5.png}
\caption{Effect of indomethacin on number of cultured tumor cells. Tumor line 4526 or 410 is plated in tissue culture dishes \((5 \times 10^4 \text{ cells/dish})\) in the presence or absence of various concentrations of indomethacin, and the cell number in randomly selected dishes is determined at daily intervals. Data are expressed as the percentage increase in the cell number of dishes with indomethacin in comparison to cells cultured in the presence of vehicle control \(\times, 10^{-6} \text{ M}; \textcircled{O}, 10^{-7} \text{ M}; \textcircled{C}, 10^{-8} \text{ M}; \textcircled{I}, 10^{-9} \text{ M}.\) Each point represents 3 dishes.}
\end{figure}
a positive correlation existed between high PGE levels and metastatic potential. Similar relationships have been reported for human breast cancer (1, 2, 20, 21). The present experiments show that, in spite of the heterogeneity in prostaglandin levels, the s.c. growth of 3 of these tumors is inhibited in the presence of indomethacin. Whether the differences in effectiveness of treatment for the different tumors are due to lower levels of PGE in Tumor 410 compared to Tumors 4501 and 4526, the high immunogenicity of this tumor, or its low metastatic capacity compared to the other 2 cannot be answered at this time. Studies to determine the effect of indomethacin on highly metastatic, high-PGE, immunogenic tumors are in progress.

Kollmorgen et al. (15) have shown recently that oral indomethacin could inhibit the growth rate of transplantable mammary tumors in lymph nodes of Wistar-Furth rats. Interestingly, this tumor is poorly transplantable in rats receiving standard (low-fat) laboratory chow, whereas the incidence of successful transplantation is increased with a high-fat diet. This dietary effect could be blocked by administering indomethacin. Carter et al. (4) have shown that the tumorigenic effects of 7,12-dimethylbenz(a)anthracene are enhanced in rats that are fed a high-fat diet, and indomethacin could block this dietary effect on tumorigenesis.

The inhibition of primary tumor growth by the cyclooxygenase inhibitor indomethacin suggests that one or more arachidonic acid metabolite plays a biological role in the progressive growth of these mammary tumors. We have reported that these tumors contain 2 primary prostaglandins, PGE2 and PGF2α (6). We have found that material that cochromatographs on thin-layer gels with PGA (a nonenzymatic breakdown product of PGE2) is identifiable in tumor homogenates. Both tumor parenchymal cells (7) and macrophages isolated from these tumors synthesize PGE (17). Thus, the mediation of tumor inhibition by indomethacin may be through either or both of these cell types. The effect of indomethacin on the phenotype of tumor-associated macrophages is being investigated. Future studies will be carried out using more sensitive methods to describe other possible cyclooxygenase metabolites synthesized by tumor cells and tumor-associated macrophages.

In contrast to our findings, some studies have shown that prostaglandins inhibit tumor cell proliferation. Favalli et al. (5) have reported that administration of a PGE2 analogue (16,16-dimethyl-PGE2 methyl ester) inhibited growth of the B-16 melanoma in vivo and in vitro, whereas indomethacin accelerated tumor growth. Jubiz et al. (13) have shown that PGF2α has an inhibitory effect on a hormone-dependent rat mammary adenocarcinoma. Many studies in vitro have shown that various prostaglandins inhibit tumor cell replication (reviewed in Ref. 12) and that inhibition of endogenous prostaglandin synthesis has the opposite effect.

It is clear that the response of different tumors to prostaglandins and prostaglandin inhibitors will differ, and this may be due to the unique prostaglandin profiles of different tumors (e.g., the B16 melanoma synthesizes high levels of PGD2). The elucidation of the relative importance of individual cyclooxygenase products awaits the development of specific inhibitors and/or long-acting analogues of these products.

It is unlikely that the tumor-inhibitory effects of indomethacin that we see are a result of inhibition of tumor cell proliferation, because our studies in vitro (Chart 5; Ref. 7) and those of others (23) show that indomethacin, at concentrations which inhibit the synthesis of PGE in vitro, has a stimulatory effect or lack of effect on tumor cell division in vitro. Conversely, addition of several different prostaglandins to cultured tumor cells inhibits cell growth (12). Interestingly, although our tumors synthesize large amounts of prostaglandins that generally inhibit proliferating lymphocytes, they apparently do not shut themselves off. We are exploring the possibility that these tumor cells are relatively unresponsive to prostaglandins, due to a paucity of prostaglandin receptors.

A more likely explanation for the effects of indomethacin in vivo is blockage of prostaglandin-mediated immunosuppression. The immunosuppressive effects of prostaglandins, particularly PGE, are well documented (11). Plescia et al. (19) first suggested that tumor-associated prostaglandins are immunosuppressive, and this notion has been supported by a number of subsequent studies (reviewed in Ref. 11). Thus, the more marked inhibition of Tumor 410 may be due to the fact that this tumor, in contrast to Tumors 4501 and 4526, is very immunogenic.

It seems unlikely that the inhibitory effects of indomethacin in our system are due to blocking of a tumoricidal macrophage, as we have found that the tumors with the highest PGE levels and highest metastatic potential are more likely to contain cytotoxic macrophages than those of the nonmetastatic tumors (16). We have found that tumor-associated macrophages are phenotypically heterogeneous with respect to size, density, and ectoenzyme and PGE levels (17). While both macrophages from metastatic and nonmetastatic tumors release PGE, the peak activity in different tumors is associated with macrophages of different sizes and, therefore, it is possible that the net antitumor effect of these macrophages is due to a balancing of macrophages with different activities. Other macrophage products such as thromboxane and prostacyclin may also contribute to these effects. Other targets of indomethacin action might be suppressor macrophages, T-lymphocytes, or suppressors of natural killer function.

In summation, although indomethacin stimulates cell division in vitro (7), our studies show that the net effect of indomethacin administration in vivo is inhibition of primary tumor growth. Therefore, if prostaglandins act in vivo to inhibit tumor cell replication, as has been shown in vitro, they must also inhibit an otherwise effective antitumor mechanism. We hypothesize that the dominant action of prostaglandins in vivo is an immunosuppressive one that allows for protection and subsequent tumor growth.

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