ABSTRACT

The levels of two prostaglandins (prostaglandins E and F) have been determined in a series of murine mammary lesions ranging from preneoplastic, hyperplastic alveolar nodules to highly metastatic adenocarcinomas. A highly positive correlation was seen between high levels of prostaglandin E and high tumorigenicity and metastatic potential. In addition, spontaneous metastasis of two highly metastatic tumors was partially inhibited by p.o. administration of indomethacin from the time of s.c. tumor transplantation until removal of the primary tumor at a limited size. Further, mammary tumor cells of differing metastatic potential were susceptible to polyinosinic-polycytidylic acid activated spleen lymphocytes in vitro. Cells of metastatic tumor lines (410.4 and 66) were more resistant to killing than were cells of two non-metastatic tumor lines (168 and 410). The sensitivity of all target cells was increased when endogenous prostaglandin synthesis was prevented by the addition of indomethacin (1 μM) but was not affected by the lipoxygenase inhibitor nordihydroguaiaretic acid.

INTRODUCTION

That tumor subpopulations are heterogeneous in many characteristics is now well established (3). Among numerous phenotypic traits that have been described, a number are associated with the ability to metastasize, including relative insensitivity to natural killer cells (4, 5), increased surface sialylation of cell surface glycoconjugates (6), increased platelet aggregating activity (7, 8), and increased collagenase secretion (9). Many tumors have been shown to have high levels of one product or more of the cyclooxygenase pathway of arachidonic acid metabolism (10). Although PGE has been most thoroughly studied, elevated levels of PGF, PGD, and other prostanoids have also been reported. That these products have a biological function in the growth and metastasis of tumors is suggested by our studies (11, 12), and those of many others (reviewed in Refs. 10, 13, and 14), showing that the growth of many experimental tumors is inhibited by prostaglandin synthesis inhibitors such as indomethacin.

The present study was carried out to determine the role of prostaglandins in the early steps of metastatic dissemination of murine mammary adenocarcinomas.

1 Supported by Concern Foundation, The E. Walter Albachten Bequest, The United Foundation of Metropolitan Detroit, NIH Grant 27437, and NIH Grant 37943. A preliminary report was presented previously (1, 2). To whom requests for reprints should be addressed.
2 The abbreviations used are: PGE, prostaglandin E; PGF, prostaglandin F, other prostaglandins are designated similarly; NK, natural killer cell, polycl(C), polyinosinic-polycytidylic acid; HAN, hyperplastic alveolar nodule.
3 Received 12/11/84; revised 6/13/85; accepted 6/19/85.

MATERIALS AND METHODS

Mice. Male and female BALB/cMCF mice 4 to 6 weeks old were bred in the Animal Care Facility of the Michigan Cancer Foundation from cesarean derived breeding pairs originally obtained from the Cancer Research Laboratory, University of California, Berkeley, CA. Mice are maintained on standard laboratory chow ad libitum.

Tumor Cell Lines. The C,HAN line was isolated and described by Medina (15). It is a benign tissue that will not grow outside the mammary fatpad, but it is preneoplastic. Transplantation of C,HAN into the cleared mammary fatpads of 3-week-old BALB/c mice, as described by DeOme et al. (16), gives rise to C tumors at a frequency of approximately 80%. The mean latent period for tumor formation is 27 weeks (15). The derivation of the mammary tumors used here has been described previously (17). Tumor lines 410, 66, 168, and 68H were derived from a spontaneously arising mammary tumor of a BALB/cfC3H mouse. Lines 410 and 68H are rarely metastatic, line 168 does not metastasize spontaneously from a s.c. site but will form lung colonies following tail vein injection, and line 66 is frankly metastatic. Line 410.4 was derived from the fourth transplant generation of the 410 cell line which was derived from a metastatic nodule isolated from the lung of a BALB/cfC3H mouse bearing the 10th s.c. passage of the original tumor. Lines 4501 and 4526 are cloned subpopulations of the uncloned 410.4 line. Lines 4501, 4526, and 410.4 metastasize spontaneously from s.c. implants at a frequency of >80% (17). The metastatic properties of the tumor cell lines have remained stable for periods of 2 to 5 years of cultivation in vitro as determined by transplantation and in vitro tests (17). Tumor 410.4 is immunogenic whereas tumor line 66 is nonimmunogenic in BALB/c mice. All tumor cell 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 μg/ml) and were buffered with NaHCO3. 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 tumor cell cultures were treated with 0.25% trypsin:EDTA, washed, counted, and resuspended to appropriate concentrations in Dulbecco's phosphate buffered saline prior to s.c. injection of 5 x 106 cells into the inguinal region of syngeneic mice. Tumor 68H is transplanted by trocar of tumor pieces.

Prostaglandin Assays. Isolation and measurement of tumor associated prostaglandins have been described previously (18). Tumor bearing mice were sacrificed by cervical dislocation and nonnecrotic tumors (<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 Wilems Polytron (Brinkman Instruments, Inc.). H-Labeled PGE was added to tumor homogenates to determine recovery efficiency. The entire homogenate was extracted with three 4-mL volumes of ethyl acetate. The three extracts were pooled and evaporated to dryness under a stream of nitrogen. The residue was resuspended in 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 three 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 microlumins (16 x 1 cm; New England Nuclear, Boston, MA) containing a mixture of silicic acid and Hyflo Super Cell (10:1, w/v) supported by a glass fiber disc. Recovery of radioactive PGE2 was estimated as 85 ± 3.3% (SE). The fractions were collected and evaporated to dryness and the levels of PGE2 and PGF2α 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%). PGF2α was measured with a commercially prepared anti-PGF2α antibody (Clinical Assays, Cambridge, MA). This antibody expresses cross-reactive binding with PGI2 (28%).

Indomethacin. Immediately after tumor transplantation mice were transferred to cages containing water bottles with 0.25% absolute ethanol (vehicle) or indomethacin, dissolved in ethanol, and diluted in water to achieve a final concentration of 7 μg/ml. Ingestion of 3 ml of water/day results in a drug dose of approximately 1 mg/kg/day. Water was changed daily and mice were maintained on standard laboratory chow. This dose of indomethacin has been shown to reduce levels of PGE in situ by 50–60% (11). Twice weekly two tumor diameters were determined by vernier caliper measurement. Tumors growing s.c. were removed on an individual basis when they had achieved a size predetermined to be associated with lung metastases (17). Surgical removal of tumors was carried out under sodium pentobarbital anesthesia, blood vessels were cauterized, and wounds were closed with 3–4 wound clips. At this time mice were removed from drug treatments. Three weeks post-surgery mice were sacrificed by cervical dislocation and internal organs were examined for the presence of macroscopically visible metastases.

Natural Cytotoxicity Assay. The 18-h cytotoxicity assay was performed as described by Hanna and Fidler (5). Tumor target cells were trypsinized, washed, and resuspended in Eagle’s minimal essential medium containing 8–10 μCi of [3H]proline (New England Nuclear, Boston, MA). After 18 h these labeled cells were trypsinized, washed three times, resuspended in RPMI 1640 supplemented with 10% fetal calf serum (Hy Clone; Sterile Systems, Logan, UT), and plated in flat-bottomed wells (1 × 104 cells/well) of 96-well microtiter plates. These target cells were allowed to adhere for 24 h. Single cell suspensions of spleen effector cells from normal mice or from mice receiving 100 μg of poly(l-C) (Sigma) were dissolved in absolute ethyl alcohol, filter sterilized, and diluted to the appropriate concentrations in RPMI 1640. Recovery of radioactive PGE in situ by 50-60% (11). Twice weekly two tumor diameters were tested contained measurable amounts of PGE and PGF. Due to the cross-reactivity of the anti-PGB2 with PGB1 and the anti-PGF2α with PGF1α, results are reported as PGE and PGF. When the various lesions are ranked in order of increasing tumorigenicity and metastatic potential (Table 1), a highly positive correlation is seen between these properties and in situ levels of PGE. The preneoplastic C4 HAN has low levels of PGE (9 ng/g) whereas tumors which arose from it have higher levels (35 ng/g). Tumors of line 68H, which are extremely slowly growing and nonmetastatic, have similar low levels as do tumors of the 410 line which are progressively growing tumors that do not metastasize. Line 168 tumors do not metastasize spontaneously but will form lung colonies following i.v. injection. Line 410.4 tumors, which are highly metastatic tumors exhibiting high levels of PGE, NK SENSITIVITY AND METASTASIS.

PGE, NK SENSITIVITY AND METASTASIS

RESULTS

Prostaglandin Content of Mammary Lesions with Varying Tumorigenic and Metastatic Potential. All tumor homogenates tested contained measurable amounts of PGE and PGF. Due to the cross-reactivity of the anti-PGB2 with PGB1 and the anti-PGF2α with PGF1α, results are reported as PGE and PGF. When the various lesions are ranked in order of increasing tumorigenicity and metastatic ability (Table 1), a highly positive correlation is seen between these properties and in situ levels of PGE. The preneoplastic C4 HAN has low levels of PGE (9 ng/g) whereas tumors which arose from it have higher levels (35 ng/g). Tumors of line 68H, which are extremely slowly growing and nonmetastatic, have similar low levels as do tumors of the 410 line which are progressively growing tumors that do not metastasize. Line 168 tumors do not metastasize spontaneously but will form lung colonies following i.v. injection whereas tumors 410.4, 66, 4501, and 4526 are highly metastatic tumors exhibiting high levels of PGE (321–689 ng/g). A similar albeit less perfect correlation is seen between PGF levels and metastatic ability.

Effect of Indomethacin p.o. on Spontaneous Metastasis of Line 410.4 and 66 Tumors. We have shown previously that indomethacin p.o. inhibits the s.c. growth of tumor lines 410, 4501, and 4526 (11). In order to determine if a prostaglandin inhibitor can affect the metastatic rate of two related tumors, mice were given indomethacin p.o. or vehicle control beginning on the day of tumor transplantation. As shown in Table 2, when mice are given injections of 5 × 10⁴ line 410.4 cells s.c., tumors of control mice reach a mean diameter of 15 mm on day 45 compared to indomethacin-treated tumors which reach this size 53 days posttransplantation. In the second experiment using a lower tumor cell dose, no inhibition of the primary tumor was seen at early stages of growth. This is because the effects of indomethacin on growth inhibition are more apparent as tumors enlarge. All mice had detectable lung metastases but the mean number was significantly reduced (P < 0.05) by drug treatment (39 and 55%) in both experiments. The number and distribution of extrapulmonary metastases was not affected significantly by drug treatment (data not shown).

Table 1

<table>
<thead>
<tr>
<th>Prostaglandin levels in mouse mammary neoplasms</th>
<th>Sample</th>
<th>Characteristics</th>
<th>PGE (ng/g)</th>
<th>PGF (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4 HAN</td>
<td>Preneoplastic benign</td>
<td>9 ± 5</td>
<td>62 ± 1</td>
<td></td>
</tr>
<tr>
<td>C4 tumor</td>
<td>Preneoplastic nonmetastasizing</td>
<td>35 ± 8</td>
<td>161 ± 55</td>
<td></td>
</tr>
<tr>
<td>68H tumor</td>
<td>Preneoplastic nonmetastasizing</td>
<td>29 ± 8</td>
<td>385 ± 145</td>
<td></td>
</tr>
<tr>
<td>410 tumor</td>
<td>Preneoplastic low metastasizing</td>
<td>75 ± 22</td>
<td>58 ± 4</td>
<td></td>
</tr>
<tr>
<td>168 tumor</td>
<td>Preneoplastic low metastasizing</td>
<td>98 ± 40</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>410.4 tumor</td>
<td>Preneoplastic high metastasizing</td>
<td>321 ± 45</td>
<td>563 ± 106</td>
<td></td>
</tr>
<tr>
<td>66 tumor</td>
<td>Preneoplastic high metastasizing</td>
<td>337 ± 70</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>4501 tumor</td>
<td>Preneoplastic high metastasizing</td>
<td>580 ± 163</td>
<td>489 ± 122</td>
<td></td>
</tr>
<tr>
<td>4526 tumor</td>
<td>Preneoplastic high metastasizing</td>
<td>689 ± 142</td>
<td>434 ± 112</td>
<td></td>
</tr>
</tbody>
</table>

* Each value is the mean ± SE for 6–10 samples.
  * Does not metastasize spontaneously; will form lung colonies following i.v. injection.
  * ND, not done.

Unpublished observations.

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Tumor Lines to Natural Cytotoxic Effector Cells In Vitro. We have begun to test the role of NK cells in our system by comparing the sensitivity of metastatic versus nonmetastatic mammary tumor cells to killing by nonsensitized spleen lymphocytes. Chart 2 shows the relative cytotoxicity detected against two tumor lines using an 18-h [3H]proline assay and poly(C) activated spleen effector cells at an effector:target ratio of 200:1. Cytotoxicity against the nonmetastatic line (410) is higher than for the metastatic line (410.4). These are results from one representative experiment. Mean cytotoxicities using an effector:target ratio of 200:1 for five or six experiments were 14.1 ± 2.25% (SE) against 410 targets and 4.0 ± 1.48% for line 410.4 targets. When these [3H]proline labeled target cells are precultured for 24 h in the presence of indomethacin, increased cytotoxicity is seen for both targets precultured with 1 µM indomethacin and the difference in sensitivity between the metastatic and nonmetastatic lines is lost. Lower concentrations of drug have no effect.

When similar studies are carried out using four different targets and the lipoxigenase inhibitor nordihydroguaiaretic acid, again one sees greater sensitivity to cytotoxicity by the nonmetastatic lines (lines 410 and 168) versus the metastatic lines (lines 410.4 and 66) (Chart 3). Although high dose nordihydroguaiaretic acid (2 × 10⁻⁴ M, 1 × 10⁻⁴ M) leads to apparent increased cytotoxicity, at these concentrations most cells are killed by drug alone and the addition of lymphocytes kills the remaining targets. Drug concentrations that are not cytotoxic (≤ 1 × 10⁻⁵ M) have no effect on natural cytotoxicity.

**DISCUSSION**

Experimental and human tumors have been shown to be heterogeneous for many traits. We have shown that murine
PGE, NK Sensitivity and Metastasis

Chart 3. Cytotoxicity of poly(I-C) activated spleen effector cells for tumor targets 410, 168, 410.4, and 66 at an effector-target ratio of 200:1. Tumor targets were preincubated in the presence of nordihydroguaiaretic acid (NDGA) for 24 h before the addition of effector cells.

Bennett et al. (21) have shown that human breast tumors with high levels of PGE-like material are more often associated with skeletal metastases than are low PGE tumors and survival time postsurgery is inversely correlated to in situ prostaglandin levels (22). Rolland et al. (23) concluded that high prostaglandin synthetic activity is associated with histologically aggressive human breast tumors. Bishop et al. (24) showed a positive correlation for high PGE and higher grade tumors that have a poor prognosis. In a prospective study of primary human breast tumors we found that higher levels of PGE and PGF are associated with less differentiated tumors, and PGE levels are negatively associated with tumor diameter. Higher PGE levels were seen in tumors of postmenopausal women and in estrogen receptor positive tumors (18). Others have reported high levels of prostaglandins in human breast cancer (24–26) in prospective studies. The significance of these findings and the relationship to the clinical prognosis for these patients is not yet known. Watson et al. (26) report that levels of PGE2 and PGF2 are not related to the presence of estrogen or progesterone receptor.

In contrast some studies have shown no relationship between PGE in mammary tumors and other biological characteristics (27–29). Malachi et al. (29) found that some malignant breast tumors had high levels of PGE2 in comparison to benign tumors or normal breast tissue but that these levels were not related to clinical stage, histological classification, or survival time during a relatively short 12–36-month follow-up period. In the follow-up study of 17 patients no relationship was seen between PGE2 levels and presence of metastases. It is possible that significant differences would be seen during longer term follow-up or with larger groups of patients.

Hortobagyi (27) studied the production of PGE2 by metastatic breast carcinoma lines maintained in vitro. Seventeen of 21 lines synthesized PGE2 but no correlation was seen between this activity and patient survival time. Karmali et al. (30) analyzed prostaglandin levels in tissues and prostaglandin synthetase activity in microsomal preparations from 24 human mammary carcinomas. She found that prostaglandin content was higher in neoplastic tissues than in uninvolved areas from the same specimen but when the value for normal tissue was subtracted from the tumor yield, no relationship was seen between these levels and tumor size, lymph node involvement, or distant metastases. The exception was thromboxane B2 yields which were associated with tumor size and number of involved lymph nodes. Uncorrected PGE levels were higher with advanced stage in agreement with the findings of Rolland et al. (23). Interestingly the microsomal prostaglandin synthetase activity was comparable for tumor and noncancerous tissues. Kubey et al. (28) found no correlation between metastasis and PGE2 in a series of four rat mammary tumors.

These discrepancies suggest that the role of prostaglandins in tumor growth and metastasis is complex. Some of the discrepancies in the clinical findings are due to: (a) differences in patient populations, e.g., some studies include only patients preselected to have known metastatic disease; and (b) differences in methodology, e.g., tissue prostaglandin yields during homogenization of whole tissues versus measurement of specific enzyme activities in subcellular fractions. In addition it is clear from experimental studies that different prostaglandins can have markedly diverse effects depending in part on the tumor studied. While the present report and previous studies (reviewed in Ref. 10) show that indomethacin inhibits s.c. and metastatic growth of many transplantable tumors, a number of studies have shown that rat mammary tumors, which are hormone dependent for growth, are inhibited by prostaglandin F2α (31–33). It is likely that some of these effects are mediated by interactions of prostaglandins and hormones whereas the present study of hormonally independent tumors argues for a growth promoting role of prostaglandins related to their effects on the immune system. PGE2 is a likely candidate for the indomethacin inhibitable effect due to its presence in high levels in these tumors and to its immunosuppressive qualities (34, 35).

Although much evidence suggests that the association of PGE with tumor metastasis is related to immunosuppression, altered platelet function (mediated by prostanoids) may also be an important parameter. In other murine tumor systems Gasic et al. (7) have shown a direct correlation between the ability of a variety of tumor cells to cause platelet aggregation in vitro and the ability to metastasize to the lung but not to other organs. These workers also showed that aspirin treatment prevents the appearance of lung metastases in mice bearing a mammary tumor but has no effect on the primary tumor growing at a s.c. site. Ambrus et al. (36) found that three different inhibitors of platelet aggregation are able to decrease the number of pulmonary metastases after the i.v. injection of Ehrlich ascites cells. Stringfellow and Fitzpatrick (37) found the malignant potential of B16 melanoma sublines to be inversely correlated with the levels of prostaglandin D2, a cyclooxygenase product that inhibits platelet aggregation. Culture of B16 cells in the presence of indomethacin prior to i.v. injection increased the number of
experimental lung metastases, whereas preculture in the presence of PGD\(_2\) reversed the indomethacin effect. Although the authors hypothesize that the effects seen are probably due to interference with the formation of platelet-tumor emboli, indomethacin might have stimulated the cells to divide, giving them a selective growth advantage immediately after arrest in the lung (20). The fact that PGD\(_2\) is a major product of B16 melanoma cells but is rarely seen in other tumors suggests that the role of these products will be diverse depending on the proaggregatory mechanism (generation of thrombin?) and that elevation of prostacyclin (an antiaggregatory product) inhibits metastasis. On the basis of this hypothesis our high PGE tumors would be expected to be poorly metastatic (due to the antiaggregatory action of PGE). To explain our results based on a platelet effect one would have to postulate that a second, overriding proaggregatory mechanism (generation of thrombin?) is present which negates the antiaggregatory action of PGE. Studies are in progress to determine if these tumors do have platelet aggregatory activities.

In a beginning attempt to elucidate the mechanism of indomethacin mediated inhibition of metastasis, we have proposed that tumor associated prostaglandins may be immunosuppressive (38). However, metastasis of both an immunogenic (line 410.4) and nonimmunogenic tumor (line 66) are inhibited, suggesting that classical immune effector mechanisms are not involved. Hanna and Fidler (5) and Gorelik et al. (4) have shown a role for NK cells in controlling metastatic dissemination of B16 melanoma and Lewis lung carcinoma. We have confirmed their findings that metastatic tumors are more resistant to killing by natural effector cells. Our finding that mammary tumor cells are more sensitive to natural killing when endogenous prostaglandin synthesis is inhibited confirms the findings of Droller et al. (39) that natural cytotoxicity against bladder tumor cells is enhanced in the presence of indomethacin. Our finding also suggests a possible mechanism for in vivo inhibition of metastasis, namely by blocking the PGE mediated inhibition of natural killer activity. The fact that indomethacin was active only at 1 \(\mu\)M, a dose which has been shown to inhibit 50–90% of PGE synthesis (20), is troubling to this interpretation. Even in the presence of indomethacin, cytotoxicity never reaches high levels, which might explain why lung metastases are only reduced in number, not completely eliminated by indomethacin. Leung and Koren have shown, however, that poly(I-C) activated NK cells are relatively resistant to the suppressive effects of PGE in comparison to unsensitized NK cells (40). Interestingly Gorelik et al. (41) have shown that the ability of heparin or prostacyclin to inhibit lung colonization by i.v. injected B16 cells is dependent on intact NK function. The fact that metastases of both an immunogenic (line 410.4) and a nonimmunogenic (line 66) tumor are inhibited argues against a role for specific (T-lymphocyte) effector mechanisms. We have shown that macrophage mediated cytosisis is also more effective against nonmetastatic than metastatic tumor lines and that indomethacin pretreatment increases target sensitivity (42). Determining the relative importance of these two non-specific effector cells (NK and macrophages) and the mechanisms of indomethacin mediated tumor inhibition will require further study.

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REFERENCES


Relationships of Prostaglandin E and Natural Killer Sensitivity to Metastatic Potential in Murine Mammary Adenocarcinomas

Amy M. Fulton and Gloria H. Heppner