Lack of Effect of in Vivo Prostacyclin on the Development of Pulmonary Metastases in Mice following Intravenous Injection of CT26 Colon Carcinoma, Lewis Lung Carcinoma, or B16 Amelanotic Melanoma Cells

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ABSTRACT

Honn et al. [Science (Wash. DC), 212: 1270, 1981] have recently reported a 93% reduction in the development of metastases of B16 amelanotic tumor cells given i.v. following a single dose of prostacyclin (PGI2) (100 µg) and theophylline (100 µg) 30 min prior to the injection of tumor cells. We have been unable to reduce pulmonary metastases induced by the i.v. injection of CT26 colon adenocarcinoma, Lewis lung carcinoma, or B16 amelanotic melanoma cells with a similar regimen. Thus, PGI2 and theophylline given prior to injection of tumor cells and 2 hr postinjection had no effect on the number or volume of pulmonary tumor nodules for CT26 cells, using 15 experimental and 14 control animals; Lewis lung cells, using 14 experimental and 13 control animals; or B16 amelanotic cells, using 26 experimental and 12 control animals. The PGI2 used was shown to be active in vitro, inhibiting tumor-induced platelet aggregation by all three tumors at 10^-9 M; and in vivo by inhibition of Lewis lung-induced thrombocytopenia at 1 hr, using 100 µg PGI2 prior to the injection of tumor cells.

INTRODUCTION

In 1968, Gasic et al. (8) reported a reduction in tumor metastases following the induction of thrombocytopenia in the host. This observation prompted several early studies on the ability of antiplatelet agents to inhibit metastases with conflicting results (5, 10, 15, 19, 30).

The resurgence of interest in the role of platelets in tumor cell metastases (1, 3, 12, 18, 20, 21, 23, 25, 26, 28), as well as the pivotal role of prostaglandins (endothelial cell PGI2 versus platelet thromboxane A2) in the blood vessel-platelet axis, stimulated the recent study of Honn et al. (16) on the effect of in vivo PGI2 on the prevention of B16 amelanotic melanoma (B16a) metastases in mice. These workers noted a 70% decrease in the number of metastatic foci in the lungs, as well as a total inhibition of foci in other organs, following a single 100-µg dose of PGI2, given i.v. 10 min prior to the i.v. injection of tumor cells. A 93% reduction in metastases was noted following the addition of a phosphodiesterase inhibitor (theophylline, 100 µg) given i.p., 30 min prior to the PGI2 injection. In addition, an inhibitor of PGI2 synthesis, 15-hydroperoxyarachidonic acid, increased the number of metastases by 163%.

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2 The abbreviations used are: PGI2, prostacyclin; PBS, 0.01 M phosphate-buffered saline, pH 7.4.

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The purpose of this communication is to report our experience with the effect of PGI2 and theophylline on the development of pulmonary metastases in mice using 3 different tumors: CT26 undifferentiated adenocarcinoma of the colon; Lewis lung carcinoma; and B16 amelanotic melanoma. Our results indicate that PGI2 and theophylline given 5 min prior to and 2 hr post-i.v. injection of tumor cells had no effect on the prevention of pulmonary metastases.

MATERIALS AND METHODS

Tumor Cell Lines and Tissue Culture Medium. All cell lines were grown in tissue culture medium supplemented with 2 mM glutamine, penicillin (100 units/ml) and streptomycin (100 µg/ml) and harvested at or near confluence, prior to utilization for in vivo use. Tumor cells were harvested from tissue culture dishes with brief trypsin-EDTA treatment (23). They were washed 3 times, suspended in PBS, and quantitated microscopically with a hemocytometer. Trypan blue exclusion for viability was greater than 90%. Tissue culture supplies were obtained from Grand Island Biological Co., Grand Island, NY.

CT26, a N-nitroso-N-methylurethan-induced mouse undifferentiated colon carcinoma (20), was obtained through the courtesy of Dr. M. H. Goldrosen at Roswell Park Memorial Institute, Buffalo, NY, and was grown in RPMI 1640 containing 10% fetal bovine serum.

Lewis lung carcinoma cells were obtained from Mason Laboratories, Wooster, MA, and grown in the same media as the CT26 cell lines. B16 amelanotic melanoma was obtained through the courtesy of Dr. C. Maniglia, Yale University Medical School, and was grown in Dulbecco’s minimal essential medium-glucose (0.45 g/100 ml) plus 10% fetal bovine serum.

The Lewis lung and B16 tumor cell lines were maintained s.c. in syngeneic mice. Prior to their use in vivo, tumor cells were grown for 5 to 7 days in tissue culture. CT26 was maintained in tissue culture and replenished from frozen stocks. This line was used for experiments between 4 and 12 weeks following defrosting.

Platelet Aggregation. Platelet-rich plasma and platelet-poor plasma were prepared using heparin, 5 units/ml final concentration (Liquamid; Organon, Inc., West Orange, NJ). Platelets were obtained from New Zealand White rabbits, since they have been shown to have aggregating properties similar to those of mouse platelets (22).

Aggregation was performed turbidometrically, using a Bio-Data aggregometer (Bio-Data, Willow Grove, PA). Apyrase (Grade 1 from potato; Sigma Chemical Co., St. Louis, MO) was used at 40 µg/ml platelet-rich plasma to eliminate the effect of ADP leakage from tumor cells.

Experimental Protocol. Animals (approximately 18 to 20 g) were given i.p. injections of 100 µl of PBS, Dulbecco’s PBS, or theophylline (100 µg in 100 µl PBS) 30 min prior to the i.v. injection of freshly prepared tumor cells. One hundred µl of the vehicle for PGI2 (25 mM glycine-25 mM NaCl, pH 10.1) or 100 µg PGI2 in its vehicle were then given i.v. 5 min prior to the injection of tumor cells. Tumor cells (25,000 to 75,000/µl) were removed from subconfluent tissue culture dishes with trypsin-

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EDTA and washed in PBS immediately prior to their in vivo injection in a volume of 100 µl.

Enumeration of Metastases. Animals were sacrificed at 21 days by cervical dislocation, and the pulmonary parenchyma was removed and fixed in Perifix (Fisher Scientific Co., Springfield, NJ) for 24 hr prior to enumeration of metastases. The number of size of nodules were classified into 3 categories, <0.5 mm, 0.5 to 2 mm, and >2 mm in diameter. The volume of each nodule was calculated from its diameter, assuming a sphere. The mean nodule volume for each mouse was calculated by dividing the total pulmonary nodule volume for each group of mice by the number of mice in each group.

RESULTS

Effect of in Vitro Incubation of Tumor Cells at 0° on Cell Viability and Metastatic Potential. Because preliminary studies had suggested a loss of metastatic potential with time, we determined the effect of in vitro incubation of tumor cells on metastatic potential. Table 1 (Experiments 1 to 3) depicts losses of metastatic potential for CT26 cells of 47, 92, and 68%, respectively, following 135 to 150 min of incubation in a test tube immersed in an ice bucket for mean number of pulmonary nodules and 63, 95, and 66%, respectively, for total tumor nodule volume. In vitro viability, determined by trypan blue exclusion, declined slightly for Experiment 1 but did not decline for Experiments 2 and 3. Similar results were obtained for Lewis lung and B16a tumor cells (Experiments 4 to 6). In vitro viability was not affected.

Further experiments were therefore specifically designed to avoid this loss of metastatic potential following in vitro incubation of cells. Accordingly, each control and experimental animal was consecutively alternated for each i.v. tumor injection.

Effect of PGI2 and Theophylline on CT26 Pulmonary Metastases. One hundred µg of the theophylline were given i.p. followed by 100 µg of PGI2 i.v. 20 min later, followed by 50,000 CT26 tumor cells given i.v. 10 min later into female BALB/c mice; theophylline and PGI2 were again given 2 hr later. This regimen had no effect on the development of pulmonary metastases per lung as well as total tumor volume per lung (Table 2).

Effect of PGI2 and Theophylline on Lewis Lung Pulmonary Metastases. Similarly, an identical regimen of theophylline and PGI2 also had no effect on the development of pulmonary metastases following the i.v. injection of 175,000 Lewis lung tumor cells into female C57BL/6J mice.

Effect of PGI2 and Theophylline on B16a Pulmonary Metastases. In this experimental protocol, 2 sets of experimental animals were used: one which received 2 injections of PGI2 and theophylline as with the previous 2 tumors; and one which simply received a single injection of PGI2 and theophylline, as reported by Honn et al. (16). Again, PGI2 and theophylline had no effect on the development of pulmonary metastases in male C57BL/6J mice.

Effect of PGI2 on In Vitro Platelet Aggregation Induced by CT26, Lewis Lung, and B16a Melanoma Cells. Chart 1 demonstrates the effect of PGI2 on platelet aggregation induced by 50,000 CT26, 500,000 Lewis lung, and 250,000 B16a tumor cells. Complete inhibition of aggregation could be achieved with 10⁻⁸ M PGI2 for CT26 and B16a cells (equivalent to 0.33 ng/ml platelet-rich plasma) and at 10⁻⁷ M for Lewis lung cells (equivalent to 3.3 ng/ml platelet-rich plasma).

Effect of PGI2, Given In Vivo, on Lewis Lung-Induced Thrombocytopenia. Table 3 depicts the reduction of tumor-induced thrombocytopenia by preinjection of mice with theophyline and PGI2. In Experiment 1, 1.5 x 10⁶ Lewis lung cells given i.v. reduced the platelet count to 59% of the base-line value at 1 hr and 85% of this value at 2 hr. Pretreatment with theophylline plus PGI2 completely prevented the induction of thrombocytopenia (Experiment 1). Similarly, 4 x 10⁶ Lewis lung cells reduced the platelet count to 28% of base line at 1 hr. Pretreatment with PGI2 and theophylline partially prevented the induction of thrombocytopenia to 44% of base line p < 0.05 (Experiment 2).
DISCUSSION

Although considerable evidence exists for a role for platelets in the development of tumor metastases in both the older (2, 4–9, 13, 14, 17, 19, 27, 29) and recent (1, 3, 10, 12, 18, 20, 21, 23, 25, 26, 28) literature, there is controversy regarding the efficacy of antiplatelet agents in the prevention of metastases. Thus, although Gasic et al. (5) reported beneficial effects of the efficacy of antiplatelet agents in the prevention of metastases, 23, 25, 26, 28) literature, there is controversy regarding the use of aspirin in mice given injections of MCA2 and T241 fibrosarcoma cells and Kolenich et al. (19) made similar observations on BW10232 adenocarcinoma of rabbits, Wood and Hilgard (30) obtained negative results with a V2 carcinoma of rabbits, and Hilgard et al. (15) obtained negative results in a careful study with Lewis lung carcinoma in mice using various antiplatelet agents: aspirin, bencyclane, and RA 233 (a dipyrindamole derivative). Furthermore, although Gordon et al. (10) obtained positive results with a Wilms' tumor of Wistar-Furth rats, equivocal results were obtained with a neuroblastoma (C1300) of mice, and negative results were obtained with an NIH renal adenocarcinoma of mice, using pentoxifylline (a phosphodiesterase inhibitor) as an antiplatelet agent.

Therefore, the recent report of Honn et al. (16) on the dramatic reduction of B16a metastases in mice by the use of a combination of PGI2 and theophylline was very encouraging. Our inability to reproduce their results, with the use of 3 different mouse tumors, including the same B16a pedigree line utilized by Honn et al. (16), was disappointing.

Several possibilities should be considered which might explain the discrepant results obtained from both laboratories: (a) the PGI2 which we used was inactive. This was shown not to be the case by our in vitro studies using platelet aggregation and by in vivo studies using tumor-induced thrombocytopenia; (b) different tumors were used. One of our tumor cell lines, B16a, was also used by Honn et al. (16); (c) the experimental pharmacological protocol was different. Although we used 2 dosages of PGI2 and theophylline for the 3 tumor cell lines used, an identical pharmacological protocol (single dose of PGI2 and theophylline) was also used with the B16a tumor cell line; (d) method of preparation of the tumor cell lines for injection in vivo. The 3 lines that we used were obtained by trypsin treatment of subconfluent tissue culture dishes, whereas the B16a cell line used by Honn et al. was removed directly from animals bearing the tumor, diced, dispersed, treated with collagenase and DNaSe, exposed to soybean trypsin inhibitor, and collected through cheesecloth prior to centrifugation and washing of cells. This preparation may have included macrophages which have recently been shown to dramatically increase the number of i.v. administered metastases if injected prior to or immediately after the tumor cell inoculation (11). It is conceivable that PGI2 may have inhibited this macrophage-enhancement effect; (e) Honn et al. (16) injected approximately 10 times as many B16a tumor cells (3 x 105) as were injected in our experiment (2.5 x 104) and obtained approximately 6 times as many control pulmonary nodules as in our experiments. However, one would expect a greater effect for an antimetastatic pharmacological agent(s) if the number of control pulmonary nodules were decreased rather than increased; (f) finally, the manner in which the tumor cells were injected into the control and experimental animals is not stated in the paper of Honn et al. (16). In our experience, considerable systematic error can be introduced if control animals are given injections first as a group, followed by the injection of experimental animals. It is necessary to remove the injected macrophages, which can be introduced if control animals are given injections first as a group, followed by the injection of experimental animals. It is necessary to remove the injected macrophages, which can be introduced if control animals are given injections first as a group, followed by the injection of experimental animals.
not clear whether this may have contributed to the differences in results obtained by both laboratories.

The proposal that PG\(\text{I}_2\) may be an effective antimetastatic agent (16) is reasonable if platelet aggregation plays a role in the development of tumor metastases, since PG\(\text{I}_2\) is a potent inhibitor of platelet aggregation at 10\(^{-9}\) M and 100 \(\mu\)g of PG\(\text{I}_2\) inhibited the in vivo induction of thrombocytopenia in our animals. Unfortunately, PG\(\text{I}_2\) plus theophylline, given prior to the injection of tumor cells and 2 hr postinjection, had no effect on pulmonary metastases. If platelet aggregation and release do play a role in the development of tumor metastases, then it is possible that the short half-life of PG\(\text{I}_2\) (relative to the duration of the platelet contribution to metastasis) may have contributed to the negative results. In addition, it is possible that some property of platelets that is not affected by PG\(\text{I}_2\) may be responsible for the role of platelets in tumor metastases. In the following paper (24), we present evidence that platelets are required for the development of optimum tumor metastases.

### REFERENCES


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