Production of Prostaglandin E₂ by Tumor Cells in Vitro

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ABSTRACT

Our previous observations on the production of prostaglandin E₂ (PGE₂) by bladder tumor cell lines in vitro and the enhancement of tumor cell PGE₂ production upon exposure to purified peripheral blood lymphocytes from normal human donors prompted us to examine this interaction in an animal model in order to further define conditions that determine the occurrence of this phenomenon. Cell lines derived from carcinogen-induced bladder and mammary tumors and from embryonic fibroblasts in Fischer rats were exposed to purified peripheral blood or splenic lymphocytes in the presence or absence of indomethacin (10⁻⁷ M). After varying times at 37°C, supernatants were harvested for determination of PGE₂ by radioimmunoassay. Time course studies demonstrated rapid PGE₂ production with plateau levels appearing at 8 hr. Increased tumor cell PGE₂ production occurred in the presence of increased numbers of lymphocytes. Indomethacin partially inhibited PGE₂ production. Preincubation studies suggested that the contribution of lymphocytes to overall PGE₂ production in the present system was minimal. On the basis of previous observations of PGE₂-associated inhibition of lymphocyte cytotoxicity against tumor cells in vitro, the present results suggest that tumor cell PGE₂ production may reflect a response of the tumor cells to challenge by effector lymphocytes and may represent a mechanism whereby tumor cells subvert an immune response mounted against them.

INTRODUCTION

Studies of the host immune response to the development of neoplasia have led to the description of a variety of mechanisms by which effector cells might be cytotoxic to tumor target cells in vitro (20, 21, 24). As these mechanisms have been suggested to underlie the host defense against tumor development in vivo, we thought it of interest to explore the possibility that tumor cells might react to these host defense mechanisms and, in so doing, alter interactions that are seen.

Previous studies have shown that tumor cells could modulate immune effector mechanisms directed against both neoplastic cells in vitro (9, 32) and infecting microbes (6, 29). Other studies have demonstrated that tumor cells could produce prostaglandins in vitro (11, 17, 18, 25). Since prostaglandins have been found in many instances to inhibit lymphocyte cytotoxicity directed against tumor cells in vitro (1, 16, 27, 31), it appeared to us that the production of prostaglandins by tumor cells might constitute a defense mechanism whereby tumor cells could subvert the cellular immune response mounted against them.

In testing this hypothesis, we found that a variety of bladder tumor cell lines produced prostaglandins in vitro and, when challenged with effector lymphocytes, could markedly increase their production of these prostaglandins (3, 4). Furthermore, prostaglandins at levels approximating the concentrations produced by the tumor cells in these studies inhibited lymphocyte cytotoxicity directed against the same target cells in vitro (7), whereas inhibition of tumor cell prostaglandin production appeared to enhance the expression of lymphocyte cytotoxicity (5).

As our recent emphasis had been on the definition of cellular immune response mechanisms that might be active during the development of carcinogen-induced bladder cancer in an experimental animal model, we thought it of interest to explore this model for the production of prostaglandin by bladder tumor target cells and their response to challenge by effector lymphocytes. The present work describes our observations on conditions that determine the occurrence of this phenomenon.

MATERIALS AND METHODS

Target cells used in these investigations were from established cell lines derived from carcinogen-induced tumors in Fischer rats. Bladder transitional epithelial cell tumor lines induced by the carcinogen N-[4-(5-nitro-furyl)-2-thiazolyl]formamide (2, 8) were the generous gifts of Dr. Samuel Cohen (University of Massachusetts Medical School, Worcester, Mass.) and Dr. Eugene Ornellas and Dr. George Prout, Jr. (Massachusetts General Hospital, Boston, Mass.) and are referred to as Ay and W₂. A mammary carcinoma cell line induced by the carcinogen (7,12-dimethylbenz(α)-anthracene was the gift of Dr. Mary Bordelon (Baylor University, Houston, Texas). A Fischer rat embryo fibroblast line was established by routine methods in our own laboratory (28). Cells were maintained in monolayer in Roswell Park Memorial Institute TCM supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), gentamicin (50 µg/ml), and heat-inactivated 10% FBS (Grand Island Biological Co., Grand Island, N. Y.). Prior to use, cells were trypsinized with 0.025% trypsin (Grand Island Biological Co.) containing 0.2 mM EDTA and were then washed prior to plating in 24-well Linbro plates (Flow Laboratories, Inc., Hamden, Conn.). Approximately 1 to 2 × 10⁵ cells were plated per well in 2 ml TCM and were then incubated at 37°C in a 5% CO₂ humidified atmosphere. At confluence, 4 to 5 × 10⁵ tumor cells were present in each well.

Lymphocytes, obtained from the spleen or peripheral blood of 200- to 250-g male Fischer rats, were purified by centrifugation over a Ficoll-Isopaque layer (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), washed, and resuspended in TCM: 10% FBS for use either immediately or after overnight incubation at room temperature. Cell suspensions from both spleen...
and blood were found to contain approximately 15 to 20% monocytes.

At time "0," TCM was aspirated from confluent target cell monolayers and replaced by fresh TCM either with or without purified lymphocytes. Parallel samples contained indomethacin at 10^{-7} M final concentration. Incubation was then continued at 37°C for 16 to 20 hr after which supernatants were harvested and rapidly frozen until assayed for prostaglandin E_2. Initially, triplicate wells were used for each experimental condition examined. As the high degree of reproducibility became apparent, however, duplicate wells often were used in later experiments. Preliminary studies with ^{31}Cr suggested that geometric conditions used in the present system were not conducive to significant cell lysis during the 16- to 20-hr incubation.

Initial studies were designed to define conditions in which prostaglandin E_2 was produced. In time course studies, incubation lasted from 2 through 48 hr, and supernatants were harvested at various times within these intervals. In dose response studies, different numbers of lymphocytes were added to cell monolayers to produce final lymphocyte:target cell ratios of 1:5, 1:3, 1:1, 3:1, and 5:1.

Preincubation studies were performed to determine whether prostaglandins produced under these experimental conditions were derived from the tumor target cells or from the purified lymphocytes. In the first type of study, confluent tumor cell monolayers were incubated with TCM:10% FBS containing 10^{-7} M indomethacin for 6 to 8 hr. The monolayers were then washed twice with fresh TCM and incubated with or without lymphocytes for 16 hr prior to harvesting the supernatants for prostaglandin E_2 determination. Controls for these studies consisted of tumor cell monolayers preincubated with fresh TCM:10% FBS without indomethacin. Parallel samples of tumor cell monolayers and lymphocytes contained indomethacin throughout the 16 hr of final incubation.

In related studies, purified lymphocytes were preincubated with 10^{-7} M indomethacin for 8 to 12 hr. They were then washed with fresh TCM:10% FBS prior to their addition to tumor cell monolayers that had not been preincubated with indomethacin. Control samples in these studies contained lymphocytes that had not been preincubated with indomethacin but that had undergone similar washing procedures. Other samples contained indomethacin during the entire 16-hr period of incubation.

Radioimmunoassay procedures similar to those previously described were used to assess the degree of prostaglandin E_2 production in the supernatants harvested in these studies (19, 22). Results are expressed as pg/ml and are similar to observations when expressed in pg/10^6 tumor cells (the approximate number of cells/well at confluence).

RESULTS

Each cell line produced prostaglandin E_2 shortly after initial change of TCM, and these levels were generally found to increase throughout 24 hr of incubation (Chart 1). Plateau levels were generally reached by 8 hr of incubation after which only slight increments were seen.

The presence of lymphocytes led to significantly higher levels of prostaglandin production by tumor cell monolayers at each time interval examined. Increments were apparent as early as 2 hr after the onset of incubation and continued during the full 48-hr time period studied (Charts 1 and 2). Plateau levels were generally seen after 8 hr of incubation.

Indomethacin (10^{-7} M) in parallel samples inhibited prostaglandin production. Control samples containing lymphocytes alone demonstrated negligible levels of prostaglandin production.

The number of lymphocytes present appeared to determine levels of prostaglandin that occurred in the supernatants. Generally, lymphocyte:target cell ratios of less than 1:1 led to no increment in production of prostaglandins by the tumor cells higher than that seen in samples in which tumor cells had been

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incubated alone. Ratios higher than 1:1, in contrast, led to the production of significantly higher levels (Chart 3). The presence of indomethacin in these samples led to partial inhibition of prostaglandin production at each ratio examined.

Lymphocyte source also appeared to affect the ultimate production of prostaglandins by tumor cell monolayers. Blood lymphocytes routinely led to the production of higher levels than did splenic lymphocytes (Chart 4). Preliminary studies have suggested that this phenomenon may be correlated with the number of lymphocytes with F₄₅₁ receptors present in these different populations. This phenomenon is described in further detail in a separate communication (23).

Preincubation studies were designed to assess the possible source of prostaglandin in these experiments. When tumor cell monolayers were preincubated with indomethacin (10⁻⁷ M) and then incubated with lymphocytes, lower levels of prostaglandins subsequently appeared in the supernatant (Chart 5). When the same tumor cells that had been preincubated with indomethacin were then incubated with lymphocytes in the presence of indomethacin throughout the 16-hr incubation period, even lower levels of prostaglandin production were seen. Control samples in which tumor cells were processed in an identical fashion but were not exposed to indomethacin suggested that indomethacin inhibited prostaglandin production through its presumed inhibitory effect on tumor cells.

In contrast, studies in which lymphocytes were preincubated with indomethacin demonstrated the production of levels of prostaglandins that were either the same or occasionally even higher than levels seen when lymphocytes that had not been preincubated with indomethacin were used (Chart 6). However, the same lymphocytes were incubated with tumor cell monolayers and indomethacin was present throughout incubation. In these controls, inhibition of prostaglandin production was observed. Throughout these experiments, lymphocytes incubated alone either in the presence or absence of indomethacin were used as controls. In these samples, minimal prostaglandin production occurred. This was in marked contrast to control samples in which tumor target cells were incubated alone in either the presence or absence of indomethacin. In these controls, prostaglandin E₂ was produced in significant amounts, and an

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**Chart 3.** Effect of lymphocyte number on the production of prostaglandin E₂ by tumor cells. Tumor cell lines Ay or W₂ at confluence (5 × 10⁶ cells/well) were incubated with purified splenic lymphocytes adjusted to the ratios indicated. After 16 hr at 37°, supernatants were harvested and assayed for prostaglandin E₂. Control wells contained indomethacin at 10⁻⁷ M as indicated. Results from duplicate samples, pg/ml.

**Chart 4.** Comparison of effects of blood versus splenic lymphocytes on tumor cell prostaglandin E₂ production. ○, Tumor cells alone. Tumor cell lines Ay and W₂ were incubated with purified splenic lymphocytes (SPL) or peripheral blood (BL) lymphocytes at a lymphocyte:target cell ratio of 2:1 for 16 hr at 37°. Indomethacin (10⁻⁷ M) was added to appropriate tubes as indicated. Conditions were tested in duplicate or triplicate, and results are expressed as pg/10⁶ tumor cells/ml with the mean ± S.E. □, control; □, indomethacin (10⁻⁷ M).

**Chart 5.** Effect of preincubation of tumor cells with indomethacin on tumor cell prostaglandin production. Tumor cell monolayers Ay and W₂, after reaching confluence at approximately 4 to 5 × 10⁶ cells/well, were incubated with TCM either with (T) or without (T) 10⁻⁷ M indomethacin. After 6 to 8 hr at 37°, the monolayers were washed twice and then incubated with or without purified splenic lymphocytes (L) in the presence or absence of indomethacin (10⁻⁷ M) for 16 hr at 37°. Supernatants were harvested and assayed for prostaglandin E₂. Results of triplicate samples, pg/ml with the mean ± S.E. □, control; □, indomethacin (10⁻⁷ M).

**Chart 6.** Effect of preincubation of lymphocytes with indomethacin on tumor cell prostaglandin production. Purified splenic lymphocytes were incubated for 12 hr at 37° with 10⁻⁷ M indomethacin. These lymphocytes (L) and parallel nonpreincubated lymphocytes (L) were washed 3 times with fresh TCM:10% FBS and then added at a lymphocyte:target cell ratio of 2:1 to confluent monolayers of Ay and W₂ (T) for incubation for 16 hr at 37°. Control samples contained indomethacin (10⁻⁷ M) throughout incubation of the tumor cell lines with the lymphocytes. The results represent triplicate samples for each experimental condition with the mean ± S.E. Of 4 experiments, 2 showed increased production of prostaglandin in response to lymphocytes that had been preincubated, and 2 showed no enhancement of prostaglandin production. No experiments demonstrated decreased production of prostaglandins by the tumor cells. □, control; □, indomethacin (10⁻⁷ M).
target cells were necessary for prostaglandin production to different levels of tumor cell prostaglandin production that adhere to target cells in vitro (10) and that direct contact with
the tumor target cells often demonstrated higher levels of prostaglandin production, presumably 
by findings that lymphocyte prostaglandin production was 5ev 
comparable to levels observed in previous studies (3, 14). 
Somewhat higher levels have been observed with macrophages 
(12, 13).

That the occurrence of increased levels of prostaglandins did not represent production by lymphocytes was suggested by findings that lymphocyte prostaglandin production was sev-
eral orders of magnitude less than the production seen by the 
tumor target cells when each was incubated alone. The quan-
tities of prostaglandins produced by these lymphocytes were 
comparable to levels observed in previous studies (3, 14). 
A somewhat higher level has been observed with macrophages 
(12, 13).

Although it is impossible to state with certainty on this basis 
alone that the increments of prostaglandin production observed 
by mixing lymphocytes with tumor cells were not the result of 
increased effector cell prostaglandin production, preincubation 
was suggested for the inhibition of tumor cell prostaglandin 
production. That the occurrence of increased levels of prostaglandins prior to their addition to the tumor target cells often demonstrated higher levels of prostaglandin production, presumably 
by the tumor cells, rather than lower levels as might have been 
expected were the lymphocytes the major source of prosta-
glandins. Support for this interpretation was provided by find-
ings that when indomethacin was present throughout incuba-
tion, the lower levels of prostaglandin production that might 
have been expected were seen.

It appeared that direct interactions between effector and 
target cells were necessary for prostaglandin production to 
occur. Several factors supported this suggestion: (a) the num-
ber of lymphocytes present was associated with the production 
of different levels of prostaglandins in a dose-dependent man-
ner; and (b) the source of lymphocytes seemed to play a role 
in determining absolute levels of prostaglandins produced. 
Previous studies have shown that cytotoxic T-lymphocytes 
cling to target cells in vitro (10) and that direct contact with 
target cells is important for the expression of natural 
killing (15, 26). Because subpopulations of lymphocytes that 
comprise peripheral blood may differ substantially from sub-
populations of lymphocytes that comprise the spleen, we rea-
sioned that a direct interaction between one of these subpopu-
lations and the tumor target cells might be responsible for the 
different levels of tumor cell prostaglandin production that 
occurred. The possibility that a lymphocyte product may also 
be responsible for the induction of tumor cell prostaglandin 
production, however, is presently being explored.

The significance of this phenomenon is still unclear. The 
apparent necessity of a direct interaction between effector 
lymphocytes and tumor target cells in the present experiments 
supports the hypothesis that the production of prostaglandins 
by tumor target cells may represent a defense mechanism whereby tumor cells subvert the cellular immune response that 
is presumably mounted against them in vitro. Circumstantial 
evidence in support of this concept has been offered by Plescia 
et al. (25) who, working on an in vivo model of neoplasia, 
demonstrated that the use of indomethacin for the inhibition of 
prostaglandin production led to diminished growth of trans-
planted tumors. Because these tumors had been found to 
produce prostaglandins, it was suggested that inhibition of 
prostaglandin production permitted the full manifestation of the 
immune response directed against the transplanted tumor and 
a resultant diminution of tumor growth. Other studies by Spi-
talny and North (29) suggested that the production of sub-
stances in vivo by tumor cells was associated with the sup-
pression of the ability of sensitized T-lymphocytes and activ-
ated macrophages to eliminate an inoculum of the bacterial parasite Listeria monocytogenes. 
In our own studies, we ob-
erved the production of prostaglandins in response to lym-
phocyte challenge (3), inhibition of both spontaneous cytoto-
icity and antibody-dependent lymphocyte cytotoxicity by pros-
taglandins (7), and enhancement of cytotoxicity by inhibition of 
tumor cell prostaglandin production (5). Each of these obser-
vations strongly supports the possibility that tumor cells may 
respond to an environmental challenge by the production of 
prostaglandins which may in turn inhibit the expression of a 
cellular immune response directed against the prostaglandin-
producing cell. Additional support for this hypothesis is evi-
denced by the putative increase in tumor cell killing that occurs 
in the presence of activated lymphocytes. This would theoreti-
cally make more prostaglandin precursors available for the 
tumor cells that remain to produce still higher levels of inhibitory 
prostaglandins.

It is also possible that a portion of the prostaglandins seen 
originated from the effector cells when they came into contact 
with the tumor targets. Indeed, an unexpected finding in the 
present experiments was the increased production of prosta-
glandins when lymphocytes preincubated with indomethacin 
were mixed with tumor target cells. As a possible explanation 
that indomethacin may have prevented the production of 
prostaglandins by the lymphocytes. If lymphocyte prostaglan-
din production is involved in the modulation or inhibition of 
lymphocyte activity (16, 31), the inhibition of lymphocyte pros-
taglandin production may have permitted more direct contact 
between lymphocytes and tumor cells which, in turn, may have 
led to increased stimulation of tumor cell prostaglandin pro-
duction. The interaction of these interrelated events may pro-
foundly influence the ultimate expression of the immune re-
sponse that may occur to the development of neoplasia. Cer-
tainly, further studies along these lines appear to be indicated.

In our previous studies of lymphocyte cytotoxicity in a human 
system, indomethacin was found to enhance both spontaneous 
and antibody-dependent lymphocyte cytotoxicity (5), and we 
suggested that enhancement may have been the result of 
inhibition of tumor cell prostaglandin E2 production. In view of 
the present findings, however, it is possible that inhibition of 
lymphocyte prostaglandin E2 production by indomethacin may 
have been the factor responsible for this enhanced cytotoxicity 
through the promotion of more direct lymphocyte-target cell
contact. Although our lymphocyte preincubation studies were inconclusive in documenting this possibility, this question requires further investigation.

In sum, these studies suggest the possible presence in our animal model of a mechanism by which tumor cells may modulate the cellular immune response in a manner similar to that previously described in the human system. In addition, there is evidence as well for the possible existence of a mechanism of control within lymphocytes that may modulate their own effect in the cellular immune response. Our present efforts are directed towards the further characterization of these interactions and how each may be necessary for the expression of this phenomenon. Additional study is indicated to examine the possible existence of these phenomena in vivo as they may influence the development of neoplasia.

REFERENCES

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