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Biochemistry

Prostaglandin D_2 formation by malignant melanoma cells correlates inversely with cellular metastatic potential

(B16 F_1 cells/B16 F_{10} cells/platelet–tumor interaction/prostaglandin synthesis deficiency)

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ABSTRACT **B16 malignant melanoma cell lines transform arachidonic acid and its transient metabolite, prostaglandin endoperoxide H_2, into prostaglandin D_2. The highly metastatic line, B16 F_{10}, forms less prostaglandin D_2 compared to the moderately metastatic parent line, B16 F_1. Since platelet aggregation may be one factor involved in B16 metastasis and since prostaglandin D_2 inhibits platelet aggregation, this prostaglandin could affect the outcome of platelet–tumor interactions, which may contribute ultimately to metastasis. Arachidonic acid metabolism may be another one of the intrinsic biochemical properties of tumor cells that affects their metastasis. Our results suggest that quantitative release of unusual prostaglandins must be considered in this context.**

Fidler (1–3) has developed a series of malignant melanoma cell lines that are useful for studying tumor metastasis. By comparing the moderately metastatic parent line, B16 F_1, to the highly metastatic derived line, B16 F_{10}, one averts the problem of selecting a potentially inappropriate control cell. After repeated subculturing *in vitro* the cells remain stable and retain their distinct metastatic characteristics *in vivo*. Experiments with these cells indicate that metastasis is not a random event (4, 5, 6, 7). Instead, tumor cells survive within the host because they have inherent biochemical and biological properties, guaranteeing their inevitable multiplication. These properties include surface enzymes (4); interactions with host immune cells (2, 5, 8) with host platelets (6), or with host endothelial cells (9); and reduced adhesiveness (7).

Prostaglandins influence tumor cell function and proliferation; however, their exact role is vague (10). We examined arachidonic acid and prostaglandin H_2 metabolism in B16 F_1 and B16 F_{10} cells. In contrast to other transformed or malignant cells that are thought to produce mainly prostaglandin E_2 or prostaglandin $F_{2\alpha}$ (11–16), both lines of malignant melanoma cells produced mainly prostaglandin D_2. When incubated with either arachidonic acid or prostaglandin endoperoxide H_2, the moderately metastatic line, B16 F_1, produced more prostaglandin D_2 than the highly metastatic line, B16 F_{10}, did. This suggests that qualitative and quantitative aspects of arachidonic acid metabolism may be one of the inherent biochemical properties of the tumor cell that can affect metastasis.

EXPERIMENTAL

Materials. Arachidonic acid (Nu Chek Prep, Elysian, MN), [1-^{14}C]arachidonic acid (60 mCi/mmol, 1 Ci = 3.7×10^{10} becquerels) (New England Nuclear), and prostaglandin standards (Upjohn) of verified purity were used. Prostaglandin endoperoxide H_2 was prepared biosynthetically (17). Organic solvents distilled in glass (Burdick and Jackson, Muskegon, MI)—pentafluorobenzylbromide (Aldrich), pentafluorobenzylhydroxylamine hydrochloride, methoxylamine hydrochloride, bistrimethylsilylacetamide (Anspec, Ann Arbor, MI)—for gas chromatography and thin-layer chromatography plates coated with silica gel G (Analtech, Newark, DE) were used.

Cell Culture Conditions. B16 F_1 and B16 F_{10} cells, kindly supplied by I. Fidler, Frederick Cancer Research Center, were grown as described (5) in Eagle's minimum essential medium supplemented with 10% fetal calf serum and penicillin/streptomycin. Cells planted in plastic wells (30 mm diameter) at a density of 1×10^5 cells per ml reached confluency within 3 days. For counting, cells were washed once with Hanks' balanced salt solution (2.0 ml) and then removed by incubating with 0.1% trypsin in isotonic, serum-free phosphate buffer. Cell viability was measured by trypan blue exclusion.

Metabolism of [1-^{14}C]Arachidonic Acid by B16 F_1 and B16 F_{10} Cells. Cellular metabolism of [1-^{14}C]arachidonic acid was determined according to Hammarström (13). At confluency, the cells ($1–2 \times 10^6$ cells per well) were washed twice with Hank's balanced salt solution (1.0 ml) and then incubated at 37°C for 15 min with [1-^{14}C]arachidonic acid [0.5 μg (2×10^5 dpm; 1 dpm = 16.7 mBq) of [1-^{14}C]arachidonic acid/0.4 ml of Hank's balanced salt solution per well]. After incubation, the medium was removed, cells were washed twice with Hanks' balanced salt solution (0.5 ml), standard nonradioactive prostaglandins were added as carriers, and the combined aqueous fractions were acidified (pH 3) and extracted three times with ether (5.0 ml). The combined ether extracts were reduced in volume and spotted on silica gel G thin-layer plates (20×20), which were developed in the organic phase of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (110:50:20:100). The prostaglandins present from arachidonic acid metabolism were monitored with a radiometric thin-layer chromatogram scanner. The identity of the radioactive zones was established by localization of prostaglandin standards, added as carriers, which were chromatographed on the same plate. Quantitative measurements were made by accurately dispensing the sample (100 μl) onto the plate and scraping the zones containing prostaglandins $F_{2\alpha}$, E_2, and D_2 and arachidonic acid into a vial containing scintillation counting fluid. Radioactivity determined by scintillation counting was corrected for quenching. Results of quantitative radiometric thin-layer chromatography are expressed as percentage conversion of arachidonic acid into prostaglandins.

Metabolism of Prostaglandin Endoperoxide H_2 by B16 F_1 and B16 F_{10} Cells. Cellular metabolism of prostaglandin endoperoxide H_2 was determined by open tubular glass capillary gas chromatography with electron capture detection (18). Cells were treated as above except each well was incubated at 37°C for 15 min with prostaglandin endoperoxide H_2 (1.0 μg of prostaglandin H_2/0.4 ml Hanks' balanced salt solution per well). After washing, acidification, and extraction into ether, the metabolites of prostaglandin H_2 were converted into two dis-

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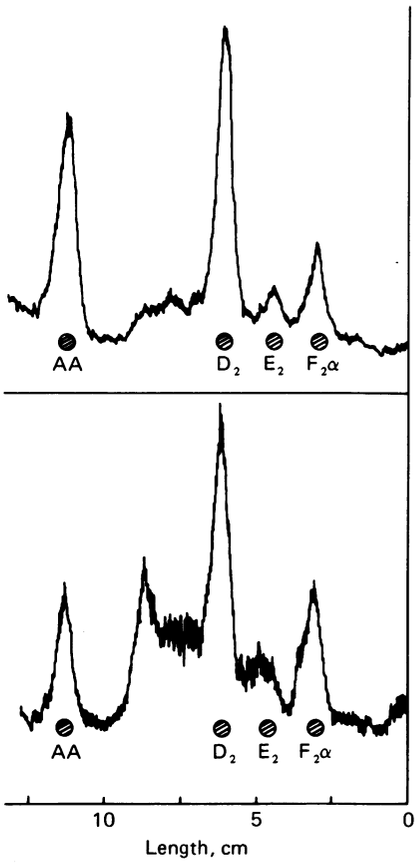

FIG. 1. Radiometric thin-layer chromatographic profiles resulting from the incubation of [1-^{14}C]arachidonic acid (AA) with B16 F_1 cells (*Upper*) and B16 F_{10} cells (*Lower*). Excess [1-^{14}C]arachidonic acid was removed by partitioning into hexane to improve the presentation of the chromatogram. Equivalent results were obtained after thin-layer chromatography in the following solvent systems: ethyl acetate/acetic acid (99:1, vol/vol) or chloroform/methanol/acetic acid (90:5:5, vol/vol). The undesignated peak migrating ahead of prostaglandin D_2 is prostaglandin A_2, formed by dehydration of prostaglandin E_2 during the extraction.

tinct perhalogenated derivatives for gas chromatography on glass capillary columns. Prostaglandin pentafluorobenzyl ester methoxime trimethylsilyl ethers, and prostaglandin methyl ester pentafluorobenzyloxime trimethylsilyl ethers were prepared and analyzed as described (18–20). The identity of the prostaglandins was established by comparing retention data of both types of perhalogenated derivatives with an equivalent prostaglandin standard. Quantitation was based on calibration curves prepared from these standards. Calibration curves were linear over the range 80–420 pg injected on-column for each prostaglandin.

RESULTS

The major radioactive metabolite formed by incubating washed B16 F_1 or B16 F_{10} cells with [1-^{14}C]arachidonic acid migrated with prostaglandin D_2 in several thin-layer chromatography systems (Fig. 1). Lesser amounts of prostaglandins E_2 and $F_{2\alpha}$ were formed. After sodium borohydride reduction the major radioactive zone migrated with prostaglandin $F_{2\alpha}$, substantiating the identity of the original peak as prostaglandin D_2. Addition of a fatty acid cycloöxygenase inhibitor (10 μM flurbiprofen) to the incubation medium prevented the conversion of arachidonic acid into the prostaglandins. The qualitative radiometric thin-layer chromatograms suggested that the B16 F_1 line transformed more arachidonic acid into prostaglandins than the B16 F_{10} line did. In subsequent experiments we confirmed that B16 F_1 cells formed 5 times more prostaglandin D_2 than B16 F_{10} cells did (Fig. 2). B16 F_1 cells also formed more prostaglandin E_2 and prostaglandin $F_{2\alpha}$ than B16 F_{10} cells did, but the differences were less pronounced than for prostaglandin D_2. At 5, 15, and 30 min, both cells contained equal amounts of cell-associated radioactivity, localized as arachidonic acid in the phospholipid fraction. There was no cell-associated radioactivity that migrated with any prostaglandins. Thus, quantitative differences in prostaglandin D_2 formation were not due to differences in substrate availability. Treatment with thrombin did not stimulate additional formation of prostaglandin D_2.

Cells transform arachidonic acid into stable prostaglandins via a transient endoperoxide intermediate, prostaglandin H_2 (21, 22). We incubated intact B16 F_1 and B16 F_{10} cells with prostaglandin H_2 directly to confirm the results with arachidonic acid. Glass capillary gas chromatographic profiles indicated that prostaglandin D_2 is a prominent metabolite of prostaglandin H_2 in these cells. Results were similar for either pentafluorobenzyl ester methoxime trimethylsilyl ether derivatives (Fig. 3) or methyl ester pentafluorobenzyloxime trimethylsilyl ether derivatives (Fig. 4). After sodium borohydride reduction the pentafluorobenzyl ester trimethylsilyl ether derivatives eluted at the same retention time as prostaglandins $F_{2\alpha}$ and $F_{2\beta}$, from the reduction of prostaglandins D_2 and E_2, respectively. There was no evidence by radiometric thin-layer chromatography, glass capillary gas chromatography, or radioimmunoassay that B16 F_1 or B16 F_{10} cells formed other arachidonic acid metabolites such as prostaglandin I_2, thromboxane B_2, or 12-hydroxyeicosatetraenoic acids.

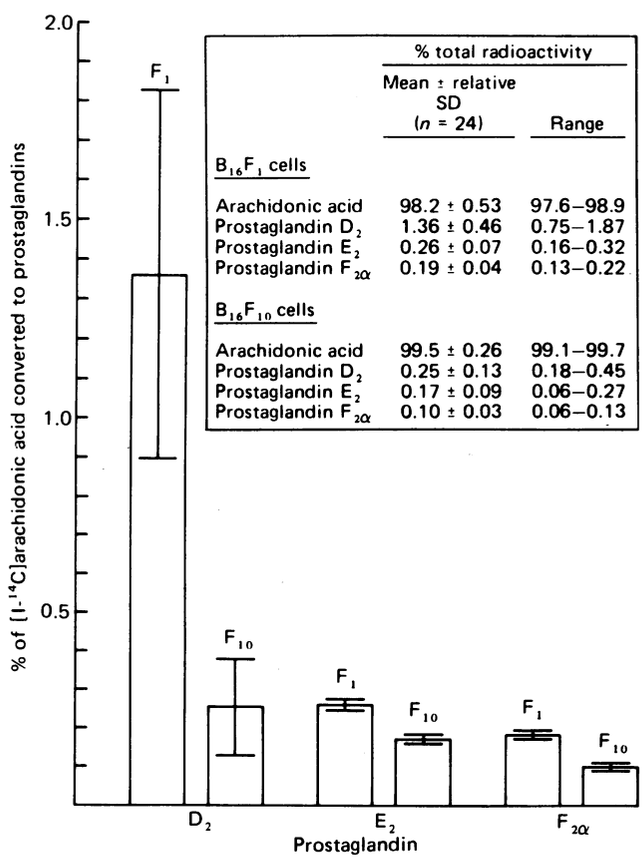

FIG. 2. Quantitative results by radiometric thin-layer chromatography. Under identical conditions, B16 F_1 cells produced significantly more ($P < 0.001$) prostaglandin D_2 than did B16 F_{10} cells.

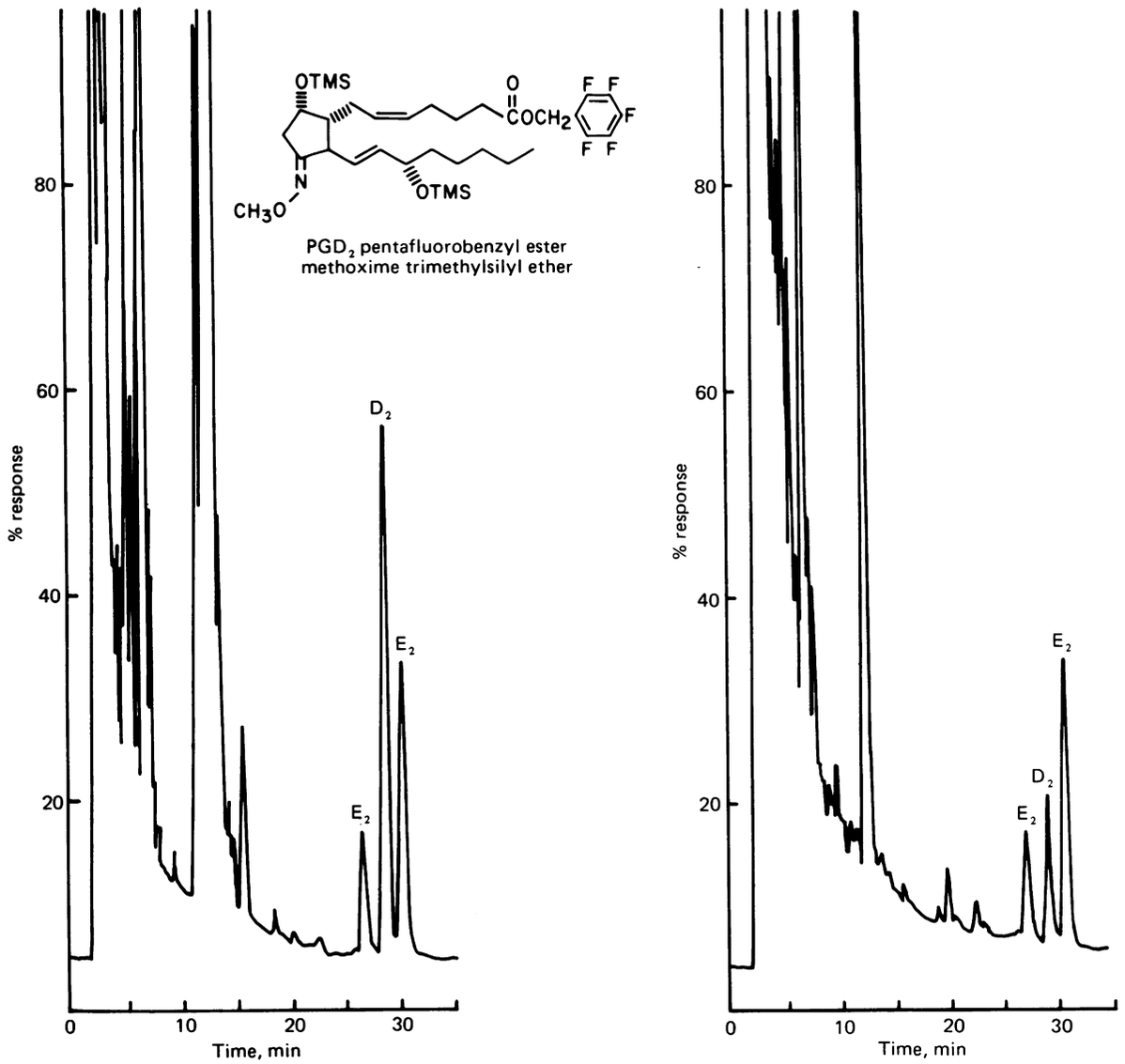

FIG. 3. Glass capillary gas chromatographic profile of prostaglandin H_2 metabolites formed by B16 F_1 (*Left*) and B16 F_{10} (*Right*) cells. Compounds were chromatographed as pentafluorobenzyl ester methoxime trimethylsilyl ethers on a methylphenylpolysiloxane glass capillary column (29 meters) operated isothermally at 265°C. Under these conditions, all of the currently known metabolites of prostaglandin H_2 are resolved. Quantitative analysis of six samples showed that B16 F_1 cells produced 0.686 ± 0.022 (SD) μg of prostaglandin D_2 and 0.223 ± 0.022 μg of prostaglandin E_2 per μg of prostaglandin H_2 per 10^6 cells. In addition, B16 F_{10} cells produced 0.310 ± 0.032 μg of prostaglandin D_2 and 0.515 ± 0.033 μg of prostaglandin E_2 per μg of prostaglandin H_2 per 10^6 cells.

We verified the enzymatic origin of prostaglandin D_2 from B16 cells, because it has been reported (23) that serum factors can promote prostaglandin D_2 formation. We isolated a soluble protein component from the high-speed (105,000 × *g*) supernatant of B16 F_1 or B16 F_{10} cell homogenates according to Christ-Hazelhof *et al.* (24). This component transformed more than 90% of exogenously added prostaglandin H_2 into prostaglandin D_2. Heating the soluble protein component at 98°C for 10 min inhibited prostaglandin D_2 formation by more than 90%, and the prostaglandin H_2 then transformed chemically into prostaglandin E_2 and (*12L*)-hydroxy-5,8,10-heptadecatrienoic acid. Although B16 F_1 and B16 F_{10} cells contain prostaglandin D_2 isomerase, some prostaglandin H_2, because of its labile nature, can transform chemically into prostaglandins E_2, $F_{2\alpha}$, or D_2. The rate constants for the enzymatic transformation and the rate constants for the chemical transformation govern the relative proportions of each prostaglandin. Essentially all of the prostaglandin E_2 in Figs. 3 and 4 was formed from the nonspecific, chemical decomposition of prostaglandin H_2. In control incubations of prostaglandin H_2 and medium alone, prostaglandin E_2 accounted for 97 ± 2% of all prostaglandins formed chemically, and prostaglandins D_2 and $F_{2\alpha}$ combined accounted for only 3 ± 2%. Prostaglandin D_2 is, therefore, the principal enzymatic metabolite of prostaglandin H_2 in B16 F_1 or B16 F_{10} cells. Analogous to our results on the quantitative transformation of arachidonic acid, we found that the B16 F_{10} cells also converted less prostaglandin H_2 into prostaglandin D_2. B16 F_{10} cells formed 0.310 ± 0.032 (mean ± relative SD, n = 6) μg of prostaglandin D_2 per μg of prostaglandin H_2 per 10^6 cells, compared to 0.686 ± 0.022 μg of prostaglandin D_2 per μg of prostaglandin H_2 per 10^6 cells for B16 F_1 cells. This reduction in prostaglandin D_2 formation was also reflected by increased chemical formation of prostaglandin E_2 by B16 F_{10} cells relative to B16 F_1 cells: 0.515 ± 0.033 (B16 F_{10}) versus 0.223 ± 0.022 (B16 F_1) μg of prostaglandin E_2 per μg of prostaglandin H_2 per 10^6 cells. Since the experiments with the prostaglandin H_2

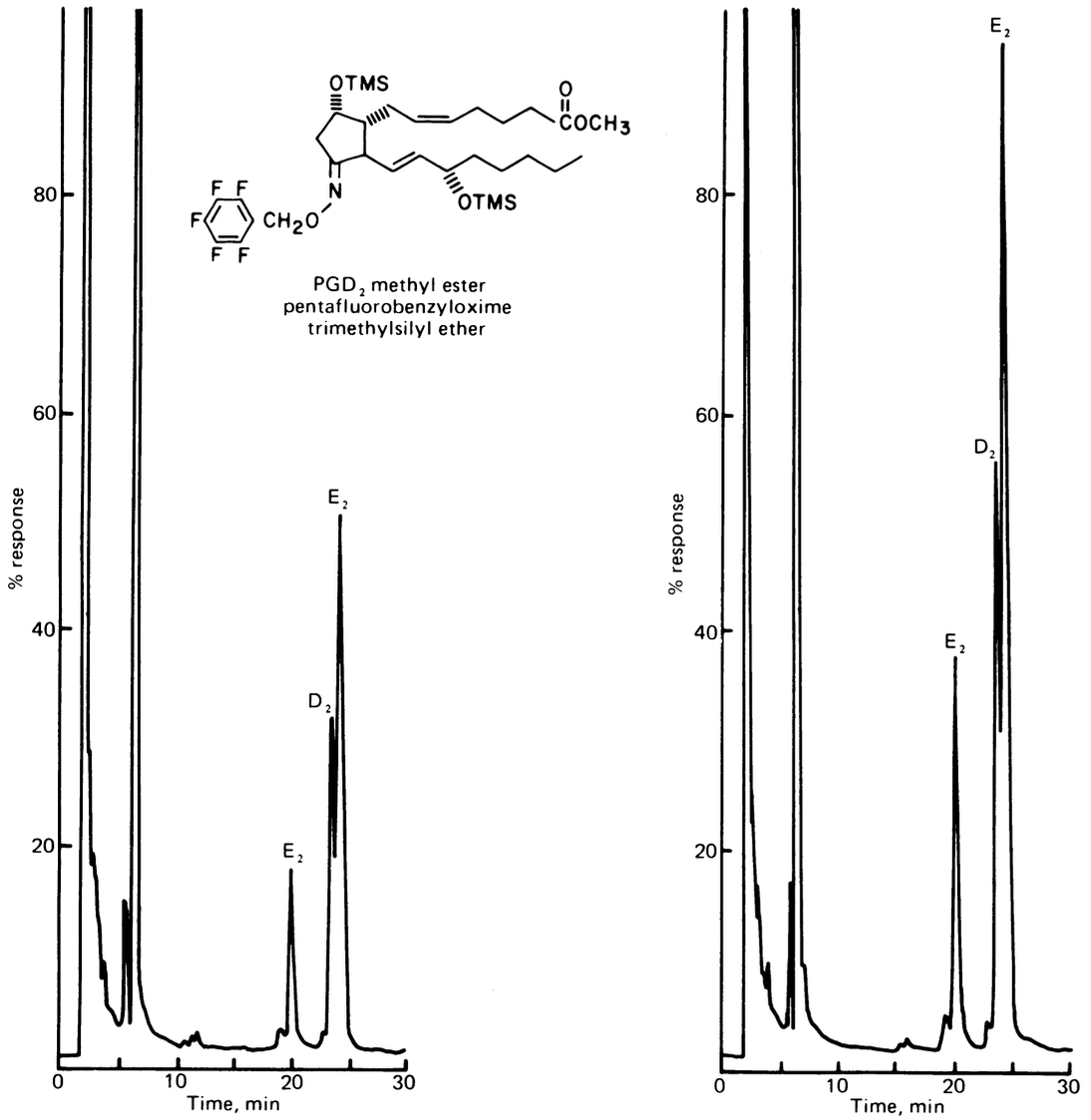

FIG. 4. Glass capillary gas chromatographic profile of prostaglandin H_2 metabolites formed by B16 F_1 (*Left*) and B16 F_{10} (*Right*) cells. Compounds were chromatographed as methyl ester pentafluorobenzyloxime trimethylsilyl ethers.

substrate bypass the cycloöxygenase enzyme step, B16 F_{10} cells must also have some quantitative or qualitative difference in prostaglandin D_2 isomerase compared to B16 F_1 cells.

DISCUSSION

Prostaglandin D_2 is the principal arachidonic acid metabolite produced by B16 F_1 and B16 F_{10} melanoma cells. Quantitative differences in prostaglandin production exist between the two lines. The highly metastatic line, B16 F_{10}, formed less prostaglandin D_2 from either arachidonic acid or prostaglandin H_2 substrate. Since the experiments were performed under conditions of equal substrate availability, we conclude that the results reflect relative differences in the two cell types in cycloöxygenase and prostaglandin D_2 isomerase. There may be different amounts of enzymes in each cell type or there may be different factors that affect the kinetics and equilibrium state of the enzymatic reactions in each cell type. There was no evidence of arachidonic acid metabolites besides prostaglandins D_2, E_2, and $F_{2\alpha}$; however, they may have been present but undetectable.

The reduced formation of fatty acid cycloöxygenase and prostaglandin D_2 isomerase products could contribute to the enhanced metastasis observed with B16 F_{10} cells. We postulate a physiologically relevant role for prostaglandin D_2 that unifies certain biological properties of B16 F_1 and B16 F_{10} cells with known biological effects of prostaglandin D_2. First, platelet aggregation by B16 cells may contribute to their metastasis (6). Second, prostaglandin D_2, in sufficient amounts, inhibits platelet aggregation in several species (25, 26), and we verified this effect in C57b16J mice. Oelz *et al.* (27) have shown that human platelets produce trace amounts of prostaglandin D_2, and they proposed that its formation could provide a mechanism for feedback inhibition of platelet aggregation. Under *in vitro* experimental conditions, it is difficult to show suppression or reversal of thromboxane A_2-mediated aggregation by the concomitant release of endogenous inhibitors such as prostaglandin D_2 or prostaglandin E_1 by platelets themselves. Aggregation is suppressed, ordinarily, only when the prostaglandin level is augmented prior to the onset of an aggregatory stimulus. In the context of these observations, prostaglandin D_2 could affect the outcome of platelet–tumor cell interactions. It is plausible that B16 F_1 cells metastasize less easily because they release sufficient prostaglandin D_2 to resist the formation of platelet–tumor emboli which could arrest and proliferate in pulmonary capillary beds. Conversely, B16 F_{10} cells may metastasize more easily because they release insufficient prosta-

glandin D_2 to resist the formation of platelet–tumor emboli. This hypothesis provides at least one theme unifying the possible role of platelets with the metastatic characteristics of B16 F_1 and B16 F_{10} cells. We stress that it is only one possibility: the recent comprehensive review on platelets and arachidonic acid metabolites by Marcus (28) testifies to the complexity of the system. Moreover, the initial observations by Gasic *et al.* (6) concerning metastasis of B16 cells and platelet aggregation could have been due to collagenlike materials or glycoproteins secreted by the cells, which aggregate platelets coincidentally *in vitro* but are less significant to metastasis *in vivo*. The shifting boundary between aggregation and adhesion, which contributes to the formation of platelet–tumor emboli *in vivo*, limits the validity of extrapolations from *in vitro* data. Nevertheless, platelet–tumor interactions are implicated as a factor contributing to metastasis in other malignant cells (29–32). Precedents also exist to support the notion that inherent quantitative or qualitative differences in the biology of B16 F_1 and B16 F_{10} cells influence their distinct metastatic character (5–7).

Our results indicate that qualitative and quantitative aspects of arachidonic acid metabolism may affect tumor metastasis, and that other metabolites, besides prostaglandin E_2 and prostaglandin $F_{2\alpha}$, may be important in tumor cell biology. We cannot generalize on the significance of prostaglandin D_2 without knowing the normal progenitor cell of the B16 melanoma. Certainly, prostaglandin D_2 is enigmatic (22, 24). It occurs prominently only in the brain (33), during cardiac anaphylaxis (34), and in basophilic leukemia cells (35). Unlike most enzymes in the arachidonic acid cascade, prostaglandin D_2 isomerase occurs as a soluble, cytosolic component (24).

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