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Prostaglandin and Thromboxane Synthesis by M5076 Ovarian Reticulosarcoma during Growth: Effects of a Thromboxane Synthetase Inhibitor

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ABSTRACT

The five stable metabolites [prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), thromboxane B_2 (TXB_2), and 6-ketoprostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$)] of arachidonic acid (AA) via the cyclooxygenase pathway were measured by high-resolution gas chromatography-mass spectrometry in M5076 ovarian reticulosarcoma (M5) homogenates at various times after tumor implantation (Days 15, 18, 21, and 24). Vegetating tumor showed an active AA overall metabolism, which significantly increased during tumor growth. Synthesis of selected products (TXB_2, PGD_2, and PGE_2) increased markedly over time (up to 10.6, 3.5, and 0.9 μg/g, respectively). The overall metabolic profile was $TXB_2 \gg PGD_2 > PGF_{2\alpha} >$ 6-keto-$PGF_{1\alpha} > PGE_2$ on Day 15 and $TXB_2 \gg PGD_2 \gg PGF_{2\alpha} > PGE_2 >$ 6-keto-$PGF_{1\alpha}$ on Day 24. TXB_2 was also by far the most abundant product of *in vitro*-cultured M5 cells. Chronic treatment of M5-bearing mice with dazmegrel (UK-38,485), a selective thromboxane synthetase inhibitor (100 mg/kg p.o. daily, from Day 7 to killing), resulted in incomplete TXB_2 synthesis inhibition, AA metabolism diversion toward the other prostaglandins, and no effects of tumor growth and metastasis. More frequent dazmegrel treatment (100 mg/kg p.o. every 8 h from Day 1 to killing) resulted in complete TXB_2 synthetase inhibition, AA metabolism diversion, and increased tumor growth and metastasis. These data do not support the hypothesis of thromboxane synthetase inhibitors reducing tumor growth. However, since TXB_2 suppression was accompanied by the production of other products possibly interfering in tumor growth, no conclusions on the effective role of TXA_2 in malignancy can be drawn.

INTRODUCTION

PG[2] and TXA_2, the arachidonic acid metabolites produced through the cyclooxygenase pathway, have been proposed as modulators of a number of biological events involved in cancer growth and dissemination (1-4). However, the relationship between arachidonate metabolism and malignancy has not yet been conclusively defined.

We recently started a study aimed at characterizing AA metabolic profiles in various animal and human tumors, to help identify models for studying the roles of PG and TXA_2 in malignancy. In the first tumor examined, 3LL, we found that the major products, PGE_2 and PGD_2, were formed in increasing amounts during tumor development (5).

This paper shows the profiles (at various times after implantation) with homogenates of M5, a tumor metastasizing to the liver, uterus, and ovary (6). Since preliminary experiments had shown that in this tumor the main AA metabolite was TXB_2 (the stable metabolite of TXA_2), we decided to study the effect of the thromboxane synthetase inhibitor dazmegrel (UK-38,485) on tumor development, with the aim of verifying the current hypothesis (7) that reduction of tumor TXA_2 could reduce tumor growth and metastasis.

MATERIALS AND METHODS

Materials. PG standards were a generous gift from Dr. John Pike of the Upjohn Co., Kalamazoo, MI. Dazmegrel (UK-38,485) was obtained through the courtesy of Dr. M. J. Randall of the Pfizer Co., Sandwich, Kent, United Kingdom.

Animals and Tumor. M5076 ovarian reticulosarcoma, a tumor of spontaneous origin, was maintained *in vivo* by i.m. implant [5×10^5 viable cells/0.1 ml/mouse] in female C57BL/6 mice [body weight, 20 ± 2 (SE) g] from Charles River, Calco, Italy. The M5 tumor was also cultivated *in vitro*. M5 cells were suspended in RPMI 1640 (Grand Island Biological Co., Grand Island, NY) supplemented with 10% heat-inactivated equine serum (Flow Laboratories, Rockville, MD), 2 mM L-glutamine, and 1 mM sodium pyruvate, as described previously (8). The cells were harvested by tapping the flask; the dislodged cells were then washed twice in phosphate-buffered saline. Viable tumor cells were centrifuged and the pellet was frozen on solid CO_2 and stored at −20°C until analyzed for AA metabolite production.

In a first experiment, mice bearing M5 tumor received 100 mg/kg p.o. daily of dazmegrel or vehicle from the seventh day after implantation until killing. Groups of treated and control mice were killed by cervical dislocation 15, 18, 21, and 24 days after M5 implant. In a second experiment, mice received vehicle or dazmegrel, 300 mg/kg p.o. daily (100 mg/kg every 8 h), from the first day after implant until killing (Day 25). Minimum (*a*) and maximum (*b*) tumor diameters were measured with calipers. Tumor size was calculated as $(a^2 \times b)/2$. The livers were examined for macroscopic metastatic nodules.

Sample Preparation. Vegetating sections of the primary tumor were rapidly dissected, weighed, frozen on solid CO_2, and stored at −20°C. Frozen samples were directly homogenized in 10 volumes of 50 mM for phosphate buffer (pH 7.4), incubated at 37°C for 15 min, and then spun at 200,000 × *g* for 30 min. The supernatants were kept frozen until analyzed. This procedure allows the *ex vivo* metabolism of endogenous AA to give characteristic and reproducible profiles as reported previously (9). *In vitro*-cultured M5 cells were homogenized and incubated in the same conditions (3×10^6 cells/ml buffer).

The 5 stable metabolites of AA (PGE_2, PGD_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, and TXB_2) were quantified by high-resolution gas chromatography-mass spectrometry as described previously (10).

RESULTS

AA Metabolic Profiles in M5 Tumor. Table 1 shows the arachidonic acid metabolic profile in M5 homogenates during tumor development. By Day 15, when the tumor was palpable, TXB_2 was the predominant metabolite, with $TXB_2 \gg PGD_2 > PGF_{2\alpha} >$ 6-keto-$PGF_{1\alpha} > PGE_2$. The qualitative profile did not change much during tumor growth, since at Day 24 it was $TXB_2 \gg PGD_2 \gg PGF_{2\alpha} > PGE_2 >$ 6-keto-$PGF_{1\alpha}$. However, a difference was noted in the absolute amounts of metabolites formed at various times after implant, with a 2-fold increase in TXB_2. Total metabolite formation increased with time.

The AA metabolic profile was also examined in M5 cells cultivated *in vitro* to rule out a major contribution of host

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[2] The abbreviations used are: PG, prostaglandin; AA, arachidonic acid; TXA_2 and TXB_2, thromboxanes A_2 and B_2, respectively; $PGF_{2\alpha}$, PGD_2, PGE_2, prostaglandins $F_{2\alpha}$, D_2 and E_2, respectively; 6-keto-$PGF_{1\alpha}$, 6-ketoprostaglandin $F_{1\alpha}$; 3LL, Lewis lung carcinoma; M5, M5076 ovarian reticulosarcoma.

Table 1 *Synthesis of AA metabolites by M5 tumor after vehicle or dazmegrel treatment*

Tumors were dissected at various times after implant. Vegetating tissue was homogenized and incubated at 37°C for 15 min. AA metabolites were measured by high-resolution gas chromatography-mass spectrometry as described in "Materials and Methods."

Treatment	Day	AA metabolites (μg/g)					
		TXB_2	PGE_2	PGD_2	6-Keto-$PGF_{1\alpha}$	$PGF_{2\alpha}$	Total metabolite
Vehicle	15	5.07 ± 1.31[a,b]	0.52 ± 0.17	1.33 ± 0.44	0.86 ± 0.18	1.16 ± 0.18	8.96 ± 2.17
	18	5.55 ± 0.98	0.53 ± 0.06	1.22 ± 0.37	0.79 ± 0.14	1.51 ± 0.32	9.59 ± 1.61
	21	6.26 ± 3.94	0.84 ± 0.30	1.69 ± 0.32	0.61 ± 0.09	1.31 ± 0.41	11.92 ± 2.47
	24	10.65 ± 4.17[b]	0.94 ± 0.25	3.51 ± 2.12[b]	0.58 ± 0.12	1.11 ± 0.47	16.79 ± 3.49[c,d]
Dazmegrel							
24 h	15	5.46 ± 2.04	0.57 ± 0.19	1.50 ± 0.05	0.77 ± 0.17	1.61 ± 0.41	9.92 ± 2.39
24 h	18	5.14 ± 0.53	0.69 ± 0.05	1.43 ± 0.33	0.80 ± 0.23	1.24 ± 0.17	9.29 ± 0.30
6 h	21	4.55 ± 0.19[e]	2.40 ± 0.48[e]	4.81 ± 0.87[e]	0.90 ± 0.07	2.97 ± 0.33[e]	11.54 ± 1.68
15 h	24	6.66 ± 3.23	1.28 ± 0.72	4.22 ± 1.00	0.69 ± 0.22	1.39 ± 0.67	14.25 ± 0.70

[a] Mean ± SD ($n = 4$ for all groups, except $n = 3$ for Day 24, dazmegrel). Data were analyzed by two-way analysis of variance. Statistical differences were determined by Tukey's multiple comparison test.
[b] $P < 0.05$ for Day 24 *versus* Days 18 and 15.
[c] $P < 0.01$ for Day 24 *versus* Days 18 and 15.
[d] $P < 0.05$ for Day 24 *versus* Day 21.
[e] $P < 0.01$ for dazmegrel *versus* vehicle (Day 21).

infiltrating cells in determining the profile observed with the *in vivo*-growing tumor. TXB_2 was the major product of M5 cells (2 ng/10^6 cells), other products being below the assay detection limit (0.1 ng/10^6 cells).

Thromboxane Synthetase Inhibition and AA Metabolic Profile Diversion by Dazmegrel: Effect on Tumor Growth and Metastasis. To check the inhibition of thromboxane formation throughout treatment (daily doses of 100 mg/kg), we examined AA metabolite synthesis by tumor homogenates on Days 15, 18, 21, and 24 from implant (Table 1). Mice were killed at various times (6, 15, or 24 h after the last treatment). Six h after the last dose, TXB_2 synthesis was almost completely inhibited (94% reduction), with AA metabolism diverted to the increased production of PGD_2, PGE_2, and $PGF_{2\alpha}$. A slight increase was seen in 6-keto-$PGF_{1\alpha}$ synthesis. Overall production of AA metabolites (expressed as the sum of $PGF_{2\alpha}$, PGD_2, PGE_2, TXB_2, and 6-keto-$PGF_{1\alpha}$) was the same as for controls. By 15 h from the last dose (Day 24), there was a nonsignificant trend toward TXB_2 inhibition and consequent AA metabolism diversion. Total production of AA metabolites was again similar to that of controls. Synthesis of TXB_2 and other metabolites was not affected by dazmegrel 24 h from the last dose (Days 15 and 18).

As shown in Table 2, tumor growth was not influenced by chronic dazmegrel (daily doses of 100 mg/kg). Liver metastases were also unaffected (data not shown).

Since complete thromboxane synthesis inhibition was not observed during the whole treatment period, we made a second experiment to obtain conclusive data on the effect of complete thromboxane synthesis suppression on tumor growth and metastasis. Groups of 15 M5-bearing mice were treated with vehicle or dazmegrel (100 mg/kg p.o.) every 8 h from the first day after implant until killing. Five mice in the treated and 3 in the control group died before the end of the experiment. Four mice per group were killed 8 h after the last dose (Day 25) to confirm TXB_2 synthesis inhibition in tumor homogenates.

Fig. 1 shows the AA metabolite profile data in M5 tumors from dazmegrel-treated mice. These data confirm those of the first experiment, showing almost complete (90%) TXB_2 synthesis suppression with consequent diversion of AA metabolism toward PGE_2, PGD_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$. In this experiment, total AA metabolite production was higher ($P < 0.01$, Student's t test) in dazmegrel-treated tumors (33.78 ± 2.09 μg/g) than in controls (18.32 ± 2.32 μg/g). AA metabolic profiles

Table 2 *M5 tumor growth in mice treated with dazmegrel*

Dazmegrel (100 mg/kg) was given p.o. once daily from the seventh day after implant until killing.

	Tumor wt (g)			
	Day 15	Day 18	Day 21	Day 24
Vehicle	1.26 ± 0.18[a] (5)[b]	2.31 ± 0.22 (5)	3.41 ± 0.20 (4)	4.29 ± 0.27 (4)
Dazmegrel	1.34 ± 0.13 (5)	2.17 ± 0.31 (5)	3.84 ± 0.35 (4)	4.29 ± 0.36 (3)

[a] Mean ± SD. Data were analyzed by two-way analysis of variance. No differences were found between dazmegrel and control mice at any time.
[b] Numbers in parentheses, number of determinations.

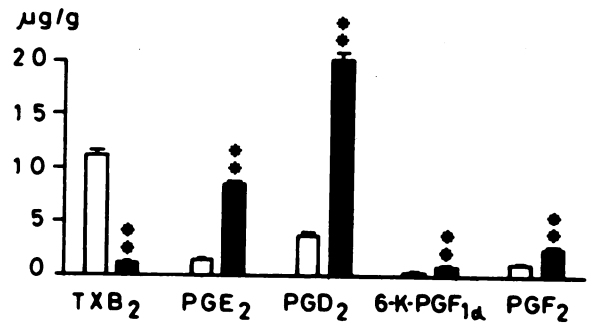

Fig. 1. Synthesis of AA metabolites ($PGF_{2\alpha}$, PGD_2, PGE_2, TXB_2, and 6-keto-$PGF_{1\alpha}$) by M5 tumor after vehicle (□) or dazmegrel (■) treatment (100 mg/kg p.o. every 8 h, from the first day after implant until killing). Mice were killed 8 h after the last treatment. Data are expressed as μg/g. Statistical differences were evaluated between control and dazmegrel values (** $P < 0.01$) by Student's t test. *Columns*, mean of 4 determinations. *Bars*, SE.

of controls (Day 25) were, however, similar to those obtained in the first experiment (Day 24): TXB_2, 11.08 ± 0.94 *versus* 10.65 ± 2.09 μg/g; PGE_2, 1.55 ± 0.25 *versus* 0.94 ± 0.13 μg/g; PGD_2, 3.94 ± 1.19 *versus* 3.51 ± 1.06 μg/g; 6-keto-$PGF_{1\alpha}$, 0.48 ± 0.07 *versus* 0.58 ± 0.06 μg/g; $PGF_{2\alpha}$, 1.27 ± 0.09 *versus* 1.11 ± 0.24 μg/g, respectively.

Table 3 shows M5 tumor size (cm^3) at various times from implant in dazmegrel-treated (100 mg/kg every 8 h) and control mice. The more frequent dazmegrel schedule did not slow the growth of M5 tumor in mice and, in fact, even significantly increased it. On Day 25 tumor weight was also recorded for controls (4.904 ± 0.252 g, $n = 12$) and for treated mice (6.301 ± 0.318 g, $n = 10$; different from controls at $P < 0.01$, Student's t test). A slightly larger number of mice with macroscopic metastases was found among treated (7 of 10) than among control (5 of 12) mice.

Table 3 *M5 tumor size in mice treated with dazmegrel*

Dazmegrel (100 mg/kg) was given p.o. every 8 h from the first day after implant.

	Tumor size (cm^3) at following times after implant					
	Day 15	Day 17	Day 19	Day 21	Day 23	Day 25
Vehicle	1.27 ± 0.33[a] (10)[b]	1.81 ± 0.30 (10)	2.11 ± 0.28 (10)	2.60 ± 0.35 (10)	2.81 ± 0.42 (10)	3.19 ± 0.59 (9)
Dazmegrel	1.57 ± 0.22 (10)	2.08 ± 0.31 (10)	2.34 ± 0.34 (10)	2.84 ± 0.33 (8)	3.43 ± 0.46 (8)[c]	4.02 ± 0.73 (8)[d]

[a] Mean ± SD. Data were analyzed by two-way analysis of variance. Statistical differences were determined by Tukey's multiple comparison test.
[b] Numbers in parentheses, number of determinations.
[c] $P < 0.05$.
[d] $P < 0.01$.

DISCUSSION

This paper describes the metabolic pattern of AA via the cyclooxygenase pathway in growing M5 tumor and confirms our previous findings in 3LL of a preferential increase of selected AA metabolites during tumor growth. In both cases the main metabolites (PGE_2 and PGD_2 in 3LL, TXB_2 and PGD_2 in M5) increased substantially during tumor development. The AA metabolic profiles in M5 were qualitatively and quantitatively different from those of 3LL, as could be expected in cells of different histological origin. AA metabolic profiles from 3LL and M5 successive (several months apart) implants were well reproducible.

TXB_2 was the most abundant AA metabolite also of *in vitro*-cultured M5 cells. This rules out a major contribution of host infiltrating cells to the AA metabolic profile observed with the *in vivo*-growing M5 tumor.

The increased overall AA metabolism observed in 3LL and M5 seems to indicate that growing tumors might, in general, have an elevated cyclooxygenase activity (11) or a greater availability of AA (possibly due to a higher AA phospholipid content and/or a more active phospholipase A_2). Hammarstrom *et al.* (12), reviewing the mechanism of altered endogenous PG production by transformed fibroblasts, concluded that virus transformation of mammalian cells could enhance their basal phospholipase A_2 activity, leading to release of AA from cellular lipids with consequent increased synthesis of AA metabolites. AA levels in cellular lipids have been shown, in fact, to govern the amounts of PG synthesized by cells growing in culture (13).

The preferential increase of selected products in each tumor might, on the other hand, reflect one or more of the following events: (*a*) the redistribution of PG endoperoxide metabolism through the most active enzymatic pathway(s) in the presence of increasing substrate availability; (*b*) the preferential growth of selected cell subpopulations with different amounts of the AA cascade enzymes and/or AA availability; and (*c*) a selective AA cascade enzyme induction, together with an increase in available precursor.

These considerations and the scarcity of profile data on most experimental tumors do not support the hypothesis of any particular AA metabolite(s) being produced in excess by tumors. In fact, PGE_2, often regarded as typically abundant in many tumors and studied as representative of tumor PG production (14–17), accounted for only about 6% of the total AA metabolites produced by M5 at any time considered. A systematic view of this metabolic pattern in tumors should therefore be of considerable utility when studying the roles of these products in malignancy. Focusing on selected metabolites might be misleading since each product could influence many of the mechanisms leading to cancer growth and spread, with different intensity and direction.

In view of these considerations, we decided to test the effect of chronic dosing with dazmegrel, a thromboxane synthetase-selective inhibitor, on M5 tumor growth and metastasis, while examining its overall effect on cyclooxygenase AA metabolic pattern. Structurally unrelated thromboxane synthetase inhibitors have been reported previously to reduce tumor cell growth (7), tumor size (18), and experimental and spontaneous metastases (7, 19).

In our first experiment, the dazmegrel dosage was chosen on the basis of the work of Drago and Al-Mondhiry (18), who showed an effect on prostate tumor growth and metastasis with a daily dose of 10 mg/kg to rats. We used 10 times this dose, with the aim of obtaining more pronounced effects on both parameters. With this treatment schedule we observed complete inhibition of TXB_2 formation up to 6 h after dazmegrel but could not demonstrate any effect of this drug on M5 tumor growth and metastasis.

While this experiment was in progress, Vicenzi *et al.*[3] reported that the daily administration of 300 mg/kg p.o. (150 mg/kg every 12 h, from the day of implant) to mice bearing a thromboxane-producing fibrosarcoma had no effect on tumor development and spread. These authors described marked TXB_2 synthesis inhibition in tumor cells from dazmegrel-treated mice (2 h after the last dose) as we did in tumor homogenate (6 h after the last dose) but could not demonstrate complete thromboxane synthesis inhibition during the whole treatment period.

For this reason we decided to administer 100 mg/kg of dazmegrel every 8 h from the day of implant, but in spite of the complete inhibition of thromboxane synthesis throughout the treatment period, we did not observe any reduction in tumor growth and metastasis. If anything, there was a significant increase in tumor size (on Days 21 and 25) and a slightly larger number of animals with metastases.

This increase in tumor size might account for the higher overall AA metabolite synthesis in dazmegrel-treated tumors (observed only with the higher dosage), should the increased tumor AA metabolism be related more to tumor growth rate than tumor "age." Other possible explanations for the increased cyclooxygenase AA metabolism observed with the higher dazmegrel dosage are: (*a*) a direct effect of this drug on the enzymes which make AA available for further metabolism; (*b*) a direct effect on the lipoxygenase pathway, causing in turn a diversion of AA metabolism toward the cyclooxygenase system.

Our data thus do not support the hypothesis of thromboxane synthetase inhibitors reducing tumor growth and spread, at least with this particular tumor. However, in the presence of a metabolic rearrangement such as that described here, one cannot draw any firm conclusion on a specific role of TXA_2 in malignancy, since other products were formed which might have had opposing or similar effects on the same parameters.

[3] E. Vicenzi, M. G. Lampugnani, A. P. Bolognese Dalessandro, A. Niewiarowska, G. de Gaetano, and M. B. Donati. Dissociation between thromboxane generation and metastatic potential in cells from a murine fibrosarcoma. Studies with a selective thromboxane synthase inhibitor, submitted for publication.

It must also be considered that no direct evidence is available on the *in vivo* levels of AA metabolites released by tumors, since most studies dealt with *in vitro* or *ex vivo* synthesis by tumor cells or homogenates.

The pharmacological manipulation of AA metabolism through the simultaneous use of possible selective inhibitors of the various products, together with a careful evaluation of their overall *in vivo* effect on metabolic profiles, may prove useful in future studies to clarify the roles of PG and TXA_2 in malignancy.

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