Inhibition of Hepatic Metastasis From a Human Pancreatic Adenocarcinoma (RWP-2) in the Nude Mouse by Prostacyclin, Forskolin, and Ketoconazole

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Metastasis is a multistep phenomenon in which platelets appear to play an important role. This study examined several compounds for their effects on experimental hepatic metastasis and on human pancreatic tumor cell–platelet interactions. Prostacyclin (PGI_2) and forskolin (stimulators of platelet adenylate cyclase) and ketoconazole (inhibitor of lipoxygenese and thromboxane synthetase) were used in order to investigate their effects on hepatic metastases from a human pancreatic tumor cell (RWP-2) in the nude mouse. The tumor cells were injected intrasplenically and the animals were divided into control, prostacyclin (PGI_2 200 μg), forskolin (150 μg), and ketoconazole (180 μg) groups. All three drugs were administered intraperitoneally 30 minutes before and 24 hours after the tumor cell injections. Statistically significant differences were observed between control and treated groups in tumor surface area ($P < 0.001$), percentage of liver surface area occupied by tumor ($P < 0.001$), and number of tumor colonies ($P < 0.004$ for prostacyclin, $P < 0.005$ for forskolin, and $P < 0.001$ for ketoconazole). These agents also strongly inhibited RWP-2-induced platelet aggregation in human platelet-rich plasma.

***Cancer* 65:446–451, 1990.**

PANCREATIC CANCER is a rapidly progressive and fatal disease with an overall 5-year survival rate of less than 1%.[1] Currently, existing therapeutic modalities are largely ineffective, and metastases are responsible for the fatal outcome in the vast majority of cases. Metastasis is a multistep process in which vascular integrity and platelets play an important role.[2–5]

About 20 years ago, Gasic *et al.*[5] suggested that platelets enhance tumor cell survival and adhesion in the circulation. Later they also demonstrated that a number of tumor cell lines can induce platelet aggregation both *in vitro* and *in vivo*.[6–9] After this work, many laboratories have shown that several antiplatelet agents can prevent tumor-induced platelet aggregation and tumor metastasis in animal models.[6,10–16] Arachidonic acid metabolites through both the cyclooxygenase and lipoxygenase pathways may alter vascular tone and produce platelet aggregation.[17–19] Thromboxane A_2 (TxA_2) and prostacyclin (PGI_2) exert antagonistic effects on platelet aggregation mediated by opposing effects on platelet cyclic AMP (cAMP).[20,21] Prostacyclin inhibits aggregation by increasing platelet cAMP levels. Leukotrienes are powerful modulators of inflammation with significant effects on vascular integrity.[19,22] Selective inhibition of thromboxane synthetase and pretreatment with exogenous prostacyclin significantly decrease hematogenous metastases in murine tumor models.[23,24] Ketoconazole, an antifungal agent which inhibits both the thromboxane synthetase and 5-lipoxygenase, was found to reduce significantly the incidence of spontaneous pulmonary metastases in the B16-F10 melanoma tumor mouse model.[25]

Forskolin, a diterpene isolated from an Indian plant, *Coleus forskohlii*,[26] has been shown to block human platelet aggregation induced by a wide variety of aggregation stimulators.[27,28] Agarwal and Parks demonstrated that forskolin strongly inhibited B16 melanoma cell-induced platelet aggregation and significantly reduced tumor colonization in the lungs in a B16-F10 tail-vein injection model.[29]

The current study was undertaken in order to determine the effects of prostacyclin, forskolin, and ketoconazole on platelet aggregation and on hepatic metastases from a human pancreatic adenocarcinoma (RWP-2) in the nude mouse.

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Supported by a Grant from Veterans Administration, Washington, DC and USPHS, CA13943.

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The authors thank Robert H. Emma for expert assistance in preparation of the illustrations, Ms. Ashley Youngman for assistance in preparing the manuscript, and Dr. Kenneth G. Mann, University of Vermont, Burlington, Vermont, for providing dansylarginine N-(3-ethyl-1,5-pentanediyl)amide (DAPA).

Accepted for publication August 11, 1989.

Materials and Methods

Cell Culture

A human pancreatic adenocarcinoma (RWP-2) cell line established from a hepatic metastasis[30] was used in this study. The RWP-2 cells were cultured in an RPMI 1640-based medium (Gibco, Grand Island, NY) which was buffered to pH 7.3 with hepes (10 mmol/l final concentration), tricine (10 mmol/l), and sodium bicarbonate (33 mmol/l) (all buffers obtained from Gibco). The buffered RPMI 1640 was supplemented with heat-inactivated fetal bovine serum (12.5% final concentration, Gibco). Antibiotics added to the medium included penicillin (100 U/ml, Gibco), streptomycin (100 μg/ml, Gibco), fungizone (amphotericin 2.5 μg/ml, Gibco), and garamycin (20 μg/ml, Elkins-Sinn, Richmond, VA).

The monolayer cultures grew in a humidified 5% carbon dioxide (CO_2) atmosphere at 37°C in 25 cm tissue culture flasks (Nunclon, Thomas Scientific, Philadelphia). The cells were fed at least once a week. When the cells became confluent (about 1/20 of the culture was passaged before trypsinization), the cultures were washed with 0.9% saline (containing 100 U/ml penicillin and 100 μg/ml streptomycin). The cells were trypsinized with 0.25% trypsin in 0.1% ethylenediamine tetraacetic acid (EDTA) (pH 7.3) (both from Gibco) for 15 minutes at 37°C. Trypsin was inactivated by addition of the above medium containing 12.5% fetal bovine serum. The cells were immediately centrifuged at 200 × *g* for 5 minutes and resuspended in the fresh medium.

The RWP-2 tumor cells were grown in 100-mm diameter petri dishes (Nuclon). Cultures were harvested as above and resuspended after centrifugation into Hanks balanced solution (Sigma Chemical Co., St. Louis). Cells were counted using a hemocytometer and viability assessed with trypan blue dye exclusion.

In Vivo *Experiments*

Athymic NCI nude mice (NCr-nu/nu), 6 weeks old, were used as experimental animals in this study. The animals were maintained in a positive-pressure isolation room at the animal care facility of the Providence Veterans Administration Medical Center, Providence, Rhode Island. The food, water, and bedding for the mice were sterilized and changed at least once weekly. Surgical gowns and gloves were used when working in the room to prevent pathogenic contamination.

We used the model of intrasplenic tumor cell injections as described previously.[31] Briefly, after the establishment of satisfactory anesthesia, the abdomen was entered through a midline incision approximately 1 cm in length. The spleen was exteriorized by applying gentle traction and 2×10^6 cells suspended in 0.02 ml of Hanks balanced salt solution were injected just under the capsule of the lower pole of the spleen, using a tuberculine syringe with a 30-gauge needle (Ethicon, Somerville, NJ). Hemostasis was obtained by applying pressure on the injection site with a sterile swab for a period of 1 minute. The spleen was then inspected and, if hemostasis was satisfactory, it was returned to the peritoneal cavity and the incision was closed in two layers using a continuous 4-0 Vicryl suture (Ethicon) for the muscles and clips (American Hospital Supply, Beford, MA) for the skin. The animals were allowed to recover and the skin clips were removed 10 days after the operation.

Prostacyclin from Sigma Chemical Co., forskolin from Hoechst-Roussel Pharmaceuticals (Somerville, NJ), and ketoconazole from Janssen Pharmaceutica, (Piscataway, NJ) were obtained. A total of 51 animals were divided into four groups. The control group (11 animals) received 0.1 ml normal saline solution intraperitoneally 30 minutes before and 24 hours after the tumor cell injections. Each mouse in the treated groups (13 or 14 animals/group) received intraperitoneally 0.1 ml containing prostacyclin (200 μg, in 0.05 M Tris buffer, pH 9.37), forskolin (150 μg in 40% dimethyl sulfoxide), or ketoconazole (180 μg in 20 mmol/l hydrochloric acid [HCl]) 30 minutes before and 24 hours after the tumor cell injections.

The animals were killed by craniocervical dislocation 8 weeks after the operation. Autopsies were performed and the findings were recorded. The livers were harvested and placed in phosphate-buffered 10% formalin (Fisher, Pittsburgh).

Tumor colonies in both superior and inferior aspects of the liver were counted, their surface areas were calculated using a Don Santo Microplan II image analyzer (Don Santo Corp., Natick, MA), and expressed as a percent of the liver surface occupied by tumor. The results were analyzed statistically using the independent samples *t* test, and considered significantly different from controls when $P \leq 0.05$.

Isolation of Blood Platelets

Blood was drawn from healthy donors who had not ingested drugs known to affect platelet functions for at least 8 days. Blood was collected in 0.05 volume of saline (NaCl 0.9%) containing heparin to give a final concentration of 5 U/ml of blood. Platelet-rich plasma (PRP) was obtained by centrifuging the whole blood at 277 × *g* for 5 minutes and platelet-poor plasma (PPP) was obtained by centrifuging the remaining blood at 1530 × *g* for 5 minutes.

Platelet Aggregation

Platelet aggregation was carried out in heparinized PRP. Washed tumor cell suspension (1×10^7 cells/ml) was

TABLE 1. Effects of Prostacyclin, Forskolin, and Ketoconazole on Hepatic Metastases of Human Pancreatic Cancer (RWP2) in the Nude Mouse

Group	No. of animals per group	Liver surface area mm^2 (mean ± SD)	Tumor surface area mm^2 (mean ± SD)	Percentage of liver surface area occupied by tumor (% ± SD)	*P* value	Tumor liver nodules/mouse (mean ± SD)	*P* value
Control	11	1453 ± 325	1048 ± 393	71.6 ± 21.0		11.6 ± 4.9	
Prostacyclin	13	829 ± 185	113 ± 145	11.6 ± 12.6	<0.001	6 ± 3.5	<0.004
Forskolin	14	821 ± 130	137 ± 152	14.7 ± 14.4	<0.001	5.9 ± 4.4	<0.005
Ketoconazole	13	1011 ± 103	48 ± 41	4.7 ± 3.9	<0.001	4.9 ± 2.5	<0.001

Each mouse was injected with 2×10^6 cells into the spleen. Antiplatelet agents were given intraperitoneally 30 minutes before and 24 hours after tumor-cell injections (see "Materials and Methods").

treated with apyrase (0.5 U/ml) to degrade any ADP released from the tumor cells. This cell suspension was maintained at 4°C and employed within 1 hour for platelet aggregation studies. Fifty microliters of the tumor cell suspension containing 0.5×10^6 cells were added to PRP (450 μl) to induce aggregation.

Results

Hepatic Metastases

The results of the effects of prostacyclin, forskolin, and ketoconazole on hepatic metastases are shown in Table 1. All agents cause significant reduction of the number of metastatic tumor colonies. The mean number of tumor colonies in the control group is 11.6, whereas in the treatment groups this number is reduced to 6, 5.9, and 4.9 for the prostacyclin, forskolin, and ketoconazole groups respectively. The differences between the control and each of the three treated groups are statistically significant with *P* values <0.004 for the prostacyclin, <0.005 for the forskolin, and <0.001 for the ketoconazole groups. In each of the three treated groups there is a statistically significant reduction of the mean tumor surface area from 1048 mm^2 in the control group to 113 mm^2 in the prostacyclin, 137 mm^2 in the forskolin, and 48 mm^2 in the ketoconazole group ($P < 0.001$ for all groups) (Fig. 1).

Similarly, there are significant differences in the percentage of liver surface occupied by tumor between the control and the treated groups. In the control group, 71.6% of the liver surface is occupied by tumor, whereas in the prostacyclin, forskolin, and ketoconazole groups this percentage is decreased to 11.6%, 14.7%, and 4.7% respectively ($P < 0.001$).

Findings From Platelet Aggregation Studies

Suspensions of washed tumor cells were employed to examine their platelet aggregation activity in human PRP. The cell number giving optimum platelet aggregation ranged from 0.5×10^6 to 1×10^6 cells/ml of PRP. After the addition of RWP-2 tumor cells to PRP, a lag period of 1 to 2 minutes is seen before the induction of platelet aggregation, which reaches optimum in about 7 minutes, followed by clotting. Dansylarginine N-(3-ethyl-1,5-pentanediyl)amide (DAPA) (25 μmol/l), a specific inhibitor of thrombin with a Ki of 0.1 μmol/l for fibrinogen,[32,33] blocks the clotting but only moderately affects platelet aggregation, suggesting that RWP-2 tumor cells initiate the production of thrombin. Prostacyclin, forskolin, and ketoconazole block both platelet aggregation and clotting induced by RWP-2 tumor cells (Fig. 2). No clotting is seen for at least 30 minutes when platelet aggregation is blocked by these agents.

Discussion

The results of this study demonstrate that prostacyclin, forskolin, and ketoconazole significantly inhibit hepatic metastases from the human pancreatic cancer cell line RWP-2 in the nude mouse. There is a significant reduction in the number of metastatic colonies, the tumor surface area, and the percentage of liver surface area occupied by tumor in the three treated groups as compared with the control group. In addition, our studies suggest that platelet–RWP-2 tumor cell interaction causes the thrombin production of human PRP. Prostacyclin, forskolin, and

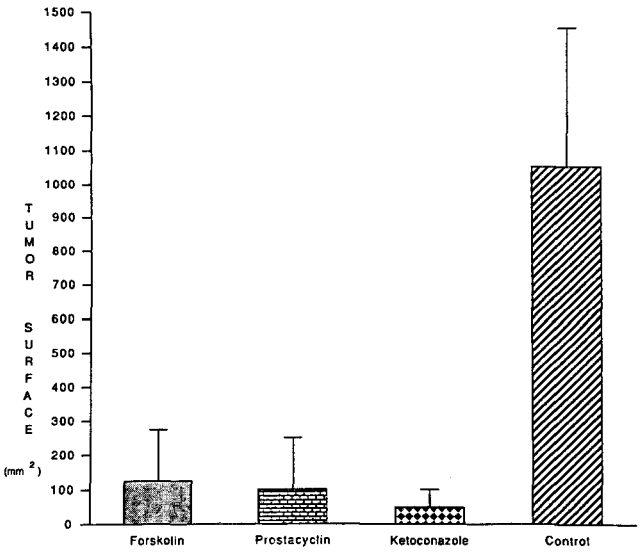

FIG. 1. Mean tumor surface area mm^2 in control, prostacyclin, forskolin, and ketoconazole groups. $P < 0.001$ for all treated groups compared with the control.

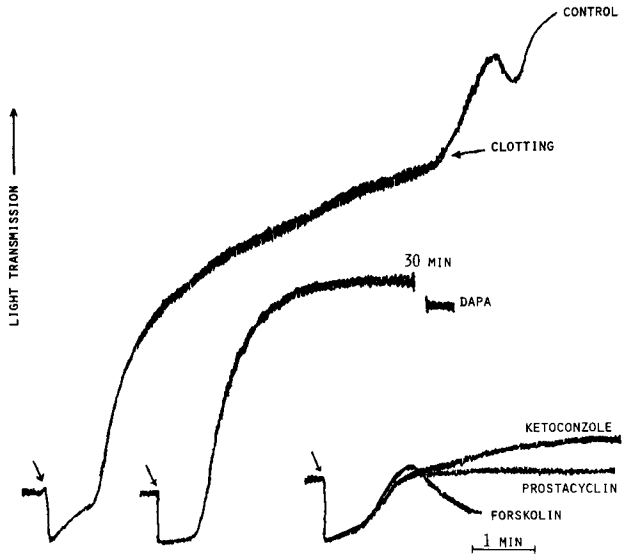

FIG. 2. Effect of prostacyclin, forskolin, ketoconazole, and DAPA on human platelet aggregation induced by RWP-2 cells. Human platelet-rich plasma was incubated with prostacyclin, (1 μmol/l), forskolin (3 μmol/l), ketoconazole (200 μmol/l), or DAPA (25 μmol/l) for 5 minutes, and platelet aggregation was stimulated by the addition of RWP-2 tumor cells (0.5×10^6).

ketoconazole strongly inhibited both platelet aggregation and clotting.

There is ample evidence that platelets play an important role in metastasis.[34–40] However, the exact mechanism through which platelets enhance metastasis remains unresolved. It has been proposed that a principal factor responsible for tumor cell-induced platelet aggregation, and possibly even for tumor cell procoagulant activity, is a tumor cell-derived cathepsin B like cysteine proteinase.[41–43] The ability of proteinase inhibitors to reduce platelet aggregation paralleled their ability to inhibit cathepsin B activity in B16a melanoma tumor cells.[43] Honn and Sloane suggested that cathepsin B induces platelet aggregation by stimulating biosynthesis of thromboxane A_2, and this effect is inhibited by PGI_2 and thromboxane synthetase inhibitors.[44]

Tumor cell-induced platelet aggregation is directly affected by the prostaglandins, which are generated from platelets and blood vessels. In the vessel wall, arachidonic acid is converted to cyclic endoperoxides PGG_2 and PGH_2, and then to prostaglandins of E, D, F, and I series.[45] Prostacyclin is the major prostaglandin produced by the vessel wall, and has potent vasodilatory as well as platelet inhibitory actions.[46] In platelets, arachidonic acid is metabolized to PGH_2 and then primarily to thromboxane A_2, a most potent platelet-generated vasoconstrictor and platelet proaggregant.[46] It has been suggested that PGI_2 and thromboxane A_2 play an antagonistic and pivotal role in the control of thrombosis, centered upon their bidirectional effect on platelet cAMP levels.[41] The interaction between prostacyclin and thromboxane A_2 is thought to be largely responsible for thromboresistance.[46] However, this view has recently been challenged by observations that in some cases the endoperoxide PGH_2 can initiate platelet aggregation independent of its conversion to thromboxane A_2.[47] In addition, platelet lipoxygenase products such as 12-HETE may play a role in secondary platelet aggregation.[48]

There is substantial evidence that arachidonic acid metabolites are involved in local tumor growth and metastasis.[23,24,41,44,49,50] Alterations in the levels of the thromboxane A_2 and prostacyclin were found in the plasma of tumors of patients with cancer of the ovary, breast, bone, and lung.[51–54] An inverse correlation between tumor cell PGI_2 production and metastatic potential was demonstrated in several tumor sublines derived from the murine mFS6 sarcoma.[55]

The results of the current study suggest that prostacyclin, forskolin, and ketoconazole exert an antimetastatic effect through interference in platelet–tumor cell interaction. Prostacyclin has been proven to be a most potent inhibitor of aggregation and stimulator of adenylate cyclase.[21,56] In our study PGI_2 at 1 μmol/l completely inhibited platelet aggregation. Forskolin is also a potent stimulator of platelet adenylate cyclase and inhibitor of human platelet aggregation. It has been shown that a single dose of forskolin reduced B16-F10 cell pulmonary colonization by more than 70%.[29] Forskolin is unique in its action and perhaps interacts directly with the catalytic submit of adenylate cyclase.[27,57] In addition to its direct effects, forskolin in low concentration was found to augment markedly the efficacy and potency of adenosine and prostaglandins (PGE_1 and PGI_2) in inhibiting platelet aggregation.[27,28] The physiologic effects of forskolin on human platelets thus appear to be mediated by both direct stimulation of adenylate cyclase and through a marked enhancement of receptor-mediated stimulation of the enzyme.[28] In this study forskolin significantly reduced hepatic metastases from the human pancreatic cancer cell line RWP-2, and strongly inhibited both platelet aggregation and clotting *in vitro*.

Ketoconazole, an imidazole derivative, selectively inhibits not only thromboxane synthetase, but also 5-lipoxygenase.[22,58,59] Pretreatment with ketoconazole significantly reduced leukotriene-induced stimulation of thromboxane A_2 by 5-lipoxygenase inhibition *in vitro*.[60] It has been recently shown that ketoconazole inhibited pulmonary metastases in the B16-F10 melanoma model.[25] The current study demonstrates a significant inhibitory effect of ketoconazole on hepatic metastasis from the human pancreatic carcinoma cell line RWP-2 in the nude mouse and on platelet aggregation *in vitro*.

Favorable alteration of the metastatic process is a significant objective in cancer research. Platelet activity may

play an important role in allowing tumor cells to implant and proliferate. This study demonstrates that three inhibitors of platelet aggregation produced significant inhibition of hepatic metastasis from a human pancreatic carcinoma. Further investigations to define the exact role of platelet aggregation, cyclic AMP accumulation and arachidonic acid metabolites in human tumor metastasis appear to be a worthwhile endeavor.

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