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Platelet-tumor cell interaction: effect of prostacyclin and a synthetic analog on metastasis formation*

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Summary. The antimetastatic effect of the antithrombotic agents exogenous prostacyclin (PGI_2) and a synthetic analog, Iloprost, on experimental metastasis formation was studied by injecting BL6 melanoma cells into C57BL/6 mice. Suitable in vivo treatment conditions were selected according to the known properties of the two drugs, including their pharmacokinetics. Iloprost showed a greater ability to inhibit platelet aggregation induced by BL6 melanoma cells. PGI_2 displayed a limited antimetastatic activity, largely dependent on the tumor cell load and treatment schedule. Iloprost showed a far superior activity, its antimetastatic effect lasting longer and remaining detectable up to 6 h after tumor cell inoculation. The present data complex provides further support to the concept of a crucial role for platelet-tumor cell interaction in the process of metastasis formation.

Introduction

Several lines of evidence suggest that tumor cells can induce platelet aggregation which, in turn, may play a role in their hematogenous dissemination. This is true, at least, for several selected experimental tumors. Thus, for instance, tumor cells injected IV into animals aggregate platelets and induce thrombocytopenia [3, 6, 9, 12, 13, 15]; or, drug- or antiplatelet antibody-induced thrombocytopenia can impair both the organ-colonizing ability of IV-injected tumors and the spontaneous potential of primary growths to metastasize to distant organs [2, 7, 11, 13]. Furthermore, the ability of IV-injected tumor cells to form lung nodules correlates fairly well with their capacity to induce thrombocytopenia in vivo and aggregate platelets in vitro [3, 13].

In agreement with the assumption that the platelet-tumor cell interaction is a crucial step in the process of metastasis formation, Honn and co-workers [7, 8] have shown that the administration of exogenous prostacyclin (PGI_2), the most potent naturally occurring platelet-aggregation inhibitor, would reduce lung colony formation by IV-injected tumor cells. Moreover, therapeutic synergism was found to result from the combined use of PGI_2 and a phosphodiesterase inhibitor, which apparently potentiated the platelet-inhibiting action of PGI_2 and thus its antimetastatic effect [7]. Different results, however, were obtained by Karpatkin et al. [10], who found no effect of PGI_2 treatment on the prevention of pulmonary metastasis formation.

These seemingly conflicting data might be reconciled on the basis of the different experimental designs and models used, when the pharmacokinetics of exogenous PGI_2 is taken into account. Along this line, the purpose of this study was to test whether, following in vivo treatment with the stable prostacyclin analog Iloprost [14], significant inhibition of pulmonary metastases would occur in mice injected IV with B16/BL6 melanoma cells. This drug (Fig. 1) is apparently endowed with an antithrombotic activity that is more potent than that of PGI_2 and is sustained over a longer period of time after administration [14]. A preliminary assessment was made of the realtive potencies of PGI_2 and Iloprost on the inhibition of platelet aggregation induced by B16 melanoma cells.

Materials and methods

Animals. Male C57BL/6 mice 10–16 weeks old were obtained from Charles River Breeding Laboratories, Calco, Milan, Italy.

Tumors. The B16/BL6 (BL6) cell line, a subline of the B16 melanoma selected for its ability to penetrate the mouse bladder wall [5], was obtained from the Mario Negri Institute (Milan, Italy) and was maintained as an in vitro tissue

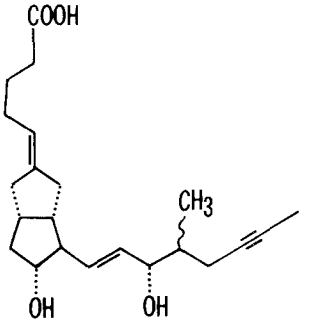

Fig. 1. Molecular structure of Iloprost

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culture in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 m*M* glutamine, 4% nonessential amino acids, 1 m*M* sodium pyruvate, MEM vitamin solution, and antibiotics (hereafter referred to as complete medium).

Platelet aggregometry studies. Human platelet-rich plasma (PRP) was prepared from samples freshly drawn from the antecubital vein of normal subjects who had not taken aspirin or related drugs in the previous 10 days. Blood was drawn using heparin (final concentration, 5 IU/ml; Eparina Vitrum, Bonomelli Farmaceutici, Italy), and PRP was separated from red blood cells by centrifuging for 15 min at 120 *g*. Platelet concentrations were adjusted to $3 \times 10^5/\mu l$ with platelet-poor plasma (PPP) obtained by blood centrifugation at 2000 *g* for 20 min. All platelet counts were done with a Thrombocounter-C (Coulter Electronic Ltd, Luton, UK).

Tumor cells, harvested from tissue culture flasks by means of brief 2 m*M* EDTA treatment, were washed three times and resuspended in PBS, and a viable cell count was done. Cell viability always exceeded 90%, as determined by trypan blue exclusion. Platelet aggregation assays were carried out turbidometrically using an Elvi 840 two-channel aggregometer after the addition of 50 µl tumor cells (final concentration, 10^6/ml) to 450 µl PRP. Values of aggregation were expressed as the percentage value of the theoretical maximal amplitude of the aggregation curve [1]. All experiments were run in duplicate on PRP preparations obtained from three different donors.

Modification of the aggregation response. In studies of the mechanism(s) of tumor cell-induced platelet aggregation, Apyrase (grade V, Sigma, St. Louis, Mo., USA) was added at a final concentration of 250 µ*M* to either tumor cells for 30 min or PRP for 3 min at 37° C. In studies of the possible role of the development of procoagulant activity, 50 µ*M* Leupeptine (Sigma), an inhibitor of cysteine protease, or 100 kIU Aprotinine (Sigma), a serine proteinase inhibitor, was incubated with BL6 melanoma cells for 30 min at room temperature. Incubation with these substances was carried out between the first and second washings of tumor cells.

Assessment of drug antithrombotic activity in vitro. In selected experiments, platelets were preincubated with 10-µl various concentrations of PGI_2 (Flolan, Wellcome Foundation Ltd, London, UK) in glycine buffer (pH 10.1) or Iloprost (Schering S. P. A., Milan, Italy) in saline solution for 3 min at 37° C in the aggregometer cuvette prior to the addition of neoplastic cells. None of these agents per se caused aggregation on prolonged incubation with human heparinized PRP in the absence of tumor cells.

In vitro testing of growth parameters of tumor cells. Quadruplicate aliquots of control BL6 cells, with or without Iloprost, in different concentrations were plated in 60-mm-diameter culture dishes at 10^3, 5×10^3, or 10^4 cells in 4 ml complete medium. After 4–5 days in culture, the cells were washed and the resulting tumor cell colonies were stained with crystal violet (0.5% in 95% ethanol).

Experimental metastasis assay. BL6 cells (10×10^5, unless otherwise indicated) were inoculated via a tail vein into normal or drug-treated mice, and the animals were then killed and autopsied after 15 days for assessment of pulmonary metastases. In selected experiments, the effect of the tumor cell load was studied by injecting different doses (5×10^4, 10^5, 2×10^5) of tumor cells. Each experimental group contained from eight to ten mice. Results were given as the arithmetic mean ± SE.

Drug treatment in vivo. Prospective recipients of BL6 cells were pretreated IV with PGI_2 (5 or 10 mg/kg) or Iloprost (0.1 or 0.2 mg/kg) according to different time schedules. The differences in drug pretreatment times were justified by the different pharmacokinetics of the two agents [14]. PGI_2 was dissolved in glycine buffer immediately before use; Iloprost was dissolved in saline. In selected experiments, control groups were included in which mice were injected with vehicle alone. The kinetics of the antimetastatic activity of Iloprost was studied in tumor recipients pretreated with Iloprost at different times (90 min, 3 h, 6 h, 18 h, or 24 h) with respect to tumor cell challenge.

Statistical analysis. For statistical analysis of the data, Student's *t*-test was used. Differences were considered significant when *P* values were < 0.05.

Results

Platelet aggregation by BL6 tumor cells

As shown in Fig. 2A, monophasic irreversible aggregation followed platelet exposure to BL6 tumor cells. Aggregation values were 40%, 35%, and 35% for the preparations from the three different donors, respectively.

The role of adenosine diphosphate (ADP) in the aggregating effect of BL6 cells was studied by the use of Apyrase, a substance with known ADP-ase activity. Pretreatment of BL6 cells with Apyrase resulted in minimal and reversible platelet aggregation (Fig. 2A). Similarly, the presence of Apyrase in the PRP before the addition of neoplastic cells brought about a complete inhibition of platelet aggregation. Thus, both tumor- and platelet-derived ADP were apparently involved in platelet activation by BL6 cells.

A possible role for the coagulation system and particularly thrombin generation in the observed effect was studied by resorting to Aprotinine and Leupeptine (Fig. 2A). These agents failed to interfere with tumor-induced platelet aggregation.

In vitro effect of PGI_2 and Iloprost on platelet aggregation induced by BL6 melanoma cells

The addition of various amounts of PGI_2 or Iloprost to PRP preparations produced a dose-dependent inhibition of platelet aggregation (Fig. 2B). It appeared that almost complete platelet inhibition was achieved by a concentration of 10^{-8} *M* PGI_2 or 10^{-9} *M* Iloprost.

In vitro plating efficiency of BL6 cells

The possibility that Iloprost treatment might induce changes in the malignant potential of tumor cells was ruled out by the assessment of the in vitro plating efficiency of BL6 cells treated with the drug. Table 1 shows the results. It is apparent that no significant differences in plating efficiency were found between control and treated cells.

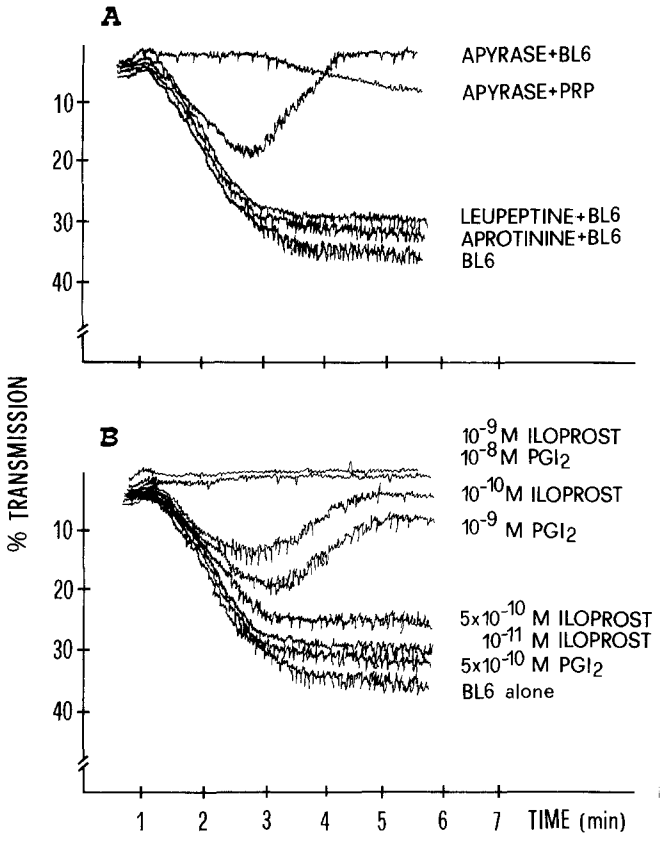

Fig. 2. (A) Characterization of platelet aggregation by BL6 melanoma cells. **(B)** Effect of Iloprost and PGI_2 on in vitro platelet aggregation induced by BL6 cells. Reported aggregation curves are representative of three different experiments

Table 1. Plating efficiency of BL6 cells treated with Iloprost at different doses

Iloprost treatment (concentration)	Plated cells		
	10^3	5×10^3	10^4
10^{-6} *M*	118*	107	121
10^{-7} *M*	122	97	88
10^{-8} *M*	115	102	114
10^{-9} *M*	101	87	91

* Control and treated cell cultures were set up in different numbers (10^3, 5×10^3, 10^4) in 60 mm plates and the number of colonies containing 20 cells or more were enumerated after 4–5 days. The data (mean of quadruplicate cultures) are expressed as % values of the number of colonies formed by the same inoculum of non-treated BL6 cells

Effect of PGI_2 or Iloprost treatment on experimental metastasis formation

According to the results shown in Fig. 3, it appears that only those animals pretreated with the higher Iloprost dose presented a significant reduction in pulmonary metastases, whereas PGI_2 was completely inactive at both dosages under the same conditions of tumor challenge.

Since it has been suggested that the antimetastatic activity of antiplatelet agents may largely be conditioned by the absolute number of pulmonary metastases [10], we also

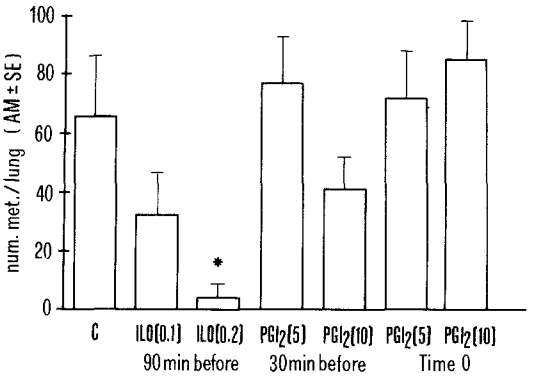

Fig. 3. Effect of PGI_2 or Iloprost (*ILO*) (mg/kg) administration at different times on experimental metastasis formation of BL6 cells. *C*, control. * $P < 0.05$, according to Student's *t*-test

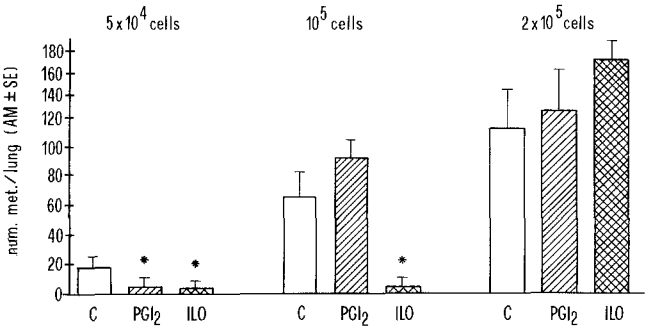

Fig. 4. Effect of PGI_2 (10 mg/kg) or Iloprost (0.2 mg/kg) administration on experimental metastasis formation of BL6 cells (5×10^4, 10^5, or 2×10^5) as a function of tumor cell load. PGI_2 was injected immediately before and Iloprost 90 min prior to tumor challenge. *C*, control mice not receiving drug treatment. * $P < 0.05$

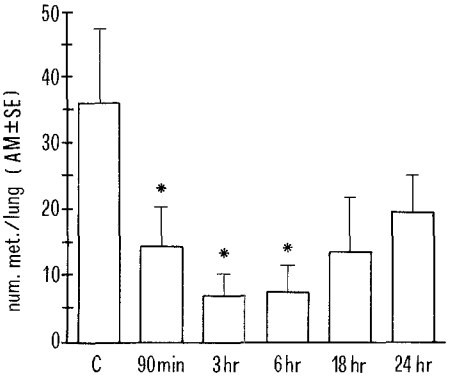

Fig. 5. Kinetics of the antimetastatic activity of Iloprost in BL6-injected mice. Iloprost was injected (0.2 mg/kg) at different times before BL6 challenge. *C*, control. * $P < 0.05$, according to Student's *t*-test

designed experiments in which the activities of PGI_2 and Iloprost were tested against different tumor cell loads. The results of the effect induced by different doses of tumor cells are shown in Fig. 4. It was found that PGI_2 exerted a significant antimetastatic activity when a tumor load as low as 5×10^4 cells was used. No effect was seen with tumor inocula as high as 10^5 or 2×10^5 cells. In contrast, Ilo-

prost showed a far superior activity, which was detectable even with a tumor inoculum size twice as large as the highest affected by PGI_2.

Kinetics of the antimetastatic activity of Iloprost in BL6-injected mice

To ascertain whether the antimetastatic activity of Iloprost could be detected over a prolonged period of time after drug treatment, tumor recipients pretreated with Iloprost at different times were used. The results are shown in Fig. 5. It is apparent that the antimetastatic ability of Iloprost treatment was detectable up to 6 h after drug treatment; when drug pretreatment was done earlier, the antimetastatic effect were no longer detectable.

Discussion

Experimental evidence supports a role for platelets in the metastatic dissemination of tumor cells, a notion reinforced by the finding that the ability of IV-injected tumor cells to metastasize in the lung correlates well with their ability to induce thrombocytopenia in vivo and aggregate platelets in vitro [3, 13]. However, conflicting results were obtained in studies aimed at ascertaining a beneficial effect of antiplatelet agents and particularly of PGI_2 [4, 7, 8, 10], the most potent naturally occurring platelet-aggregation inhibitor, on the organ-colonizing ability of experimental tumors as well as in several clinical trials on different tumor and antiplatelet agents. Thus, the importance in the metastatic process of the hemostatic system-tumor cell interaction and its possible pharmacological manipulation still remain a highly controversial issue.

In the present work we addressed the question as to whether at least some of the discrepancies resulting from studies of prostacyclin-induced manipulation of tumor metastasis might be due to the pharmacokinetic characteristics of PGI_2 as well as the pharmacological protocol adopted. As suggested by Karpatkin et al. [10], the short half-life of PGI_2 may well be an important factor contributing to the negative results obtained by these authors; it is therefore conceivable that the use of more potent and/or longer-lasting platelet-aggregation inhibitors might provide better results in terms of antimetastatic activity. The recent availability of the synthetic PGI_2 analog Iloprost, which is endowed with greater antiplatelet activity in vitro and a longer half-life in vivo [14], prompted us to evaluate its antimetastatic activity vs that of PGI_2. We tested the two agents concurrently for in vitro inhibition of platelet aggregation induced by the murine melanoma cells used in the in vivo experiments.

Our preliminary in vitro results showed that BL6 cells were indeed capable of aggregating platelets, the underlying mechanism(s) possibly involving the release of ADP from tumor cells and, possibly, from platelets. This effect was blocked by the preexposure of platelets to 10^{-8} *M* PGI_2 or 10^{-9} *M* Iloprost, a finding in keeping with a greater potency of the synthetic over the natural molecule, providing a rationale for the in vivo experiments.

According to an experimental metastasis model, BL6 melanoma cells were injected into normal or drug-treated mice, and the number of lung metastases was assessed 2 weeks later. The results showed that (a) both PGI_2 and Iloprost could induce an antimetastatic effect under selected experimental conditons and (b) the effect of Iloprost was more potent and longer lasting, being detectable at least up to 6 h after the injection of a tumor load as high as 10^5 cells. In contrast, the activity of PGI_2 was rather transient and detectable with relatively low tumor inocula.

Taken together, the data of the present study provide evidence that the antimetastatic effect of PGI_2 can only be detected when stringent experimental conditions are used, which possibly explains the failure consistently to detect the beneficial effects of this drug in other systems [10]. The mechanism of the antimetastatic activity of PGI_2 apparently involves the inhibition of platelet aggregation, and it is interesting to note that Iloprost, which has a greater activity in vitro, is also characterized by a greater antimetastatic activity in vivo. In this regard, preliminary observations in our laboratory indicate that in vivo treatment of mice with 0.2 mg/kg Iloprost results in a significant reduction in platelet aggregation induced in vitro by ADP addition (data not shown). Although mechanisms other than interference with platelet aggregation might also be implicated in the potent antimetastatic activity of Iloprost [4], our data provide further support to the concept of a crucial role for platelet-tumor cell interaction in the process of metastasis formation.

The possible therapeutic implications of these observations remain to be established. However, one might reasonably envision treatment of neoplastic patients with agents that would safely lower platelet activity to levels that are antimetastatic yet acceptably antihemostatic. More studies are needed to define the possible role of pharmacological manipulation of the hemostatic system as an adjuvant measure to conventional modalities of tumor treatment.

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