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Antimetastatic action of the prostacyclin analog Iloprost in the mouse

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The antimetastatic activity of the prostacyclin analog Iloprost has been examined in mice bearing Lewis lung carcinoma. An inhibition of lung colony formation is observed when 100 or 200 μg/kg Iloprost are administered i.v. 1 h before i.v. injection of tumor cells, which is dependent on the size of tumor inoculum. The effects of 200 μg/kg Iloprost persist for 24 h, and are of the same magnitude as those obtained with 10 mg/kg prostacyclin, which last only for 30 min. When treatment with Iloprost is followed by surgical removal of primary tumor, spontaneous metastasis formation is reduced, and the survival time of the treated animals is significantly increased over controls treated with surgery only. The antimetastatic effects of Iloprost appear dissociated from drug's effects on the hemostatic system of the host as indicated by the clot retraction assay, performed after *in vivo* treatment, using ADP or tumor cells as platelet aggregating agents. Iloprost thus appears to reduce spontaneous metastasis formation and intraoperative tumor cell dissemination, with pharmacological properties more favourable to therapeutic use than those of prostacyclin.

Introduction

The formation of hematogenous metastasis appears to involve interactions between circulating tumor cells and the hemostatic system of the host, since in laboratory animals a sufficient fibrinogen plasmatic level and thrombocyte number are necessary for the production of systemic metastases by solid malignant tumors [4–6]. Correspondingly, drugs lowering fibrinogen levels and antiplatelet drugs reduce metastasis formation in animal tumor models [7, 12, 19], and the use of anticoagulants combined with conventional forms of treatment revealed a significant, though limited, efficacy in clinical trials [27]. Physiological blood coagulation involves the prostaglandins prostacylin (PGI_2) and thromboxane A_2; these substances also appear to be involved in the pathogenetic mechanism of hematogenous metastasis, since they modify metatasis formation in laboratory animals [13, 14]. In particular, the administration of exogenous PGI_2 shortly before intravenous injection of B16 melanoma cells into syngeneic mice causes a reduction in the number of tumor lung colonies [15]; this effect was attributed to the inhibition of platelet aggregation caused by PGI_2 [13, 15, 21]. At the same time, PGI_2 is unstable in aqueous solution with a short biological half-life *in vivo*, and its antimetastatic effects were observed when PGI_2 was administered 15 min before tumor inoculation [13].

The aim of the present investigation was therefore to examine the antimetastatic properties of Iloprost, a chemically stable analog of PGI_2 endowed with platelet anti-aggregatory activity equivalent to that of PGI_2, and equally effective after

intravenous or oral administration [11, 18]. The effects of drug treatment have been determined on artificial and spontaneous metastasis formation in mice bearing Lewis lung carcinoma. The survival time of animals treated with the combination of Iloprost administration and surgical removal of primary tumor has also been examined.

Materials and methods

Tumor transplantation and evaluation

The Lewis lung carcinoma line used was originally provided by Dr J. Majo, DCT-Tumor Repository, NCI-Frederick Cancer Research Facility, MD, USA. The animals used for this investigation were female mice, weighing 18–20 g, purchased from Charles River, Calco Como, Italy. The tumor line is locally maintained by serial bi-weekly passages in C57BL mice, performed as described in the protocols of US NCI [9]. For experimental purposes, tumor inoculation was made using single cell suspensions which were prepared using 2-week-old tumors. The tumors, freed of capsule and necrotic parts, were minced with scissors, diluted with Ca^{2+} and Mg^{2+} free Dulbecco's saline (PBS), finely dispersed by gently forcing through a 2 × 38 mm disposable needle and filtered with a double layer of gauze. Tumor cell suspension was washed by centrifugation at 4°C for 10 min at 200 *g*, and by resuspension in PBS [22]; cell viability, measured by trypan blue exclusion, was 50–55 per cent, and the concentration of viable cells was adjusted to 20 $\times 10^6$ cells/ml. For i.v. tumor implantation, an injection of $2{\cdot}5 \times 10^4$–$2{\cdot}5 \times 10^5$ tumor cells in 0·1 ml of PBS was made in the lateral tail vein of BD2F1 hybrid mice; for i.m. tumor implantation, 10^6 tumor cells in 0·1 ml PBS were injected into the thigh of the left hind leg of BD2F1 mice. The number of lung colonies was determined at sacrifice 2 weeks after tumor implantation, counting by means of a low power stereo microscope the macroscopic tumor nodules present on the surface of the lungs. Surgical removal of i.m. primary tumors was performed, when indicated, on day 12 from tumor implantation, in mice under Ketalar anesthesia (125 mg/kg i.p.). After incision of the skin all around the upper thigh, the femoral and circumflex arteries were ligated with a synthetic absorbable suture (Ethicon, V303H) and the femur and muscles were cut with scissors. The wound was closed by pulling the skin into the stump and clamping it with a silk suture (Ethicon, K964H). Bleeding and fluid leaking from the tumor tissue on the normal surrounding tissue were avoided during amputation.

Drug treatment

Prostacyclin and Iloprost, kindly supplied by Schering SpA, Milan, Italy, were administered by i.v. injections through the lateral tail vein in volumes of 0·1 ml/animal. Iloprost was diluted in 0·05 M Trishydroxymethylaminomethane (pH 9·4) and PGI_2 was given in isotonic saline containing 1·25 g/l (*w*/*v*) $NaHCO_3$ (pH 8·2).

Clot retraction inhibition assay

Platelet aggregation was determined indirectly by inhibition of clot retraction, as described by De Gaetano *et al.* [3]. This assay is based on the observation that clot retraction is inhibited by previous *in vitro* platelet aggregation. Blood collection was performed by intracardiac puncture in open chested mice, previously anesthetized

with ethyl urethane (1·5 g/kg i.p.), 24 or 1 h after i.v. administration of 200 μg/kg Iloprost; nine volumes of blood were mixed directly in the syringe with 1 volume of 0·126 M trisodium citrate. Platelet rich plasma (PRP) samples were obtained, prepared and pooled from groups of three donors, as described by Gasic *et al.* [8]. Clot retraction was evaluated on PRP by adding 0·2 ml of 0·05 M $CaCl_2$ to a mixture of 0·32 ml PRP + 0·08 ml PBS containing 3·3 μM ADP or 4×10^5 Lewis lung carcinoma viable tumor cells. The measure of inhibition of clot retraction is calculated as the percentage ratio of the volumes of serum extruded in the presence of the aggregating agent over PRP samples obtained from mice not treated with drug in the absence of the aggregating agent.

Results

The i.v. administration of PGI_2 (10 mg/kg) 15 min before the i.v. inoculation of Lewis lung carcinoma cells causes a statistically significant reduction in the number of lung tumor colonies to 40 per cent of controls; a lesser reduction is observed when PGI_2 is given 30 min before tumor implantation, and the treatment performed at 1 h is completely inactive (figure 1, panel A). The i.v. injection of Iloprost (200 μg/kg) significantly and markedly reduces the formation of artificial metastases when the drug is administered 1–2 h before tumor cell inoculation; a significant reduction is still evident at 24 h (figure 1, panel B).

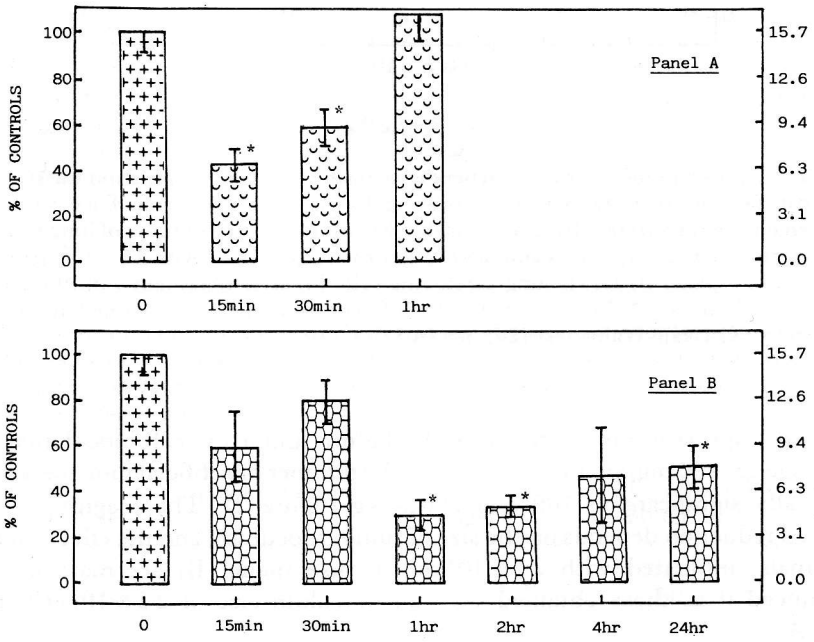

Figure 1. Time dependence of the effects of the treatment with PGI_2 and Iloprost on the number of artificial metastases in mice bearing Lewis lung carcinoma. Each value is the per cent ratio (treated over controls) ± S.E. of the average number of lung metastases determined in groups of six animals (15) controls) treated i.v. with 10 mg/kg PGI_2 (Panel A) or 200 μg/kg Iloprost (Panel B) at the time indicated prior to i.v. injection of $1{\cdot}25 \times 10^5$ Lewis lung carcinoma cells. (*) Means statistically different from the value obtained in the control group, Kruskall-Wallis test [24], $p = 0{\cdot}05$.

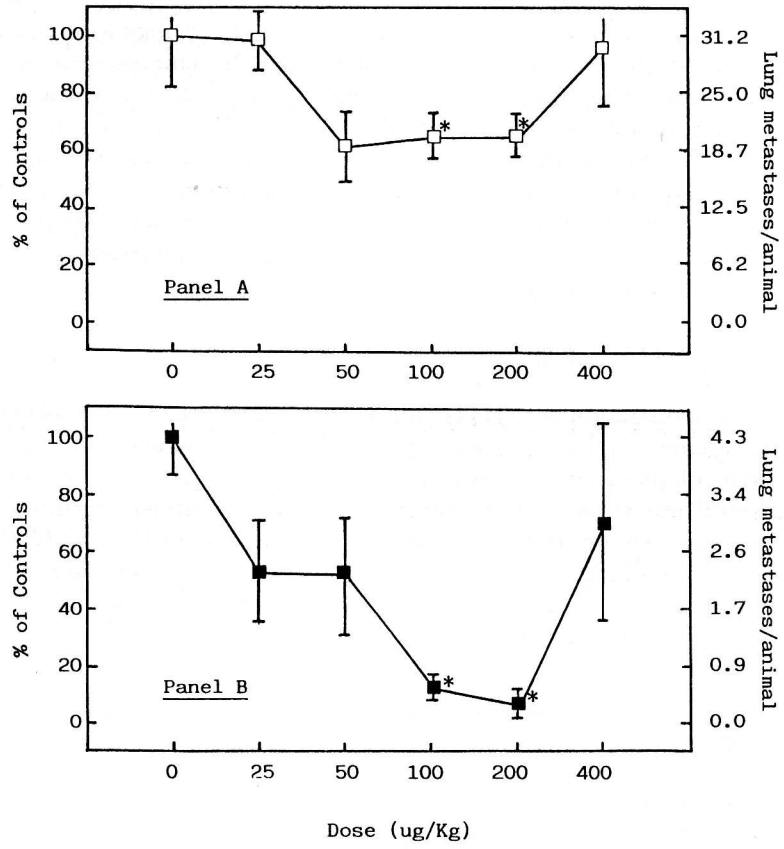

Figure 2. Dose- and inoculum size dependence of the effects of Iloprost on the number of artificial lung metastases in mice bearing Lewis lung carcinoma. Each value is the percentage ratio (treated over controls) ± S.E. of the average number of lung metastases determined in groups of six animals (12 controls) injected i.v. with $2{\cdot}5 \times 10^5$ (panel A) or $2{\cdot}5 \times 10^4$ (panel B) Lewis lung carcinoma cells 1 h after treatment with the indicated dose of Iloprost. (*) Means statistically different from the value obtained in the control group, Kruskall-Wallis test [24], $p = 0{\cdot}05$.

When Iloprost is administered i.v. 1 h before tumor i.v. cell inoculation in the dose range 25–400 mg/kg, the reduction in the number of artificial lung metastases is statistically significant at 100 and 200 μg/kg (figure 2). The magnitude of the observed reduction depends on the size of tumor inoculum, and the effects obtained in animals implanted with $2{\cdot}5 \times 10^4$ cells/mouse (panel B) are markedly more pronounced than those obtained with an inoculum size of $2{\cdot}5 \times 10^5$ cells/mouse (panel A).

The antimetastatic activity of Iloprost on the formation of spontaneous lung metastases was also examined in mice bearing intramuscular tumors. The i.v. administration of Iloprost (200 μg/kg) 1·5 h before surgical ablation of the primary tumor, causes the reduction of the median number of lung metastases to 50 per cent of controls at sacrifice, 9 days after surgery, and increases the percentage of 25 per cent long term survivors caused by surgery alone to about 60 per cent (table 1).

The effects of Iloprost on hemostasis have been determined using a clot retraction inhibition test with ADP or tumor cells as platelet aggregating agents. The use of this assay indicates that the *in vivo* treatment of platelet donor mice with Iloprost 1 h before blood collection significantly inhibits clot retraction following the addition of ADP or tumor cells. When *in vivo* treatment with Iloprost is performed 24 h before blood collection, clot retraction by both aggregating agents is the same as for platelets obtained from mice not treated with drugs (table 2).

Table 1. Effects of the preoperative treatment with Iloprost on spontaneous metastasis formation and on the survival time of mice bearing Lewis lung carcinoma with surgical removal of the primary tumor. Groups of 16 animals were implanted i.m. with 10^6 Lewis lung carcinoma cells, and were treated 12 days later i.v. with 200 μg/kg Iloprost followed 1·5 h later by amputation of the tumor bearing leg. The animals were randomized after surgery in the two groups examined for determination of metastasis number or survival time. † Means statistically different from the controls, Mann-Whitney *U*-test [25], $p = 0{\cdot}05$.

	Lung metastases[a]		survival time	
Treatment	range	median	median[b]	% cures[c]
–	0–16	6	28	25
+	0–5	3†	32†	57

[a] Number of lung metastases per animal determined at sacrifice 9 days after surgery.
[b] Days from tumor inoculation.
[c] Percentage of animals surviving and sacrificed 3 months after surgery, and free of macroscopically detectable lung metastases at necropsy.

Table 2. Inhibition of clot retraction after *in vivo* treatment with Iloprost of platelet donors mice. Groups of normal 15 mice were treated with 200 μg/kg Iloprost at the time indicated before blood collection. Each value is the mean ± S.E. of five separate determinations.

Treatment	Treatment before blood collection (h)	Aggregating agent	Clot retraction inhibition, %
—	—	ADP	35·3 ± 5·2
Iloprost	1	ADP	17·0 ± 5·1*
Iloprost	24	ADP	34·9 ± 11·7
—	—	TC[a]	37·9 ± 4·0
Iloprost	1	TC[a]	6·8 ± 3·7**
Iloprost	24	TC[a]	38·4 ± 3·7

[a] Lewis lung carcinoma viable tumor cells.
†, ‡ Mean statistically different from that of controls, *t*-test for grouped data († $p < 0{\cdot}05$; ‡ $p < 0{\cdot}01$) [26].

Discussion

Numerous papers have been published showing that interventions on the hemostatic system of the host can influence the formation of tumor metastasis [5, 6, 12, 19]. More recently, evidence was provided that the administration of exogenous PGI_2 as a platelet antiaggregating drug may reduce the formation of pulmonary lung nodules after i.v. injection of B16 melanoma cells into syngeneic mice [15]. Considering the shortness of *in vivo* biological effects of PGI_2, the aim of this study was to compare the effects of PGI_2 with those of Iloprost, a prostacyclin analog with a prolonged action *in vivo*. In this study, the activity of PGI_2 and that of Iloprost were examined in another animal tumor system commonly employed in the study of experimental metastasis (Lewis lung carcinoma), and the effects of drug treatment have been determined on spontaneous metastasis in addition to artificial metastasis obtained by i.v. injections of tumor cells.

The results obtained indicate that PGI_2 reduces the formation of artificial metastases of Lewis lung carcinoma, as already observed for B16 melanoma [15]. Under these experimental conditions, the effects of Iloprost are evident and persist when the drug is given 1 to 24 h before i.v. tumor cell inoculation, in contrast with the activity of PGI_2 which is evident for only 15–30 min. The optimum dosages of Iloprost for causing these effects (100–200 μg/kg) are markedly smaller (by a factor of 50–100) than that required for PGI_2 (10 mg/kg). At the same time, the antimetastatic effects of Iloprost appear to depend on the size of the i.v. tumor inoculum, being more pronounced against the smaller tumor burden.

The effects of Iloprost are evident on spontaneous metastases in addition to artificial metastases. The experimental conditions employed, considering the difficulty of repeating multiple i.v. injections, involved a single i.v. administration of Iloprost 1·5 h before surgical removal of the primary tumor. The median number of spontaneous metastases is decreased by Iloprost by 50 per cent, and this reduction may be considered to be of relevance. Indeed, in these conditions only intraoperative tumor dissemination can be reduced by drug treatment, since a certain amount of micrometastases are already formed at treatment, as indicated by 75 per cent of deaths at 3 months in the control group treated with surgery alone. The inhibition of intraoperative tumor dissemination is of relevance, also considering that for animals treated with Iloprost in combination with surgery, the percentage of long term survivors is significantly increased to 60 per cent, as compared to 25 per cent of controls treated with surgery alone.

The effects of Iloprost have also been determined in mice bearing B16 melanoma. On artificial metastases, the results obtained are identical to those reported above for Lewis lung carcinoma. In particular, the treatment with Iloprost followed by surgical removal of primary tumor 2 h later, significantly reduces the median number of spontaneous lung metastases to about 35 per cent, and increases the percentage of long term survivors from 12 per cent of drug untreated controls to 25 per cent (unreported results).

The results presented indicate that Iloprost requires the administration of a dose of 100 μg/kg in order to exert an inhibition in the formation of lung colonies after i.v. injection of Lewis lung carcinoma and B16 melanoma tumor cells; the antimetastatic effects of a single dose of 200 μg/kg Iloprost are evident when the drug is administered from 1 to 24 h before tumor inoculation. On the other hand, clot retraction assayed using platelets obtained from mice treated *in vivo* with Iloprost, and ADP or tumor cells as aggregating agents, indicates that the drug causes an

inhibition significant at 1 h, which is absent 24 h after treatment. These data thus indicate that the antimetastatic effects of Iloprost are dissociated from drug's effects on hemostasis. They appear also of interest considering that they were obtained using *in vivo* treatment with a pharmacologically active dosage of the drug, unlike available reports where drug treatment was performed *in vitro* [2, 20], and the platelets for the *in vitro* assays were usually obtained from species different from mice [23] and from the species of the animals used for tumor experiment [1, 16, 17, 28]. This dissociation between the effects on metastasis and hemostasis is in agreement with results reported for the antimetastatic action of the anticoagulants heparin and warfarin. At dosages highly effective on the hemostatic system of the host, the antimetastatic effects of these drugs have been shown to be exerted only in hosts with an intact functionality of their NK-cellular responses [10]; it is thus possible that Iloprost exerts its antimetastatic action modifying the interaction between tumor cells and the immune system of the host. The possibility that the drug acts inhibiting the adhesion of tumor cells to the endothelial cells of blood vessels in the target organ [21] may be of relevance, although in the present experiments it probably is of marginal importance since drug treatment is effective also when performed 24 h before tumor cells inoculation.

These observations indicate a potential use for Iloprost as an adjuvant for reducing intraoperative tumor cell dissemination. Further investigations on the effects of the preoperative treatment with Iloprost combined with pre- and post-surgical administration of cytotoxic anticancer drugs, also in relation with antitumor host responses, are in progress in animal tumors, in order to better determine the therapeutic usefulness and limitations of these adjuvant treatment combinations.

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