Inhibition of Murine Melanoma Experimental Metastasis by Recombinant Desulfatohirudin, a Highly Specific Thrombin Inhibitor

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ABSTRACT

Recombinant desulfatohirudin (r-hirudin), a highly specific inhibitor of thrombin, was examined to determine whether it would inhibit production of experimental lung metastasis by B16-F10 melanoma cells. In vitro assays using mouse plasma, the high level of procoagulant activity in B16-F10 cells was significantly inhibited by r-hirudin in a dose-dependent manner. From 15 to 120 min after s.c. administration into C57BL/6 mice, r-hirudin (10 mg/kg) markedly prolonged clotting time in a time course pattern that directly correlated with that of blood distribution of 125I-labeled r-hirudin. The production of experimental lung metastasis by B16-F10 cells was significantly inhibited by r-hirudin administered s.c. at time points ranging from 120 min before to 60 min after tumor cell inoculation with the most significant effects found in mice given r-hirudin 15 or 2 min before the i.v. injection of tumor cells. The organ distribution of [35S]IdUrd-labeled tumor cells demonstrated a clear difference in the lungs of mice treated with r-hirudin and the lungs of control mice, and these differences directly correlated with the number of lung tumor colonies found 3 weeks later. The inhibition of lung metastasis was not due to direct antitumor effects of r-hirudin. These results suggest that inhibition of coagulation events by r-hirudin significantly inhibit experimental lung metastasis during a critical time of 60 min after the entry of tumor cells into the circulation.

INTRODUCTION

That the medicinal leech Hirudo medicinalis contains a substance with anticoagulant activity was recognized in 1844. Until the discovery of heparin, this substance was the only means of preventing clotting of mammalian blood (1, 2). In 1957, Markwardt (3) showed that the anticoagulant activity is due to hirudin, a low-molecular-weight protease inhibitor present in the peripharyngeal glands of medicinal leeches. Hirudin, a 65-amino acid polypeptide, is the most potent and selective inhibitor of thrombin (1, 2, 4). This inhibition occurs subsequent to rapid formation of a highly stable and specific noncovalent 1:1 complex with thrombin independent of plasma factors such as antithrombin III (5, 6). With the application of recombinant DNA technology, the gene for hirudin has been synthesized and expressed in yeast with high yield, sufficient to allow large-scale production of recombinant hirudin (7–10). A recombinant desulfatohirudin (CGP 39393) has an amino acid sequence identical to that of the natural hirudin variant 1, but it lacks the sulfate group on Tyr4 (7). This r-hirudin has been shown to have reproducible, potent, and selective inhibitory effects on thrombin—hence its anticoagulant properties (11).

r-Hirudin might have a role in cancer therapy since blood coagulation bears on the success of cancer metastasis. During hematogenous metastasis, tumor emboli must survive transport in the circulation, adhere to blood vessels, usually capillaries and/or postcapillary venules, and invade the vessel wall (12). The vast majority of tumor cells that enter the circulation are eliminated rapidly (13) due largely to blood turbulence (13–15). There are mechanisms to increase the chance of tumor cell survival, such as tumor cell interaction with blood cells and hemostatic components (13). Tumor cells can aggregate with each other (16) or with host cells, such as platelets (17–19) and lymphocytes (20). Formation of such multicellular emboli or the coating of tumor emboli with fibrin network enhances the survival of tumor cells in the circulation.

Fibrin clots frequently occur at sites of tumor cell arrest in the microcirculation (17–19, 21). The increased coagulability observed in patients with cancer (18, 22, 23) may be related to the high levels of thromboplastin found in certain tumors (24–28), to production of high levels of procoagulant-A activity, which can directly activate factor X in the clotting process (29–31), and to the presence of phosphatidylserine in the outer leaflet of tumor cell membranes (32, 33). Since reduced blood flow could increase trapping of circulating tumor cells, and hence their survival, and fibrin deposits can facilitate and strengthen tumor cell lodgement to the microvasculature, the use of anticoagulants, such as warfarin and heparin, in the control of metastasis has been tried and has shown some promise (23, 34–36). Similarly, a reduction in the number of blood platelets (37–39) or the use of drugs such as prostacyclin (40) and forskolin (41) that prevent platelet activation both inhibit the production of experimental metastasis in rodent systems. Collectively, these observations indicate that thrombotic events play an important role in the pathogenesis of metastasis. However, the exact mechanisms by which hemostatic components participate in this process are still unclear (17–19).

Since r-hirudin has shown superior activity as an anticoagulant (9, 10), we wished to determine whether it could also inhibit the production of experimental lung metastasis. We show that r-hirudin injected s.c. can inhibit formation of B16-F10 melanoma nodules in the lungs of syngeneic mice in a time- and dose-dependent manner.

MATERIALS AND METHODS

Reagents. Recombinant desulfatohirudin (CGP 39393; activity 11,600 antithrombin units/mg) was produced by Ciba-Geigy AG (Basel, Switzerland) and purified in collaboration with GEN Therapeutica Vertriebs GmbH and Co. (10). The compound, dissolved in sterile saline for use, was free of endotoxin as determined by the Limulus amebocyte lysate assay (sensitivity limit, 0.125 ng/ml) purchased from Associates of Cape Cod (Woods Hole, MA). MTT was purchased from the Sigma Chemical Company (St. Louis, MO), and a stock solution was prepared by first dissolving 5 mg of MTT in 1 ml of PBS and then filtering the solution to remove particulates. The solution was stored at 4°C in the dark and used within a month.

Animals. Specific pathogen-free inbred female C57BL/6 mice were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility. The mice were used when...
they were 6–10 weeks old. The care and use of the animals were in accordance with institutional guidelines.

Cell Line and Culture Conditions. The B16-F10 cell line selected for high lung colonization potential (42) was maintained as adherent monolayers cultured on tissue culture plastic in EMEM supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, l-glutamine, penicillin and streptomycin, and vitamin solution ( Gibco Laboratories, Grand Island, NY) at 37°C in a humidified incubator with 5% CO₂ and 95% air. The cell line was free of Mycoplasma and the following pathogenic mouse viruses: reovirus type 3; murine pneumonia virus; K virus; Theliers virus; Sendai virus; murine minute virus; murine adenovirus; murine hepatitis virus; lymphocytic choriomeningitis virus; ectromelia virus; and lactate dehydrogenase virus (assayed by Microbiological Associates, Walkersville, MD). To ensure reproducibility of in vivo and in vitro assays, the cultures were used within 4 weeks after recovery from frozen stocks.

Preparation of Tumor Cell Suspensions. For in vivo injection, tumor cells were harvested from subconfluent cultures (50–80% confluency) by overlaying with 0.25% trypsin and 0.02% EDTA. After 1 min, the flasks were sharply tapped to dislodge cell aggregates, which were then gently pipetted into medium containing 10% fetal bovine serum. For i.v. injections, the cells were resuspended in Ca²⁺- and Mg²⁺-free HBSS. To avoid the possibility that trypsin might damage cell surface protein and that a residual EDTA might disturb the recalcification assay for in vitro assays of clotting, tumor cells were harvested by mechanical dissociation with a rubber policeman in the absence of proteases after the monolayers were washed twice with Ca²⁺- and Mg²⁺-free HBSS and the cell concentration was adjusted to 1 x 10⁶ cells/ml. Only single-cell suspensions of >90% viability as determined by trypan blue exclusion were used in the in vivo and in vitro experiments.

Determination of Direct In Vitro Effects of r-Hirudin on Cell Growth. Tumor cells were seeded into flat-bottomed 96-well plates in triplicate at the density of 2 x 10⁴ cells/38-mm² well in 0.1 ml of supplemented EMEM containing 10% fetal bovine serum. r-Hirudin in 0.1 ml of supplemented EMEM was added at final concentrations of 0.001–10 μM/0.2 ml volume/well. After 96 h of incubation, cell proliferation was determined by the MTT assay using 40 μl of MTT stock solution incubated with the cells at 37°C in 5% CO₂ for 2–4 h. The cells were then washed and lysed in dimethyl sulfoxide, and the conversion by metabolically viable cells of MTT to formazan was monitored by a Metastasis Inhibition by r-Hirudin

Determination of Radioactivity in Blood of Mice after s.c. Administration of ¹²⁵I-labeled r-Hirudin. To study the in vivo distribution and fate of r-hirudin, we radiolabeled the compound using a modification of the chloramine T method (44). For the in vivo experiments, we used the r-hirudin fraction with 34% labeling efficiency and a specific radioactivity of 68 μCi/μg. ¹²⁵I-labeled r-hirudin admixed with unlabeled r-hirudin (4.5 x 10⁶ cpm/mouse; 200 μg r-hirudin/mouse) in 0.2 ml PBS was injected i.c. into the flanks of C57BL/6 mice. Blood samples (0.2 ml/mouse) were collected by cardiac puncture from groups of mice (n = 3) at the same time points used in the ex vivo clotting assays. The samples were divided into 2 aliquots and placed in glass test tubes, and radioactivity was determined in a gamma counter. The mean count was calculated for total blood volume (2.0 ml/20 g/mouse) and expressed as the percentage of input radioactivity.

Production of Experimental Lung Metastasis. B16-F10 cells at a concentration of 25,000 cells/0.2 ml HBSS/mouse were injected into the lateral tail vein of unanesthetized C57BL/6 mice. The mice were monitored daily and killed 3 weeks later by cervical dislocation. The animals were necropsied, and the lungs were removed, washed in water, and fixed in Bouin’s solution. The number of lung nodules was determined under a dissecting microscope. The liver, kidney, spleen, lymph nodes, and pancreas were also examined for presence of melanoma.

To study the extent and the duration of effect of r-hirudin on experimental metastasis, r-hirudin solution was injected s.c. into unanesthetized mice at the dose of 200 μg/0.2 ml PBS/mouse (10 mg/kg mouse weight) at various times before or after i.v. injection of B16-F10 cells. In other experiments, different doses of r-hirudin, ranging from 3 to 400 μg/mouse (0.15–20 mg/kg mouse weight) were administered s.c. 15 min before the i.v. injection of 25,000 B16-F10 cells/mouse.

To rule out the possibility that r-hirudin mediates a direct antitumor effect, B16-F10 cells were incubated at room temperature with r-hirudin in PBS at concentrations of 0.1 to 10 μM for 30 min. The cells were then washed twice with PBS, resuspended in Ca²⁺- and Mg²⁺-free HBSS, and injected i.v. into mice (n = 5), which were killed 3 weeks later. The number of experimental lung metastases was determined under a dissecting microscope.

In Vitro Labeling of Cells with ¹³¹I-lododeoxyuridine. Tumor cells were seeded into 150-cm² tissue culture flasks at a density of 4 x 10⁶ cells/flask. Twenty-four h later, 0.3 μCi/ml of ¹³¹I-dUrd (specific activity, 2000 Ci/mmol; New England Nuclear, Boston, MA) was added to the supplemented EMEM. Twenty-four h later, the monolayers were rinsed with excess PBS and ¹³¹I-free HBSS to remove non-bound radioiodine. The cells were then harvested with 0.25% trypsin-0.02% EDTA solution as described above and suspended in medium. The cell suspension was washed and resuspended in Ca²⁺- and Mg²⁺-free HBSS at a concentration of 1 x 10⁶ cells/0.2 ml HBSS, the inoculum volume per mouse.

Distribution and Fate of ¹³¹I-dUrd-labeled Cells after i.V. Injection. Labeled B16-F10 cells (1 x 10³/mouse; 8.6 x 10⁵ cpm/mouse) were injected i.v. into unanesthetized C57BL/6 mice. To study the effect of r-hirudin on the initial organ distribution of the tumor cells, we injected PBS (group A) or r-hirudin in PBS (20 μg/mouse; 10 mg/kg mouse weight) s.c. into C57BL/6 mice 15 min before (group B) or 60 min after (group C) the i.v. inoculation of tumor cells. At various intervals after i.v. injection, groups of mice (n = 5) were killed. The lung, liver, kidneys, and spleen of each mouse were removed and placed in test tubes containing 70% ethanol. The ethanol was replaced daily for 3 days to remove all soluble ¹³¹I released from dead cells (14). In addition,
0.2 ml of blood from each mouse was collected and placed in a test tube. The radioactivity in all samples was determined in a gamma counter (TM Analytic, Elk Grove Village, IL). Triplicate tubes containing the inoculum dose were retained, and the radioactivity was determined at the same time as the sample organs.

In another set of experiments, the organs collected from each mouse were washed once in PBS and placed in test tubes, and the radioactivity was measured immediately rather than after 3 days of ethanol wash. The mean count in organs from each group of mice (n = 5) at each time point was expressed as the percentage of input counts. All measurements were corrected for radioactive decay of input cells.

Statistical Analysis. The in vivo data were analyzed for significance by the Mann-Whitney test.

RESULTS

Procoagulant Activity of B16-F10 Cells. In mouse platelet-free plasma, B16-F10 cells significantly shortened the single-stage recalcification time in a dose-dependent manner. However, in human platelet-free plasma, the procoagulant activity of B16-F10 cells was low (Fig. 1A). In contrast, tissue thromboplastin used as a positive control for production of thrombin exhibited a significant shortening of the clotting time in both mouse and human plasma (Fig. 1B). These results suggest that the B16-F10 melanoma cells possess a high procoagulant activity.

Effect of r-Hirudin on Blood Coagulation in Vitro. The clotting of mouse plasma induced either by B16-F10 cells or by tissue thromboplastin was significantly inhibited by r-hirudin. The data shown in Fig. 2 demonstrate prolongation of recalcification time by r-hirudin in a dose-dependent manner. At the higher concentrations of r-hirudin (exceeding 0.5 μM), a fibrin clot was not observed in tubes even by 1 h. r-Hirudin also inhibited the clotting of human plasma induced by B16-F10 cells or by tissue thromboplastin (data not shown).

Ex Vivo Clotting Assays after s.c. Administration of r-Hirudin. We determined the effect of r-hirudin (10 mg/kg) on mouse plasma recalcification time at various times after s.c. administration. The data, expressed as a ratio of clotting time of samples to control plasma, are shown in Fig. 3. Within 15 min after s.c. injection of r-hirudin, a significant prolongation of clotting time was observed, and this persisted at a high level for 1 h. By 2 h after s.c. injection, the prolongation of clotting time began to decrease, and by 4 h it returned to control level. Subsequent to s.c. administration of r-hirudin, the plasma clotting time induced by B16-F10 cells or by tissue thromboplastin exhibited a similar time course pattern. The extent of prolongation, i.e., the ratio of clotting time of samples to control, however, was greater in the assays using B16-F10 cells than in those using tissue thromboplastin.

Blood Distribution of 125I-labeled r-Hirudin after s.c. Administration. By 15 min after s.c. administration of 125I-labeled r-hirudin, 8.4% of input radioactivity was detected in total blood (Fig. 4). A peak of radioactivity (9.5% of input) was detected in the blood 45 min after s.c. injection, and this persisted for 90 min. Thereafter, the radioactivity in the blood decreased, and by 8 h it was less than 1% of input. These results are consistent with those obtained by ex vivo clotting assays described above (Fig. 3). Since the highest level of 125I-labeled r-hirudin in the blood was 8–9% of the injected dose (200 μg/mouse), we calculate that from 15 to 90 min after s.c. injection, r-hirudin blood concentration was 1.1–1.3 μM, a level sufficient to inhibit clot formation according to the in vitro studies. The elimination half-life of r-hirudin in rats is about 60 min (2, 5, 9), which is similar to the present results.

Effect of r-Hirudin on Formation of Experimental B16-F10 Lung Metastasis. The production of experimental lung metastasis by B16-F10 cells was significantly inhibited by the s.c. injection of r-hirudin, but the extent of this inhibition was dependent on the time of administration (Table 1). Significant inhibition of B16-F10 experimental lung metastasis was obtained with s.c. administration of r-hirudin at time points ranging from 120 min before to 60 min after tumor cell inocu-
Mетастазирование B16-F10 меланомы подавляется r-хирудином в дозозависимой степени.

**Fig. 4. Blood distribution of 125I-labeled r-hirudin after s.c. administration.** 125I-labeled r-hirudin admixed with unlabeled r-hirudin (4.5 x 10^3 cpm/mouse; 200 μg r-hirudin/mouse) in 0.2 ml was injected s.c. Blood samples were collected from groups of mice (n = 3). Radioactivity was determined in a gamma counter. Points, mean; bars, SD.

**Table 1 Inhibitory effects of r-hirudin on experimental lung metastasis produced by B16-F10 melanoma cells**

<table>
<thead>
<tr>
<th>Time of r-hirudin s.c. administration (min)</th>
<th>Number of lung nodules median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>-240</td>
<td>ND</td>
</tr>
<tr>
<td>-120</td>
<td>28 (14-39)</td>
</tr>
<tr>
<td>-60</td>
<td>12 (2-24)</td>
</tr>
<tr>
<td>-30</td>
<td>9 (3-49)</td>
</tr>
<tr>
<td>-15</td>
<td>5 (1-99)</td>
</tr>
<tr>
<td>-2</td>
<td>4 (2-94)</td>
</tr>
<tr>
<td>+15</td>
<td>12 (6-24)</td>
</tr>
<tr>
<td>+30</td>
<td>24 (1-47)</td>
</tr>
<tr>
<td>+60</td>
<td>51 (27-62)</td>
</tr>
<tr>
<td>+120</td>
<td>80 (24-106)</td>
</tr>
<tr>
<td>+240</td>
<td>ND</td>
</tr>
</tbody>
</table>

**PBS control** 83 (58-133) 49 (32-67)

- ND, not done.
- * P < 0.01.
- ** P < 0.001.

The most striking effects found in mice given r-hirudin 15 min or 2 min before i.v. injection of tumor cells (P < 0.001). In another set of experiments, r-hirudin injected s.c. at times ranging from 240 min before or 60, 120, and 240 min after i.v. tumor cell injection did not inhibit experimental metastasis (Table 1). Extrapanmonary tumor colonies were not found in any of the mice treated with PBS or with r-hirudin.

To investigate the dose relationship of r-hirudin to inhibition of experimental B16-F10 melanoma lung metastasis, we administered different doses of r-hirudin s.c. 15 min before the i.v. injection of tumor cells. The data in Table 2 show that r-hirudin inhibited experimental metastasis in a dose-dependent manner and that the dose of 10 mg/kg mouse weight used in most of our experiments achieved near-maximal effects.

**Table 2 Dose-dependent inhibitory activity of r-hirudin on formation of experimental B16-F10 lung metastasis**

<table>
<thead>
<tr>
<th>Dose of r-hirudin (mg/kg mouse weight)</th>
<th>Number of lung nodules median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>2 (1-39)</td>
</tr>
<tr>
<td>10</td>
<td>5 (1-69)</td>
</tr>
<tr>
<td>5</td>
<td>11 (1-169)</td>
</tr>
<tr>
<td>2.5</td>
<td>10 (6-199)</td>
</tr>
<tr>
<td>1.25</td>
<td>15 (7-209)</td>
</tr>
<tr>
<td>0.63</td>
<td>24 (14-32)</td>
</tr>
<tr>
<td>0.31</td>
<td>26 (18-42)</td>
</tr>
<tr>
<td>0.16</td>
<td>28 (20-40)</td>
</tr>
<tr>
<td>PBS control</td>
<td>49 (32-67)</td>
</tr>
</tbody>
</table>

- r-Hirudin was administered s.c. 15 min before tumor cell inoculation.
- B16-F10 cells (25,000/0.2 ml HBSS) were injected i.v. into syngeneic mice. The number of experimental lung metastases was determined 3 weeks later. Each treatment group consisted of 10 mice.
- ** P < 0.001.
- * P < 0.01.

In the second experiment, single-cell suspensions of B16-F10 cells were incubated *in vitro* in PBS with or without different concentrations of r-hirudin. The cells were then washed in PBS and injected i.v. into syngeneic mice. No discernible differences in the incidence of lung tumor colonies were found among the groups (data not shown), ruling out the possibility that r-hirudin directly mediated an antimetastatic effect.

**Distribution and Fate of [125I]IdUrd-labeled B16-F10 Cells after i.v. Injection.** The organ distribution and fate of [125I] IdUrd-labeled tumor cells were determined from 10 min to 24 h after i.v. injection into 3 treatment groups of mice: mice treated s.c. with PBS (group A); mice treated s.c. with r-hirudin 15 min before tumor cell injection (group B); and mice treated s.c. with r-hirudin 60 min after tumor cell injection (group C).

The most impressive changes in the distribution of labeled cells were observed in the lungs (Table 3, Fig. 5). A significant decrease in initial tumor cell arrest in the lung was found in mice treated with r-hirudin 15 min before tumor cell injection (group B). In control mice, we found 79.6% of the cells in the lungs, whereas in treated mice we found 10.7% of the cells in the lungs (P < 0.01). These results were obtained subsequent to 3 daily washes with 70% ethanol (14). When organs were washed only once in PBS and then immediately monitored for radioactivity, the difference was not found. Under these conditions, cell arrest in the lungs of mice from groups A and B was 92.8% and 85.8%, respectively. The exact reason for this discrepancy is unclear. It could be due to alcohol displacement of unarrested tumor cells in the lungs of r-hirudin-treated mice.

No significant differences in cell survival (radioactivity) in the lungs of 2 groups were found at 60 min; however, by 120 min, a clear difference emerged. By 24 h, 1.6% of injected B16-F10 cells survived in the lungs of PBS-treated mice, whereas in mice treated with r-hirudin 15 min before or 60 min after tumor cell injection, the survival was 0.06% and 0.46%, respectively (Fig. 5). The differences in the survival of [125I] IdUrd-labeled B16-F10 cells in the lungs of mice directly correlated with the number of tumor colonies found 3 weeks later (Tables 1 and 2).

The distribution of radiolabeled tumor cells in the liver differed between mice in groups A and B. As shown in Table 3, there was no difference in the radioactivity at 10 and 60 min. By 120 min, however, a higher percentage of cells was found in the liver of mice treated with r-hirudin as compared to controls. This difference in proportion may reflect the absolute decrease in viable cells arrested in the lung or present in the blood. In r-
Studies with different tumor systems have identified at least 3 different types of tumor cell-derived procoagulants: (a) a tissue factor-like substance activating Factor VII (24–28); (b) a protease that activates Factor X to Xa (29–31); and (c) a phosphatidylerosine exposed on the outer leaflet of a tumor cell’s membrane (33) that is essential to initiating the conversion of prothrombin to thrombin (53). At least 3 mechanisms by which the cells can induce platelet aggregation have also been described. These are (a) tumor cell membrane-associated glycoprotein (54–58); (b) ADP released by tumor cells (59, 60); and (c) thrombin generated by the procoagulant activity of tumor cells (24–26, 59, 60).

Since tumor cells can directly induce coagulation or induce the aggregation of platelets, the use of anticoagulants and antiplatelet agents has been advocated to inhibit or prevent hematogenous metastasis. Tumor cell procoagulants accelerate the coagulation cascade and induce hemostatic abnormalities, as well as being involved in one of the three mechanisms of platelet aggregating activity. Therefore, antithrombin agents should affect both coagulation and platelet aggregation in certain tumor systems (24, 25, 31).

Among the anticoagulants used, warfarin and heparin have shown promising results (19, 23, 34–36, 61–68). Warfarin interferes with the vitamin K-dependent, posttranslational carboxylation of glutamic acid residues (69), a modification that is essential for the biological function of many proteins other than plasma clotting factors (70). Which of these proteins accounts for warfarin’s inhibition of experimental metastasis is unclear (36, 68). Similar uncertainties are associated with the use of heparin. To exert its anticoagulant activity, heparin requires interaction with antithrombin III (71). Heparin is pleiotropic; in addition to its well-characterized anticoagulant activities, it affects lipoprotein metabolism (72), and for some cells it can inhibit proliferation (73).

To obviate the problems of interpreting data on the antimetastatic effects of warfarin and heparin, we instead chose to use a highly specific thrombin inhibitor, r-hirudin, to examine the association between blood coagulation and experimental metastasis produced by the murine B16-F10 melanoma cells. r-Hirudin forms a stable and specific noncovalent 1:1 complex with thrombin independent of other plasma factors (1, 2, 4). As such, r-hirudin is the most potent and specific inhibitor of thrombin found to date.

B16-F10 cells have been previously shown to have a procoagulant activity through direct activation of Factor X (31). In our study, B16-F10 cells exhibited a high level of procoagulant activity in mouse plasma but a low procoagulant activity in human plasma. r-Hirudin significantly inhibited clotting (in

**Table 3** Distribution and fate of $^{[125]}$IdUrd-labeled B16-F10 cells after i.v. injection

<table>
<thead>
<tr>
<th>% of input radioactivitya</th>
<th>Lungs</th>
<th>Liver</th>
<th>Spleen</th>
<th>Kidneys</th>
<th>Bloodb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Timec</td>
<td>PBS</td>
<td>H (−15)d</td>
<td>H (+60)e</td>
<td>PBS</td>
<td>H (−15)</td>
</tr>
<tr>
<td>10 min</td>
<td>79.6</td>
<td>10.7</td>
<td>100</td>
<td>0.06</td>
<td>0.10</td>
</tr>
<tr>
<td>60 min</td>
<td>58.5</td>
<td>67.0</td>
<td>100</td>
<td>0.16</td>
<td>0.06</td>
</tr>
<tr>
<td>120 min</td>
<td>50.2</td>
<td>32.7</td>
<td>58.4</td>
<td>1.0</td>
<td>3.4</td>
</tr>
<tr>
<td>240 min</td>
<td>26.6</td>
<td>43.2</td>
<td>93.9</td>
<td>0.7</td>
<td>0.3</td>
</tr>
</tbody>
</table>

*a* $^{[125]}$IdUrd-labeled viable tumor cells were injected i.v. (1 × 10⁶/mouse), and groups of 5 mice were killed at each time point.

*a* Percentage of radioactivity (mean of 5 mice) was determined after 3 daily washes of the organ samples with 70% ethanol. SD < 10% in all samples.

*b* For blood samples, radioactivity in 0.2 ml of blood/mouse was measured and expressed as a percentage of input in total blood volume, which was estimated to be 2.0 ml.

*b* r-Hirudin was administered s.c. at a dose of 10 mg/kg mouse weight (200 µg/mouse) 15 min before [H (−15)] or 60 min after [H (+60)] tumor cell inoculation.

*b* Numbers in parentheses, results obtained directly without 3 day washing with ethanol at 10 min.

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**Fig. 5.** Distribution and fate of $^{[125]}$IdUrd-labeled B16-F10 cells after i.v. injection. Mice received injections of r-hirudin (10 mg/kg) or PBS 15 min before or 60 min after the i.v. injection of radiolabeled B16-F10 cells (1 × 10⁵ cells/mouse). Mice (n = 5) were killed at the indicated time points, and the lungs were processed for monitoring of radioactivity. Points, mean percentage of cells arrested. Variation from the mean did not exceed 10%. *direct measurement of radioactivity without 3 daily washes with 70% ethanol.

For blood samples, radioactivity in 0.2 ml of blood/mouse was measured and expressed as a percentage of input in total blood volume, which was estimated to be 2.0 ml.
METASTASIS INHIBITION BY r-HIRUDIN

vitro assay) induced by either B16-F10 cells or thromboplastin in both mouse and human plasma, indicating that the potent thrombin-inhibiting activity of r-hirudin was not species specific.

The effects of r-hirudin on mouse plasma recalciﬁcation time and the blood distribution of 125I-labeled r-hirudin at various times after s.c. administration into mice were well correlated. Within 15 min after s.c. injection, 8.4% of injected 125I-labeled r-hirudin was detected in the blood, and this produced a signiﬁcant prolongation of clotting time. By 45 min after s.c. administration, the peak of anticoagulant activity correlated with the peak of r-hirudin in the blood, and high anticoagulant activity persisted for a total of 90 min. By 120 min after s.c. injection, the prolongation of clotting time began to diminish, as did the level of r-hirudin in the blood. Collectively, the data indicate that subsequent to a s.c. injection, r-hirudin can produce anticoagulant effects that last from 15 to 120 min. These findings are consistent with the reported pharmacokinetics of r-hirudin in rats, dogs, and humans (2, 5, 6, 11).

Based on these ex vivo results, we examined the ability of r-hirudin injected s.c. to inhibit or prevent hematogenous experimental lung metastasis produced by B16-F10 melanoma cells. The production of experimental metastases was signiﬁcantly inhibited by r-hirudin injected s.c., in a dose- and time-dependent manner. Significant inhibition of B16-F10 experimental lung metastasis was achieved when r-hirudin was injected s.c. at times ranging from 120 min before to 60 min after i.v. tumor cell injection. The most dramatic inhibitory effects on formation of lung tumor colonies were found in mice receiving s.c. injections of r-hirudin 15 or 2 min before the i.v. injection of tumor cells. Taken together, the kinetic ex vivo studies of r-hirudin and the in vivo inhibition of experimental lung metastasis by B16-F10 cells indicate that the critical period when blood coagulation can inﬂuence blood-borne metastasis by B16-F10 cells lasts about 60 min after the entry of tumor cells into the circulation.

Many studies with different tumor systems using different anticoagulants have implicated the coagulation events in hematogenous metastasis (19, 22, 23, 35, 36, 66). Nevertheless, detailed kinetic studies of the critical time when anticoagulation can inhibit experimental metastasis have been lacking. Fibrin or ﬁbrin-like material and platelets can be easily detected within the ﬁrst 15 min after tumor cell injection into the venous circulation; however, by 6 h, when most tumor cells were still present in the intravascular space, they disappeared (36). Moreover, while the pretreatment of mice with various anticoagulants reduced the number of experimental lung metastases, anticoagulants, with the exception of a coumarin derivative, administered 24 h after tumor cell injection did not (36). These ﬁndings suggested that microthrombosis may be a pathogenic factor only during the very early stage of tumor cell lodgement (36). Our results in the B16-F10 melanoma system are consistent with this conclusion and demonstrate more precisely the very short critical period in the early phase of experimental metastasis during which r-hirudin can inhibit the formation of experimental lung metastases. Further conﬁrmation of the critical timing necessary for r-hirudin activity came from the data on the distribution and survival of 125IIdUrd-labeled tumor cells in the lungs of mice treated s.c. with PBS, with r-hirudin 15 min before tumor cell injection, or with r-hirudin 60 min after tumor cell injection. The results correlated well with initial cell arrest and, thus, the eventual number of visible melanoma lung colonies found 3 weeks later. Thus, at least in the B16-F10 system, the coagulation events which were speciﬁcally inhibited by r-hirudin played an important role in the production of experimental lung metastases.

An ultrastructural study of the initial arrest and extravasation of B16 melanoma tumor cells in the lungs showed that by 10 min after injection, tumor cells were frequently associated with platelets and ﬁbrin in the pulmonary vasculature (74). The tumor cell-associated thrombi started to decrease by 4 h and disappeared after 24 h. By 4 h after injection, tumor cells were associated with the vascular basal lamina, and by 24–72 h, many cells were attached to the subendothelial matrix (74). The contact between the tumor and endothelial cell plasma membrane was direct (74) and preceded thrombus formation (75). These observations suggest that tumor cell-associated thrombus may protect tumor cells against mechanical forces until a more secure attachment to subendothelial matrix develops (76). This stabilization of tumor cell-endothelium contact may be an important step in the development of metastasis, and r-hirudin presumably can inhibit this stabilization.

The initial attachment of tumor cells to vascular endothelial cells may be regulated by speciﬁc interactions that include glycoproteins (77, 78) and tumor cell integrin receptors (79–82), notably IIb/IIIa (αIIbβ3) integrin that is found on the surface of metastatic melanoma cells but not on benign melanocytes (83). This glycoprotein is a known platelet surface molecule that serves as a receptor for ﬁbrinogen in platelet aggregation and can regulate platelet-melanoma interaction (84) and melanoma growth in vivo (85). Whether r-hirudin affects this glycoprotein is unknown.

The possibility that the antimitastatic effect of r-hirudin was due to direct antitumor effects was ruled out by two different studies. First, r-hirudin did not inhibit the in vitro growth of B16-F10 cells. Second, in vivo treatment of tumor cells with r-hirudin did not reduce their capacity to produce lung tumor colonies. The possibility that the antimitastatic effects of r-hirudin were due to activation of host immunity was ruled out by its short effective period.

While our results clearly demonstrate that r-hirudin can signiﬁcantly inhibit hematogenous metastasis, we do not know how this anticoagulant may interact with melanomas growing s.c. or what the relationship of this biological molecule is to the metastatic cascade as a whole. Nevertheless, at least in the B16-F10 melanoma system, this speciﬁc and potent thrombin inhibitor signiﬁcantly inhibits blood-borne metastasis.

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METASTASIS INHIBITION BY r-HIRUDIN


Inhibition of Murine Melanoma Experimental Metastasis by Recombinant Desulfatohirudin, a Highly Specific Thrombin Inhibitor

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