

Analysis of Aprotinin-induced Enhancement of Metastasis of Lewis Lung Tumors in Mice¹

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ABSTRACT

The antiprotease, aprotinin, has been reported by some workers to inhibit the growth and development of a number of different types of primary cancers in animals; however, its effects on metastasis particularly need clarification. As proteolytic enzymes are thought to be involved in some steps of metastasis, we have investigated the effects of aprotinin on the spontaneous metastasis of Lewis lung (LL) tumors in mice together with its effects on the detachment of cells from primary LL cancers, the development of lung tumors from i.v. injections of LL cells, LL cell adhesion *in vitro*, and LL cell retention in the lungs. The results suggest that the metastasis-enhancing effect of aprotinin is due partly to promotion of the retention of circulating cancer cells at the vascular endothelium. As these effects could well occur with cancers in general, we conclude that antiproteases may do more harm than good if used in cancer therapy.

INTRODUCTION

Metastasis is an extremely complex process composed of a series of sequential steps (16), some of which appear to involve proteolytic enzymes (11). A number of attempts have therefore been made to interfere with the growth and spread of cancer with antiproteases; however, considering the complexity of the situation, it is hardly surprising that the reports have been contradictory.

The object of the present study was to investigate the effects of one antiprotease, aprotinin (Trasylol), on different facets of metastasis of LL⁴ tumors in mice. In addition, as necrotic material in tumors is a source of a number of proteolytic enzymes and has been shown to affect some metastasis-related activities of cancer cells and their environments (13, 18, 19), we have also studied some of the effects of interactions between necrotic material and aprotinin.

MATERIALS AND METHODS

Tumor System and Evaluation of Metastasis Formation. LL tumors were grown in adult male C57BL/6J mice by s.c. implantation of 0.05 ml of tumor mince in the dorsolumbar region. After 21 days of growth, every animal had a large tumor at the site of implantation and extensive lung metastases.

Metastasis formation was assessed by counting and sizing the secondary deposits in formalin-fixed lungs, under a stereoscopic microscope.

Aprotinin Preparations. Aprotinin was received either dissolved in 0.9% NaCl solution containing 0.9% benzyl alcohol at a concentration of 10,000 KIU/ml (Trasylol; FBA Pharmaceuticals, New York, N. Y.) or as a lyophilized powder containing 5880 KIU/mg protein (Bayer Pharmaceuticals Ltd., London, U. K.). The former preparation was used for repeated i.p. injections as summarized in Table 1, and the lyophilized powder was used in all other experiments.

Preparation of Necrotic Extract. A necrotic extract in PBS at pH = 7.4 was prepared from the pooled necrotic portions of 10 s.c. LL tumors as described previously (17). The protein content of the necrotic extract was 19.5 mg/ml as determined by the method of Lowry *et al.* (10). The extract was stored in aliquots at -20° until required.

Measurement of Cell Detachment from LL Tumor Tissue. Cylinders of tumor tissue were punched from pieces of viable tumor using a 13-gauge trocar and cannula and divided into 1-mm lengths over a scale using a stereoscopic microscope. Four cylinders were placed into each of a number of screw-capped glass vials (4.5 × 1.3 cm) containing 2 ml of the solution under investigation. The specimens were maintained at 4° during these procedures.

The vials were clamped on a reciprocating shaker and shaken at 275 oscillations/min with an excursion of 4.5 cm for 40 min at 25°. Ten% buffered formaldehyde (0.25 ml) was next added to each vial, any macroscopic remains of the tissue cylinders were removed with forceps, and the number of single cells and cell clusters in the suspension was determined using a Fuchs-Rosenthal counting chamber (17).

Preparation of Single-Cell Suspensions from LL Tumors. One to two-g aliquots of nonnecrotic, coarsely chopped tissue were stirred in 5 ml BME, pH 7.4, containing collagenase (0.2 mg/ml) (Worthington Biochemical Corp., Freehold, N. J.) for 30 min at 37° under an atmosphere of 5% CO₂ in air. Unaggregated pieces were allowed to settle, the supernatant cell suspension was discarded, and the remaining pieces were treated again with fresh enzyme solution (7). The cell suspension from this second treatment was retained and washed 3 times with 10 ml BME containing 1% FCS. Cells were resuspended in BME plus 10% FCS at a concentration of 0.5 to 1.0 × 10⁷/ml, passed through 2 layers of a fine metal mesh (150 openings/inch; Buffalo Wire Works Co., Buffalo, N. Y.), and incubated for 1 hr at 37° under an atmosphere of 5% CO₂ in air. This incubation was repeated using fresh medium, and the cell suspension was again washed 3 times with 10 ml BME plus 1% FCS. Cells were finally resuspended in the required medium and filtered again, yielding single-cell suspensions which, by careful selection of the initial starting material, were routinely 90 to 95% viable by trypan blue exclusion.

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⁴ The abbreviations used are: LL, Lewis lung; KIU, kallikrein inactivation units; PBS, Dulbecco's phosphate-buffered saline; BME, Eagle's basal medium; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution.

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Table 1

Effect of aprotinin treatment on LL tumor-bearing mice on the number and size of lung metastases
 Values were obtained by pooling data from 3 separate experiments. Details of treatment schedules are given in the text.

Group	No. of lung metastases ^a	Size distribution of lung metastases ^b (% of total)				
		0-1 mm	1-2 mm	2-3 mm	3-4 mm	>4 mm
Control (29) ^c	43.9 ± 3.4 ^d	60.5	32.7	5.1	1.4	0.4
Treated (29)	69.2 ± 4.4	50.1	32.2	12.3	3.2	1.4

^a Control versus treated, Student's *t* test (2 tailed), *p* < 0.001.

^b Control versus treated, χ^2 test, *p* < 0.001.

^c Numbers in parentheses, total number of mice used.

^d Mean ± S.E.

Labeling LL Cell Suspensions and the Measurement of Organ Retention of Labeled Cells. LL cells were labeled with [¹²⁵I]iododeoxyuridine (Amersham/Searle Corp., Arlington Heights, Ill.) during the preparation of single-cell suspension. One μ Ci of the radiolabel was added to the medium at the start of the second hr of incubation in BME plus 10% FCS, and incubation continued for 18 hr. After washing, cells were re-suspended in BME plus 1% syngeneic mouse serum, and 10⁶ viable cells in 0.1 ml medium were injected i.v. into the tail veins of adult male C57BL/6J mice.

At specified times after the injection of the labeled cells, groups of mice were killed by cervical dislocation, and various organs were removed and placed in 2 ml 70% ethanol, which was replaced twice over 48 hr in order to remove radioactivity not associated with intact cells. The amount of radioactivity associated with each organ was determined by 10-min γ counts against a background of 250 counts per 10 min and expressed as a percentage of the original injected dose, which varied from 141.3 \times 10³ to 304 \times 10³ counts per 10 min in the different experiments.

Measurement of Cell Adhesion. Cell adhesion was measured in microtest plates (Falcon Plastics) using a modification of the technique described by Weiss (15). Aliquots (~7 μ l) of a single-cell suspension of LL cells, freshly isolated as described above, were dispensed into each of 5 or 6 replicate wells at a concentration of 0.25 \times 10⁵/ml in BME plus 10% FCS and were incubated for 30 min at 37° under a humidified atmosphere of 5% CO₂ in air and for a further 60 min after inversion of the test plate. The cells adhering to the bases of the wells were then counted. The test plate was then returned to its normal orientation, and after a further 30-min incubation, a second cell count was made. Adhesion was expressed by giving the first count as a percentage of the second count.

RESULTS

Aprotinin and Metastasis. Fragments of LL tumor were implanted s.c.; on 14 consecutive days after implantation, the mice received i.p. injections of 5000 KIU aprotinin in 0.5 ml 0.9% NaCl solution containing 0.9% benzyl alcohol. A control group of animals received the diluent only. Twenty-one days after tumor implantation, all mice were killed and assessed for lung metastases. The results summarized in Table 1 show that aprotinin treatment resulted in both a highly significant (*p* < 0.001) 58% increase in the mean numbers of lung metastases over the controls and a significant increase (*p* < 0.001) in their size distribution.

Aprotinin and the Effects of Necrotic Extract on Cell De-

tachment. Single cells and cell clusters were detached by shaking from pieces of LL tumors suspended in PBS, aprotinin in PBS, necrotic extract (1/10 dilution) in PBS, or necrotic extract (1/10 dilution) plus 125, 250, or 500 KIU aprotinin per ml PBS. As shown in Table 2, at concentrations of 125 KIU/ml, aprotinin produced no statistically significant changes in cell detachment; however, in the presence of 250 and particularly 500 KIU/ml, statistically significant increases in single-cell detachment were observed compared with controls. Exposure to the necrotic extract resulted in large increases in both the number of single cells and clumps released compared to the PBS controls, but the presence of aprotinin did not inhibit this process, and at a concentration of 500 KIU/ml, the drug potentiated the release of single cells.

Aprotinin and Tumor Transplants in the Lungs. Five min after receiving a single tail vein injection of 1 \times 10⁸ LL cells, treated mice received 5000 KIU aprotinin in 0.1 ml HBSS i.v., and control mice received 0.1 ml HBSS only. The mice were killed 17 days afterwards, and, as shown in Table 3, aprotinin treatment resulted in a 40% increase in the mean number of lung metastases, which was on the borderline of statistical significance (*p* < 0.05, 1-tailed test; *p* > 0.05, 2-tailed test), and an increase in the size distribution of the individual metastases; the number of metastatic foci with diameters greater than 2 mm increased by 70% (*p* < 0.001).

Aprotinin and Cell Adhesion. The effects of aprotinin on the *in vitro* adhesion of LL cells were studied in 4 different concentrations, ranging from 62.5 to 500 KIU/ml with appropriate controls. The results summarized in Table 4 show that the number of adherent LL cells progressively increased with increasing concentrations of aprotinin in the range 62.5 to 250 KIU/ml, and for all of these concentrations, the increases were significantly different from their controls. However, at the highest concentration of aprotinin (500 KIU/ml) investigated, no significant effect on adhesion was observed.

Aprotinin and Organ Localization of LL Cells. The effects of aprotinin "posttreatment" on organ localization of labeled LL cells 6 hr after injection were studied in mice bearing LL tumors and in non-tumor-bearing animals. In the "posttreatment" series, mice received a single i.v. injection of aprotinin (5000 KIU contained in 0.1 ml HBSS) 5 min after the injection of labeled cells, and γ counts were made on the various organs after 2 and 6 hr. The tumor-bearing mice all possessed palpable s.c. growth initiated 10 days previously. As shown in Table 5, lung and liver retention of LL cells in both tumor-bearing and non-tumor-bearing animals was significantly (0.005 > *p* > 0.001) increased by aprotinin treatment. Because of the low counts in relation to background, no effect of aprotinin on the

Table 2
Effects of aprotinin on the detachment of single cells and cell clusters from pieces of LL tumor in the presence and absence of necrotic extract
 Each value represents the mean of 5 determinations.

Aprotinin concentration (KIU/ml)	No. of single cells or clusters released/g tumor × 10 ⁻⁶			
	Control	Aprotinin	Necrotic extract (1/10 dilution)	Necrotic extract and aprotinin
125	2.9 ± 0.6 ^{a,b} 0.25 ± 0.10	2.8 ± 0.4 ^c 0.30 ± 0.06 ^c	9.8 ± 1.2 ^d 3.2 ± 0.6 ^e	10.3 ± 1.5 ^e 2.9 ± 0.4 ^d
250	2.5 ± 0.3 0.21 ± 0.09	3.3 ± 0.1 ^f 0.27 ± 0.06 ^c	10.9 ± 0.9 ^d 3.7 ± 0.5 ^d	10.9 ± 0.8 ^d 4.1 ± 0.3 ^d
500	5.2 ± 0.5 0.37 ± 0.09	8.8 ± 0.6 ^e 0.34 ± 0.10 ^c	10.6 ± 1.1 ^e 4.1 ± 0.7 ^d	16.0 ± 2.8 ^g 4.0 ± 0.3 ^d

^a Upper value, single cells; lower values, clusters.
^b Mean ± S.E.
^c Control versus treated, Student's *t* test (2-tailed), *p* > 0.05.
^d Control versus treated, Student's *t* test (2-tailed), *p* < 0.001.
^e Control versus treated, Student's *t* test (2-tailed), 0.005 > *p* > 0.001.
^f Control versus treated, Student's *t* test (2-tailed), 0.05 > *p* > 0.025.
^g Control versus treated, Student's *t* test (2-tailed), 0.01 > *p* > 0.005.

Table 3
Effect of a single dose of aprotinin on the number and size of lung tumors developing from i.v. injected LL cells
 Values were obtained by pooling data from 2 separate experiments. Details of treatment schedules are given in the text.

Group	No. of lung metastases ^a	Size distribution of lung metastases ^b (% of total)				
		0-1 mm	1-2 mm	2-3 mm	3-4 mm	>4 mm
Control (10) ^c	57.2 ± 8.2 ^d	41.6	41.6	11.9	3.1	1.3
Aprotinin treated (11)	79.9 ± 8.4	31.8	40.0	21.2	6.2	0.9

^a Control versus treated, Student's *t* test (2 tailed), *p* > 0.05; (1 tailed), *p* < 0.05.
^b Control versus treated, χ^2 test, *p* < 0.001.
^c Numbers in parentheses, total number of mice used.
^d Mean ± S.E.

retention of cells in the spleen or kidneys was demonstrable.

DISCUSSION

A large body of work has implicated proteinases in tumor invasion (11), and it is therefore reasonable to investigate the effects of antiproteinases on invasion and metastasis. As metastasis is a major problem in cancer treatment, and as drugs affecting basic metastatic process are of great interest, we have sought to clarify the action of the antiproteinase aprotinin on one well-documented, naturally metastasizing cancer in mice and have attempted to identify its levels of activity in the metastatic cascade.

Previous experiments with tumor-bearing mice have shown that administration of the antiproteinase, aprotinin, results in increased necrosis in primary cancers (9) and decreased local invasion of tissues (8). In addition, the agent inhibits the active movements of cancer cells *in vitro* (13). Reports on the effects of aprotinin on different tumors in mice and rats often appear contradictory. Thus, Giraldi *et al.* (5) and Thomson *et al.* (12) have reported that it produced a decrease in metastasis; Back *et al.* (1) found no detectable effect, and Cagliani *et al.* (3) and Clifton and Agostino (4) noted an increase in metastasis.

In the present study, treatment of mice bearing the LL tumor with aprotinin results in an increase in both the incidence and amount of pulmonary metastases (Table 1). These observations are in apparent contrast to those reported by Back *et al.* (1) for

Table 4
Effect of different concentrations of aprotinin on the adhesion of LL cells
 Values were obtained by pooling data from 2 experiments.

Aprotinin concentration (KIU/ml)	% of cell adhered	Student's <i>t</i> test (control vs. treated, 2-tailed)
0 (24) ^a	48.0 ± 1.0 ^b	
62.5 (11)	52.8 ± 2.3	0.05 > <i>p</i> > 0.025
125 (24)	55.7 ± 1.3	<i>p</i> < 0.001
250 (23)	63.0 ± 1.4	<i>p</i> < 0.001
500 (17)	46.5 ± 1.3	<i>p</i> > 0.05

^a Numbers in parentheses, total number of mice used.
^b Mean ± S.E.

the LL tumor system, that aprotinin treatment had no effect on the incidence of metastases; however, Back *et al.* used at most only one-tenth of the dose of drug reported here. Giraldi *et al.* (5) have reported that aprotinin causes a decreased incidence of LL metastases; however, their observations were made on a small number of mice, and their techniques and materials may have differed from our own.

An early step in the metastatic cascade is the release of cells from the primary cancer (17), a process thought to be enhanced by proteolytic enzymes (9), the release of which may be associated with the presence of necrosis (18) which commonly occurs in these tumors. The present results (Table 2) fail to demonstrate an inhibition of detachment by aprotinin, and enhancement of detachment by high concentrations of the agent which probably greatly exceed attainable tissue concen-

Table 5
Effect of aprotinin posttreatment on the 6-hr localization of LL carcinoma cells in normal and tumor-bearing mice

Values were obtained by pooling data from 2 or 3 separate experiments. Detailed description of treatment schedule is given in the text. Animals were killed 6 hr after the cells were injected.

Tissue	% of injected dose			
	Normal mice		Tumor-bearing mice ^a	
	Control	Aprotinin treated	Control	Aprotinin treated
Lungs	4.6 ± 0.7 ^b (18) ^c	11.1 ± 1.7 ^d (18)	7.6 ± 1.1 (14)	17.0 ± 2.5 ^d (14)
Liver	1.3 ± 0.1 (18)	2.4 ± 0.3 ^d (18)	1.5 ± 0.1 (14)	2.3 ± 0.3 ^e (14)
Spleen	0.07 ± 0.03 (12)	0.16 ± 0.05 ^f (12)	0.25 ± 0.03 (14)	0.28 ± 0.03 ^f (14)
Kidneys	0.05 ± 0.02 (12)	0.03 ± 0.01 ^f (12)	0.08 ± 0.02 (14)	0.11 ± 0.03 ^f (14)

^a Ten-day s.c. LL tumor.

^b Mean ± S.E.

^c Numbers in parentheses, number of mice used.

^d Control versus treated, Student's *t* test (2-tailed), 0.005 > *p* > 0.001.

^e Control versus treated, Student's *t* test (2-tailed), 0.02 > *p* > 0.01.

^f Control versus treated, Student's *t* test (2-tailed), *p* > 0.05.

trations in intact animals is not strictly relevant to the present discussion. In addition, while confirming that necrotic material indeed increases the release of cancer cells from primary cancers subject to mechanical agitation, the results also fail to demonstrate modification of this increase by 125 and 250 KIU of aprotinin per ml, and at aprotinin concentrations of 500 KIU/ml, cell release is increased over that in the presence of necrotic extract alone. Thus, the metastasis-promoting effects of aprotinin cannot be accounted for in terms of enhanced release of cancer cells from the primary tumor.

Our next step was to confirm that aprotinin did in fact enhance metastasis by its effect on events occurring subsequent to cell release from the primary cancer and intravasation. This was demonstrated, as shown in Table 3, by the increased incidence and shift in the size distribution of lung tumors developing from i.v. injected LL cells followed by aprotinin, than in the appropriate controls.

Another essential step in metastasis is the arrest of viable cancer cells in target organs, by their adhesion of the vascular endothelium. It is therefore of interest that the adhesion of LL cells to the protein-coated (15) microtest plate surfaces should show progressive enhancement in the presence of increasing concentrations of aprotinin (Table 4). While in many respects this *in vitro* test system is an inappropriate model for the *in vivo* situation, it nevertheless indicates that aprotinin can modify adhesive interactions, and this observation is consistent with its observed enhancement of metastasis although the present experimental data do not permit us to identify the mechanisms.

A single injection of aprotinin, given 5 min after LL cells were injected into tail veins, resulted in a highly significant increase in their pulmonary retention measured 6 hr afterwards; liver retention was also increased (Table 5). Previous work had shown differences in the distribution of i.v. injected cancer cells between tumor-bearing and non-tumor-bearing mice (22). Small differences were also observed between the lung retention patterns in the controls and aprotinin-treated animals (Table 5) in the present experiments, but aprotinin treatment produced 2.4- and 2.2-fold increases in retention over the controls in non-tumor-bearing and tumor-bearing animals, respectively. As pulmonary metastases can arise only from viable cancer cells retained in the lungs, the metastasis-enhancing effects of aprotinin are partially accounted for in terms of increased retention.

Although the precise mode of action of aprotinin in increasing the retention of arrested cancer cells at the vascular endothelium is not known, such activities are explicable in terms of its antiproteinase activities. (a) It inhibits lysosomal cathepsin G which degrades proteoglycans (2) and is thereby thought to promote invasion (11); however, by promoting cell release (17), some lysosomal enzymes may actually inhibit metastasis, and by reducing this activity, aprotinin would tend to enhance metastasis. (b) Aprotinin inhibits the serine proteinase, plasmin (2), which degrades the fibrin which temporarily stabilizes tumor emboli at the vascular endothelium (14, 23), although fibrin appears to play little or no part in the initial arrest of cancer cells (6). The release of tumor cells from the vascular endothelium is thought to be partially mediated by fibrinolysis initiated by plasminogen activators and to contribute to metastatic inefficiency (21), since cells released from temporary arrest sites in lungs have an impaired capacity to form metastases elsewhere (20). It is therefore suggested that, by inhibiting the host defense mechanism of proteolytic "purging" of arrested cancer emboli from the vascular endothelium, aprotinin thereby promotes this part of the metastatic cascade.

The results of our secondary experiments, which relate to general features of the metastatic process common to many cancers, are in accord with our primary observation that aprotinin treatment enhances metastasis from LL tumors in mice. Therefore, as far as general statements can ever be made on the basis of studies on one type of murine tumor, we conclude that antiprotease therapy of patients with at least some types of cancer may promote rather than inhibit metastasis.

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