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Inhibition by Fibrin Coagulation of Lung Cancer Cell Destruction by Human Interleukin-2-activated Killer Cells

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We examined the effect of fibrin coagulation on tumor cytotoxicity mediated by human lymphokine (IL-2) -activated killer (LAK) cells. LAK cells were induced from peripheral blood mononuclear cells (MNC) by culture with recombinant IL-2 for 4 or 5 days, and LAK cell-mediated cytotoxicity against tumor cells was assessed by ^{51}Cr release assay in the presence or absence of plasma from normal subjects and lung cancer patients. Plasma did not affect the phase of induction of LAK activity by IL-2, but dose-dependently inhibited the effector phase of LAK cell-mediated cytotoxicity against Daudi cells. Similar inhibition of LAK cell-mediated cytotoxicity was observed on pretreatment of Daudi cells and human lung cancer cell lines with human fibrinogen plus thrombin. A parallel relationship was found between the amount of fibrinogen in plasma of lung cancer patients and inhibition of LAK cytotoxicity. This inhibition was reduced by addition of anticoagulants (heparin or argatroban). These findings suggest that fibrin coagulation on tumor cells protects them from LAK cell-mediated tumor cytotoxicity in malignant lesions and that a combination of an anticoagulant drug and IL-2/LAK therapy may be effective for treatment of lung cancer patients.

Key words: Fibrin — Interleukin 2 — Lymphocyte — Lung cancer — Cytotoxicity

Antitumor cells with a wide target spectrum can be induced without antigenic stimulation by *in vitro* culture of unprimed lymphocytes with lymphokines such as interleukin 2 (IL-2[2]) for 4 to 6 days.[1-3] These cells, named lymphokine-activated killer (LAK) cells, destroy various fresh autologous and allogeneic tumor cells.[3-5] Much attention has been paid to the clinical application of IL-2 and/or LAK cells for treatment of cancer and, indeed, there is encouraging evidence that systemic IL-2/LAK therapy of patients with advanced cancer results in partial cancer regression.[6,7] Much information has been obtained on the regulatory mechanisms of LAK cell-mediated tumor cell killing by humoral and cellular factors.[5,8] But, little is yet known about the mechanisms by which LAK cells kill tumor cells *in vivo*. Cell-to-cell contact between immune effector and tumor cells is required for tumor cell killing by cytotoxic cells.[9]

The most common clotting abnormalities in cancer patients are elevated levels of fibrin/fibrinogen degradation products (FDP),[10,11] thrombocytosis[12,13] and hyperfibrinogenemia.[10,14] In lung cancer, breast cancer and lymphomas, fibrin is found in the fibrous stroma and surrounding tumor nodules and tumor cells,[15-17] suggesting that fibrin deposition associated with tumor cells may protect the cells from destruction by cytotoxic cells through cell-to-cell contact. Indeed, fibrin coagulation was found to prevent tumor cell killing by NK or other effector cells in murine systems.[18,19] Because of the clinical availability of adoptive immunotherapy with IL-2 and LAK cells,[6,7] it is important to obtain more information on the mechanism of the inhibitory effect of fibrin coagulation on human LAK cell-mediated cytotoxicity against human cancer cells.

In the present study, we found that anti-tumor expression of IL-2-induced LAK cells was inhibited in the presence of whole plasma obtained from lung cancer patients, and observed a close correlation of the fibrinogen level in the plasma with its inhibitory effect on LAK cell-mediated tumor cell killing. We also found that prevention of *in vitro* fibrin formation by an anticoagulant (argatroban) resulted in full expression of LAK cell-mediated cytotoxicity against human lung cancer cells.

MATERIALS AND METHODS

Reagents The following reagents were used: RPMI 1640 medium (Nissin Co., Japan); fetal bovine serum (FBS, Gibco Laboratories, Grand Island, NY); human fibrinogen (more than 90% clottable, Sigma Chemical Co., St. Louis, MO); thrombin for clinical use (Green Cross Co., Japan); heparin (Kodama Co., Japan); argatroban for clinical use (Daiichi Seiyaku Co., Japan) and recombinant human IL-2 with a specific activity of 3.5×10^4 U/mg as assayed on IL-2-dependent murine NKC cells[20] (Takeda Pharmaceutical Co., Japan). One unit obtained

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[2] Abbreviations used: IL-2, interleukin-2; LAK, lymphokine-activated killer; MNC, mononuclear cells.

by this method was equivalent to approximately 400 Japanese Reference Units (JRU).

Samples of plasma and serum Peripheral blood samples were taken from healthy volunteers and lung cancer patients (14 males and 1 female, aged 48 to 83 years). Plasma samples with or without 3.8% sodium citrate (9:1, v/v) were stored at −20°C until use.

Tumors Cell lines of human Burkitt lymphoma (Daudi), human leukemia (K-562), human lung small cell carcinoma (N-291) and human lung adenocarcinoma (RERF-LCK, VMRC-LCD) were purchased from the American Type Culture Collection (ATCC), Rockville, MD. A human lung adenocarcinoma cell line (PC-9) and lung small-cell carcinoma cell line (H-69) were kindly supplied by Dr. Y. Hayata (Tokyo Medical College, Tokyo) and Dr. Y. Shimosato (National Cancer Center Research Institute, Tokyo), respectively. All these cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS with gentamycin (designated as CRPMI 1640), at 37°C in a humidified atmosphere of 5% CO_2 in air. LAK cell-mediated cytotoxicity assays were performed in CRPMI 1640 medium with cultured target cells in the exponential growth phase.

Preparation of LAK cells Mononuclear cells (MNC) were separated from heparinized venous blood of healthy donors, and diluted two-fold with phosphate-buffered saline (PBS) in lymphocyte separation medium (LSM, Litton Bionetics, Kensington, MD). The cells were washed twice with PBS, and resuspended in CRPMI 1640 medium at appropriate cell densities. LAK cells were induced by incubating MNC in suspension in polypropylene tubes for 4 or 5 days in CRPMI 1640 with an optimal concentration of IL-2 (1 U/ml) at 37°C with 5% CO_2 in air, as reported previously.[21,22] Then, the cells were thoroughly washed twice with PBS and resuspended in CRPMI 1640.

LAK activity assay LAK cell-mediated cytotoxicity was assessed as described previously.[21,22] For examining the effect of fibrin clot formation on tumor cell killing by LAK cells, radiolabeled target cells and LAK cells were incubated separately with or without plasma or fibrinogen in the presence or absence of thrombin for 30 min at 37°C. Then effector cells and labeled target cells (5×10^4) were mixed in polypropylene tubes (Eppendorf 3810 microtubes) at an effector/target ratio (E/T) of 10:1. Incubations were terminated after 4 h, supernatants (0.1 ml/tube) were harvested by brief centrifugation at 1500 rpm, and their radioactivities were determined in a gamma counter. Percentage cytotoxicity was calculated as follows:

$$\%\,\text{Cytotoxicity} = 100 \times \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}}$$

Spontaneous releases from various target cells were less than 15% of maximum counts.

Statistical analysis The significance of differences between values for groups was analyzed by using Student's *t* test.

RESULTS

Effect of human plasma on LAK cell-mediated cytotoxicity We examined the effect of plasma from healthy donors on the antitumor activity of LAK cells towards NK-resistant Daudi target cells. A preliminary experiment showed that plasma or serum at dilutions of more than 1:20 was not toxic to Daudi cells or LAK cells (data not shown). First, we tested the cytotoxic activity of LAK cells against NK-resistant Daudi cells. LAK cells that had been incubated for 4 or 5 days in medium with IL-2, and Daudi cells were preincubated separately for 30 min in CRPMI 1640 medium with the indicated dilutions of plasma in the presence or absence of heparin or serum before LAK activity assay. As shown in Fig. 1A, pretreatment of target cells with plasma during cytotoxicity assay caused dose-dependent inhibition of the cytotoxic effect of LAK cells. The cytotoxicity of LAK cells was inhibited about 95% by the presence of plasma at a dilution of 1:40. Under the same experimental conditions, pretreatment of Daudi target cells with serum or with plasma plus heparin (2 U/ml) did not affect the LAK cell-mediated cytotoxicity.

Next, we examined the effects of pretreatment of LAK cells with human plasma and LAK cells mixed with human plasma just before LAK activity assay. As shown in Fig. 1B, the cytotoxicity of LAK cells was drastically inhibited in both conditions. Pretreatment of LAK effector cells with plasma at dilutions of more than 1:40 resulted in less inhibition of the cytotoxicity than pretreatment of target cells with plasma.

Blocking effect on LAK cell-mediated cytotoxicity of plasma from lung cancer patients In a parallel experiment, we examined the inhibitory effect of plasma samples from 15 lung cancer patients. The concentration of fibrinogen in these samples ranged from 269 to 869 mg/dl (mean, 444 mg/dl; normal range, 115–325 mg/dl). LAK cell-mediated cytotoxicity was tested against Daudi cells preincubated for 30 min in medium with various dilutions of plasma obtained from the lung cancer patients. As shown in Fig. 2, all the plasma samples dose-dependently inhibited expression of LAK cell-mediated cytotoxicity. We examined whether the levels of fibrinogen in the plasma were parallel with the inhibitory effects on LAK activity. As shown in Fig. 3, a significant correlation was found between the level of fibrinogen and inhibition of cytotoxicity.

Effects of fibrinogen and thrombin on LAK cell-mediated cytotoxicity To confirm that fibrin coagulation was involved in inhibition of target cell killing by LAK cells, we pretreated Daudi cells with fibrinogen and thrombin (0.01 U / ml), which converts fibrinogen into fibrin. Fibrin coagulation increased with increase in fibrinogen concentration. Pretreatment of Daudi target cells with fibrinogen in the presence of thrombin resulted in dose-dependent blocking of the cytotoxicity of LAK cells (Table I). Under these conditions, heparin, which blocks fibrin formation from fibrinogen, abolished the inhibitory effect of fibrinogen and thrombin treatment of LAK cell-mediated cytotoxicity. These data suggest that the inhibitory effect of plasma on LAK cytotoxicity is caused by fibrin coagulation.

In a parallel experiment, we also examined the effect of fibrin coagulation on induction of LAK cells by IL-2. For this, we pretreated MNC (LAK precursor cells) for 4 days in medium containing fibrinogen (0.25 mg/ml) with or without thrombin (0.01 U/ml) in the presence of IL-2 (1 U/ml) before assay of LAK activity against Daudi cells. Percent cytotoxicities of LAK cells induced by IL-2 in the presence and absence of fibrin coagulation

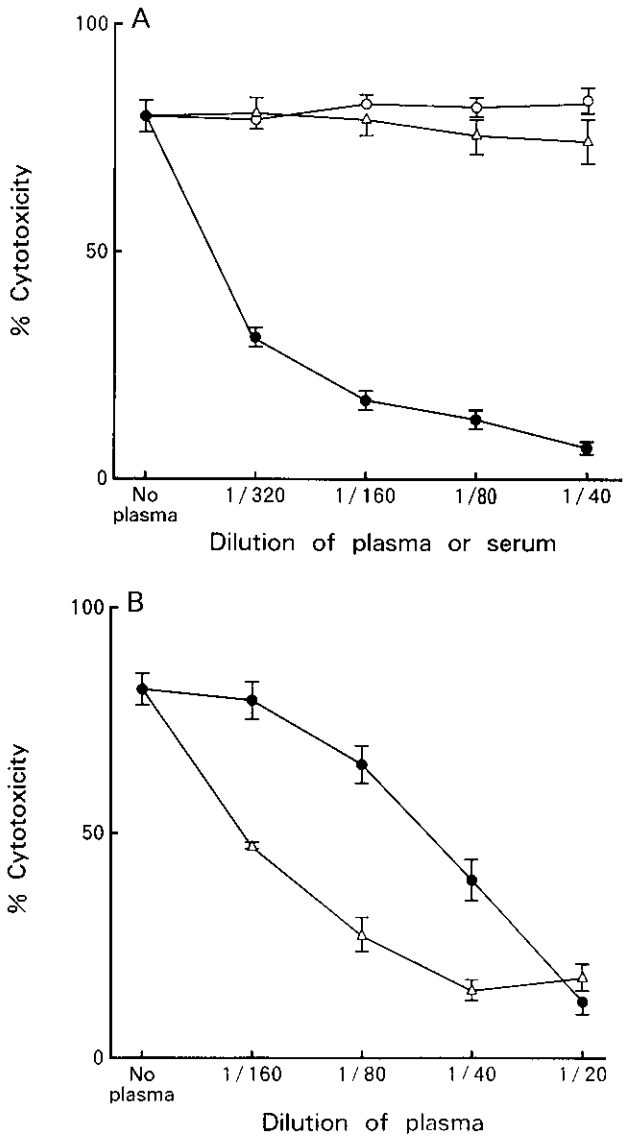

Fig. 1. Effects of normal human plasma and serum on LAK cytotoxicity. A: ^{51}Cr-labeled Daudi cells (5×10^4) were pre-incubated with the indicated dilutions of human serum (△) or plasma in the presence (○) or absence (●) of heparin (2 U/ml) for 30 min before addition of LAK cells (5×10^5). B: LAK cells were preincubated with the indicated dilutions of plasma for 30 min before addition of ^{51}Cr-labeled Daudi cells (●). ^{51}Cr-labeled Daudi cells, LAK cells and plasma were mixed simultaneously (△). LAK cytotoxicity was measured by 4 h ^{51}Cr release assay as described in "Materials and Methods." Values are means±SD for 3 independent experiments.

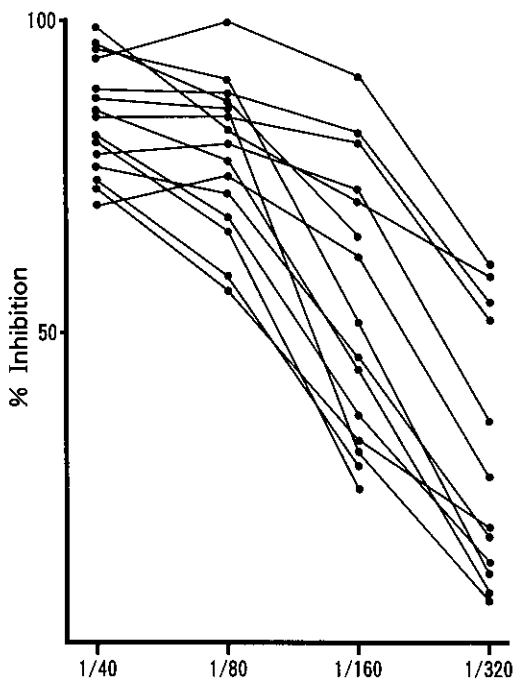

Fig. 2. Inhibitory effect of plasma from lung cancer patients on LAK cell-mediated cytotoxicity against Daudi cells. Plasma samples were obtained from 15 untreated patients with lung cancer. ^{51}Cr-labeled Daudi cells (5×10^4) were preincubated with the indicated dilutions of plasma samples for 30 min before addition of LAK cells (5×10^5). LAK-mediated cytotoxicity was measured by 4 h ^{51}Cr release assay as described in "Materials and Methods."

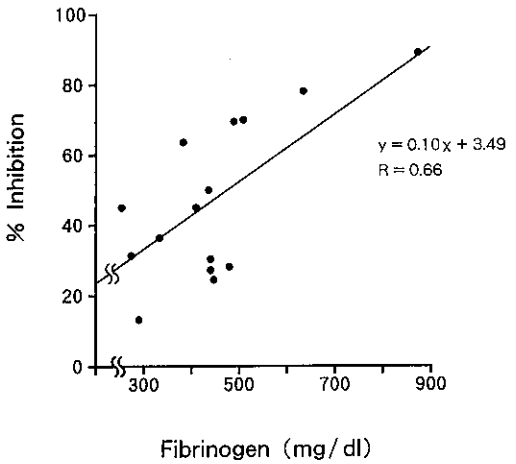

Fig. 3. Correlation of plasma fibrinogen level with inhibitory effect on LAK cell-mediated cytotoxicity. Plasma samples were obtained from 15 patients with lung cancer. Daudi cells (5×10^4) were preincubated with each plasma sample at a dilution of 1:160 for 30 min before addition of LAK cells (5×10^5). LAK cytotoxicity was measured by 4 h ^{51}Cr release assay as described in "Materials and Methods."

were 70.3±6.8 (mean±SD), and 75.6±1.9 (mean±SD), respectively, thus indicating no significant effect of fibrin coagulation on the phase of induction of LAK activity by IL-2.

Effect of fibrin coagulation on LAK cell-mediated cytotoxicity against various human lung cancer cells To extend these findings with Daudi cells to other cancer cell lines, we used various human lung cancer cells; namely, H-69, N-291, PC-9, RERF-LCK and VMRC-LCD cells. LAK cell-mediated cytotoxicity depended on the sensitivities of these target cells (the % cytotoxicity by LAK cells without fibrinogen treatment was 46–85%). The cytotoxicities of LAK cells to all 5 lung cancer cell lines were blocked dose-dependently by treatment with fibrinogen plus thrombin (Table II). A preliminary experiment indicated a linear relationship between the amount of ^{125}I-fibrinogen added and the amount of fibrinogen bound to PC-9 tumor cells or to LAK cells when these cells and radiolabeled fibrinogen were incubated for 30 min at 4°C (data not shown).

Blocking effect of argatroban on inhibition by fibrin coagulation of LAK cell-mediated cytotoxicity Finally,

Table I. Effect of Fibrinogen on LAK Cytotoxicity against Daudi Target Cells

Heparin	% Cytotoxicity on treatment with fibrinogen[a]				
	Concentration of fibrinogen (mg/ml)				
	0	0.01	0.1	0.5	1.0
(−)	79.5±2.4[b]	77.4±5.9	23.8±1.4[c]	7.1±0.2[c]	4.9±1.4[c]
(+)	79.7±4.1	86.6±4.6	89.1±2.5	86.1±1.9	86.8±3.2

a) Daudi cells (5×10^4) were incubated in medium containing fibrinogen (0, 0.01, 0.1, 0.5, 1.0 mg/ml) and thrombin (0.01 U/ml) for 30 min before addition of LAK cells (5×10^5) in the presence or absence of heparin (2 U/ml). LAK cell-mediated cytotoxicities towards these target cells were measured by ^{51}Cr release assay as described in "Materials and Methods."
b) Values are means and SDs of percent cytotoxicity in triplicate cultures.
c) Significantly different ($P<0.01$) from the value of the control group.

Table II. Effects of Fibrinogen on LAK Cytotoxicities against Various Human Lung Cancer Cells

Target cell line	% Cytotoxicity on treatment with fibrinogen[a]			
	Concentration of fibrinogen (mg/ml)			
	0	0.01	0.1	1.0
H-69	60.5±4.0[b]	50.2+0.2 (17.0)[c]	30.0±1.2[d] (50.4)	22.7±0.9[d] (62.5)
N-291	93.8±5.1	56.6±1.5 (11.3)	34.0±2.4[d] (46.7)	21.0±3.0[d] (67.1)
PC-9	47.6±2.6	34.6±1.8[d] (27.6)	28.1±0.4[d] (41.2)	ND
RERF-LCK	84.4±7.8	69.9±2.3 (17.2)	43.3±4.3[d] (48.7)	16.6±7.6[d] (80.3)
VMRC-LCD	45.9±3.0	42.7±2.8 (7.0)	23.7±5.2[d] (48.4)	13.5±2.2[d] (70.6)

ND: not determined.
a) Various target cells (5×10^4) were incubated in medium containing various amounts of fibrinogen and thrombin (0.01 U/ml) for 30 min before addition of LAK cells (5×10^5). LAK cell-mediated cytotoxicities were measured by ^{51}Cr release assay as described in "Materials and Methods."
b) Values are means and SDs for percent cytotoxicities in triplicate cultures.
c) Values in parentheses are percent inhibitions of that without fibrinogen.
d) Significantly different ($P<0.01$) from the value of the control group.

we examined the effect of the thrombin inhibitor argatroban on the blocking effect of plasma on expression of LAK cytotoxicity (Fig. 4). Argatroban is a potent and highly selective thrombin inhibitor.[23] Daudi cells were preincubated with plasma at various dilutions (1:40–1:320) in the presence or absence of argatroban at concentrations of 0.01–1 μg/ml. After 30 min, LAK cells were added and their cytotoxicity was measured after 4 h incubation. Argatroban alone did not affect the level of LAK cell-mediated cytotoxicity (data not shown), but caused dose-dependent decrease in the blocking effect of pretreatment with plasma on LAK cell-mediated cytotoxicity. Argatroban (1 μg/ml) completely abolished the inhibitory effect of plasma treatment on LAK cell-mediated cytotoxicity.

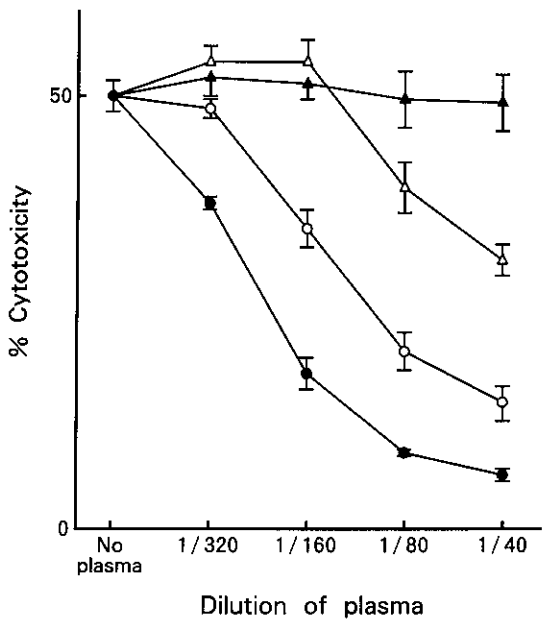

Fig. 4. Dose-dependent effect of argatroban on plasma-induced inhibition of LAK cell-mediated cytotoxicity. Daudi cells (5×10^4) were incubated with the indicated dilutions of plasma without argatroban (●) or with argatroban at 0.01 (○), 0.1 (△) or 1.0 (▲) μg/ml. After 30 min incubation, LAK cells (5×10^5) were added and their cytotoxicities were measured by 4 h ^{51}Cr release assay as described in "Materials and Methods."

Kinetics of inhibition by fibrin coagulation of the LAK cell-mediated cytotoxicity Daudi cells and PC-9 cells were preincubated with 40-fold-diluted plasma for 30 min before addition of LAK cells. Then 1 μg/ml of argatroban was added at the indicated times before and during LAK activity assay. On addition of argatroban 30 to 25 min before admixture of LAK cells and Daudi cells or PC-9 cells, fibrin formation was not visible and LAK cytotoxicity was not inhibited. When argatroban was added immediately before assay of LAK activity, LAK cytotoxicity was drastically inhibited (Table III).

DISCUSSION

Accumulating clinical and experimental evidence indicates a close relationship between blood coagulation and cancer growth.[24, 25] Up to 98% of all cancer patients have blood coagulation abnormalities, and a half of cancer patients have hyperfibrinogenemia.[10] Moreover, fibrinogen accumulation has been observed at tumor sites.[26] The substances present in malignant tissues could give rise to local thrombin production,[27] and this thrombin could convert extravasated fibrinogen of tumors to fibrin. Tumor cells surrounded by deposits of fibrin may be resistant to attack by host NK cells, because close contact between the tumor target and these effector cells is crucial for target cell killing.[9] In the present study, analysis of the *in vitro* cytotoxic activity of human IL-2-

Table III. Effect of Time of Argatroban Addition on LAK Cell-mediated Cytotoxicity against Human Lung Cancer Cells

Target cell line	% Cytotoxicity on addition of argatroban[a]					
	Time of argatroban addition (min)					
	−30	−25	−15	0	30	60
Daudi cells	59.4[b]	61.1	49.2	17.2[c]	16.3[c]	15.6[c]
PC-9 cells	39.7	36.3	30.4[c]	25.6[c]	27.9[c]	ND

ND: not determined.

a) Target cells (5×10^4) were preincubated with plasma (final dilution, 1:40) for 30 min. Then LAK cells (5×10^5) were added (time 0). Argatroban (1 μg/ml) was added to these cultures at the indicated time. LAK cytotoxicity was measured by ^{51}Cr release assay after 4 h incubation as described in "Materials and Methods."

b) Values are means and SDs for percentage cytotoxicities in triplicate cultures.

c) Significantly ($P<0.01$) different from the value at −30 min.

induced LAK cells against NK-resistant Daudi cells in the presence of whole plasma from healthy donors and lung cancer patients revealed that fibrin coagulation inhibited the cytotoxicity of LAK cells. The effect of plasma in blocking LAK cytotoxicity was mediated by fibrinogen, because 1) the level of inhibition of LAK cell-mediated cytotoxicity paralleled the level of fibrinogen in the plasma, 2) addition of heparin to plasma did not inhibit the LAK cell-mediated cytotoxicity and fibrin formation, 3) serum not containing fibrinogen showed no blocking effect at any dilution, and 4) addition of fibrinogen plus thrombin had the same blocking effect as plasma on LAK cytotoxicity. Thus, the present findings confirmed and extended previous observations on the importance of fibrin coagulation in protection of murine tumor cells from destruction by cytotoxic cells.[18,19] We concluded that the observed inhibition of LAK cytotoxicity of plasma is mediated by fibrin coagulation around target cells in the human system.

The blood coagulation mechanism is activated both locally and systemically in cancer patients.[15,27,28] Interestingly, in previous studies depositions of fibrin in the fibrous stroma surrounding nodules and around individual tumor cells were observed by electron microscopy, immunofluorescence and standard histochemical staining.[15-17] These findings suggest that the accumulation of fibrin associated with tumor masses in lung cancer protects tumor cells from host defense. Comparative *in vitro* analysis of human LAK cell-mediated anti-tumor activity in the presence of fibrinogen and thrombin showed that fibrin coagulation significantly protected human lung cancer cell lines from killing by LAK cells (Table II). The inhibitory effects against LAK cell-mediated cytotoxicity on five human lung cancer cell lines of different histological types were similar. Neither fibrinogen nor thrombin alone had any effect on tumor cell killing.

Increase of fibrinogen or of fibrin deriving from thrombin may contribute to the spreading and metastasis of cancer.[29-32] Defibrinating agents such as batroxobin have been proposed to be effective in treatment of thrombotic diseases. There are also reports of the effectiveness of defibrination in treatment of experimental cancer.[33,34] The anticoagulant drug warfarin has been shown to inhibit metastasis markedly in a number of animal models.[35,36] The protective effect of fibrin on human LAK cell-mediated killing of tumor cells was inhibited dose-dependently by addition of argatroban before fibrin formation (Table III). In contrast, addition of argatroban after fibrin formation around tumor cells did not overcome inhibition by fibrin. Argatroban also did not affect the level of LAK cell-mediated cytotoxicity. Anticoagulant drugs may prevent fibrin coagulation of human cancer cells and/or of cytotoxic effector cells, and so increase the vulnerability of tumor target cells to anti-tumor effector cells. These findings suggest that clinical treatments causing inhibition of fibrin formation arround tumor cells and/or effector cells might increase the effects of cytotoxic effector cells on lung cancer cells.

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