Role of Fibrin Coagulation in Protection of Murine Tumor Cells from Destruction by Cytotoxic Cells

Yoshio Gunji and Elieser Gorelik

Pittsburgh Cancer Institute and Department of Pathology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15213

ABSTRACT

We have previously proposed that fibrin deposition on tumor cells during their migration in the blood could protect them from elimination by natural killer (NK) or other cytotoxic cells. Anticoagulant drugs could prevent fibrin coagulation and increase the efficiency of cytotoxic effector cells in tumor cell elimination. To further investigate the protective roles of fibrin, we studied in vitro the susceptibility of various murine tumor cells to the cytotoxic activity of NK or lymphokine activated killer (LAK) cells in the presence of murine plasma or serum.

In the first set of experiments, tumor cells were incubated with plasma (at dilutions of 1:20–1:160) for 30 min before effector cells were added. Similarly, effector cells were first incubated with plasma before mixing with radiolabeled target cells for cytotoxicity assay. In some experiments target and effector cells and plasma were mixed simultaneously. The cytotoxic activity of both NK and LAK cells was inhibited if coagulation occurred around tumor-target or effector cells. Tumor cells were also protected when both target and effector cells were simultaneously mixed and trapped in the fibrin clot. Inhibition of the cytotoxic activity of effector cells against tumor cells was positively correlated with the level of fibrin clot formation. When the larger clot was formed and more radiolabeled tumor cells were trapped in the clot, the higher level of inhibition of cytotoxicity was observed. In contrast, serum did not affect the cytotoxic activity of NK or LAK cells. To exclude possible non-coagulation-related effects of plasma on LAK cells, a cytotoxicity series was performed using purified fibrinogen and thrombin. When fibrinogen and thrombin were preincubated with tumor cells or LAK cells or all components were admixed simultaneously, substantial protection of tumor cells from destruction by LAK cells was also observed. However, when heparin was added, fibrin coagulation was prevented and cytotoxic activity of LAK cells was restored.

Inhibition of LAK cytotoxicity and protection of tumor cells by fibrin coagulation were mostly due to the prevention of tumor-effector cell conjugate formation. Adding plasma at postbinding time periods (15–30 min after mixing effector and target cells) did not affect the ability of LAK cells to kill tumor cells confirming that fibrin coagulation influenced the binding rather than the lytic phase of cytotoxic cell activity.

INTRODUCTION

Tumor cells entering into the blood stream could interact with various components of the blood. As a result, an extremely high rate of elimination of tumor cells is usually observed (1). It has been considered that NK cells are mostly but not exclusively responsible for the destruction of tumor cells in the blood (2, 3). In addition, it was demonstrated that tumor cells in the blood interact with the factors of the hemostatic system resulting in the aggregation of platelets and fibrin deposition around the tumor cells (4, 5). Formation of such microthrombi has also been considered to be an important step in the metastatic process. This assumption is supported by observations that drugs which prevent platelet aggregation and fibrin coagulation also strongly inhibit the formation of metastases in experimental animals (4–9).

The ability of tumor cells to trigger the hemostatic cascade is mostly due to the presence of a recently isolated and characterized (10) cancer procoagulant factor in malignant cells. The biological significance of platelet aggregation and fibrin formation in the blood induced by migrated tumor cells and the mechanisms of antimetastatic effects of anticoagulant drugs remain unclear. It has been suggested that a platelet-fibrin-tumor cell thrombus could (a) enhance tumor cell lodgement, (b) increase adherence of tumor cells to endothelial cells, (c) induce degeneration of the endothelial cells and enhance tumor cell extravasation, and (d) activate platelets and fibrin formation that associated with release of growth factors which could stimulate tumor cell proliferation (4–9).

Previously we proposed that fibrin coagulation might have a protective effect and render tumor cells less vulnerable to the cytotoxic action of NK or other cytotoxic cells. Anticoagulant agents, by preventing the coating of tumor cells by fibrin, could increase the efficacy of NK cells in tumor cell elimination and inhibit metastasis formation (11, 12). This hypothesis was supported by the findings that elimination of the radiolabeled tumor cells in mice was substantially increased when blood coagulation was prevented by heparin or warfarin. This was paralleled by a dramatic inhibition of the experimental pulmonary metastases formation. With stimulation of NK cell activity, tumor cell destruction could be augmented and anticoagulant drugs could further potentiate the efficacy of NK cells in tumor cell elimination in these mice. However, in mice with depressed NK cell function more tumor cells were able to survive, and under these circumstances anticoagulants did not affect either tumor cell elimination or metastasis formation (11, 12). These results suggest that the antimetastatic effect of the anticoagulants is exerted only in the presence of the active NK cells, and by prevention of fibrin clot formation the anticoagulants make tumor cells more vulnerable to the cytotoxic action of NK or other effector cells (11, 12).

The present study was designed to gain more direct evidence of the protective effect of fibrin coagulation. For this purpose, we investigated in vitro the cytotoxic activity of NK or LAK cells against tumor cells in the presence of whole plasma, or only fibrinogen and thrombin under conditions when fibrin coagulation was permitted or prevented.

The results of this study clearly demonstrate that fibrin coagulation could prevent the cytotoxic action of NK and LAK cells against tumor cells. Inhibition of cytotoxicity was observed when fibrin deposition formed around either tumor or LAK effector cells. When in vitro fibrin coagulation was prevented by heparin, LAK cells were highly efficient in tumor cell destruction. These results could provide new insight into the mechanisms of the antimetastatic effects of anticoagulants and the biological significance of interactions of the hemostatic and immune systems during the metastatic process.

MATERIALS AND METHODS

Mice. C57BL/6 and BALB/c nude mice (8–12 weeks old, female), purchased from NCI-Frederick Cancer Research Facility, Frederick,
of coagulation is usually observed with plasma diluted 1:10 or less. Complete clotting of the fibrin in the tube was scored as ++++. This type of coagulation was measured as the percentage of the radioactive tumor cells trapped in the fibrin clot as

\[
\% \text{ of coagulation} = \frac{T - (S + P)}{T} \times 100
\]

where \( T \) is total cpm of the labeled tumor cells per tube; \( S \), is cpm of the whole supernatant per tube determined as cpm in 0.1 ml of supernatant multiplied by total volume of supernatants and \( P \) is cpm of the pellet.

We have found that this method of measuring the level of radioactivity of tumor cells trapped in the clot allowed us to more precisely quantitate the size of the fibrin clot in the tube.

**RESULTS**

The procoagulant activity of tumor and spleen cells was tested in the presence of human or murine plasma (Table 1). 3LL and BL6 tumor cells showed high procoagulant activity and shortened the time of blood coagulation by 4–5-fold in the presence of murine plasma. The level of procoagulant activity of these tumor cells was similar to those exerted by rabbit brain thromboplastin. In contrast, normal spleen cells slightly decreased the time of coagulation. When spleen cells were activated by in vitro culture for 3 days in the presence of IL-2 (1000 units/ml) were harvested at 37°C. Cells were centrifuged at 200 × g for 10 min and fibrin clot and pellet were separated. The number of fluorescing spleen cells binding and not binding to the nonfluorescing target tumor cells in the cell pellet and in the fibrin clot was determined under fluorescent microscope. The percentage of effector-target conjugates was calculated (14).

### Table 1 Procoagulant activity of murine normal or malignant cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Coagulation time (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Murine plasma</td>
</tr>
<tr>
<td>3LL</td>
<td>19 ± 1.3</td>
</tr>
<tr>
<td>BL6 melanoma</td>
<td>15 ± 1.2</td>
</tr>
<tr>
<td>Normal spleen cells</td>
<td>60 ± 2.3</td>
</tr>
<tr>
<td>LAK cells</td>
<td>79 ± 2.1</td>
</tr>
<tr>
<td>Thromboplastin</td>
<td></td>
</tr>
<tr>
<td>Undiluted</td>
<td>14 ± 0.9</td>
</tr>
<tr>
<td>1:10</td>
<td>39 ± 1.2</td>
</tr>
<tr>
<td>1:40</td>
<td>64 ± 0.7</td>
</tr>
<tr>
<td>Phosphate buffered saline (control)</td>
<td>84 ± 1.1</td>
</tr>
</tbody>
</table>

* NT, not tested.

---

MD, were used for these experiments. Mice were kept in specific-pathogen-free conditions and received food and water *ad libitum*.

Tumors. YAC-1, lymphoma, B16F10BL6 sublines of B16 melanoma (hereafter referred to as BL6), and 3LL Lewis lung carcinoma were maintained *in vitro* in RPMI 1640 supplemented with 10% fetal bovine serum, glutamine, streptomycin, and penicillin (hereafter referred to as complete medium), as described previously (11).

Normal Plasma and Serum. Platelet-poor citrated human plasma was obtained from the Central Blood Bank, University of Pittsburgh. Murine plasma was obtained from the blood of C57BL/6 mice by mixing with sodium citrate at a final concentration of 0.35% to prevent clotting.

In parallel, blood was drawn in the absence of anticoagulant to obtain murine serum after coagulation. Tubes were centrifuged at 400 × g for 15 min and plasma or serum were collected and stored at 4°C.

**NK and LAK Cells.** The cytotoxic activity of spleen cells of nude mice against 51Cr-labeled YAC-1 cells was tested. For induction of LAK cells, spleen cell suspensions from normal C57BL/6 mice or BALB/c nude mice were preincubated *in vitro* for 3 to 5 days with 1000 units/ml of human recombinant IL-2 (Cetus, Emeryville, CA) (13). The cytotoxic activity of NK or LAK cells was tested in a 4-h 51Cr release assay (12, 13).

**Cytotoxicity Assay.** Since coagulation is difficult to observe in the 96-well plate, the cytotoxic test in the presence or absence of whole plasma was performed in glass tubes (10 × 75 mm). Tumor cells were labeled with Na251CrO4 (5 × 106 radiolabeled cells in 0.5 ml of complete RPMI 1640 were incubated with 0.5 ml of murine plasma diluted with Dulbeccos's phosphate buffered saline (final concentrations, 1:20 to 1:160) for 30 min at 37°C in glass tubes. After incubation, NK cells or LAK cells in 0.5 ml of complete medium were added to glass tubes. In some experiments LAK cells were preincubated for 30 min with plasma and then radiolabeled tumor cells were added. Alternatively, tumor cells, plasma, and effector cells were mixed together at the same time. After 4 h incubation at 37°C, tubes were centrifuged, supernatants (0.1 ml) were harvested, and their radioactivity was measured.

The cytotoxic activity of LAK cells in the presence of fibrinogen and thrombin was studied by mixing radiolabeled tumor cells (2.5 × 10⁶/0.25 ml) with 0.25 ml of fibrinogen (Sigma Chemical Co., St. Louis, MO) diluted with Dulbeccos's phosphate buffered saline (final concentration, 1 mg/ml) and 0.05 ml of thrombin (final concentration, 0.006–0.2 units/ml). Thrombin was kindly provided by Dr. George Tuzchynski (The Johnsenau Medical Research Center, Philadelphia, PA). After 20 min of incubation at 37°C, 0.1 ml of LAK cells was added. In some experiments, fibrinogen and thrombin were first incubated with LAK cells and radiolabeled tumor cells were added 20 min later. Alternatively, tumor cells, fibrinogen, thrombin, and effector cells were mixed together at the same time and the cytotoxic activity of LAK cells was tested in the 4-h 51Cr release assay. Differences in cytotoxicity of more than 10% were significant at \( p < 0.05 \) by Student's \( t \) test.

**Procoagulant Activity.** The procoagulant activity of murine tumor cells was assessed by a one-stage plasma recalcification assay. Tumor cells (10⁶/0.1 ml of Dulbeccos's phosphate buffered saline with Ca²⁺ and Mg²⁺), 0.1 ml of human platelet poor plasma or murine plasma (1:3 dilution with Dulbeccos's phosphate buffered saline without Ca²⁺ and Mg²⁺), and 0.1 ml of 0.025 M CaCl₂ were mixed in glass tubes at a final plasma dilution of 1:9. The time of clot formation was measured. In control tubes coagulation time in the presence of tumor cells or in the presence of thromboplastin was determined. Thromboplastin extract from rabbit brain (Tate Diagnostic, Inc., Agnada, PA) was used undiluted or after 1:10 or 1:40 dilution with Hank's balanced salt solution.

The level of coagulation in the tube during the cytotoxicity assay was also tested. Tumor and effector cells were mixed with plasma at final dilutions of 1:20–1:160. The size of the fibrin clot was detected and measured according to scoring systems of +, ++, ++++, +++++. The assayed was hard clotting of the fibrin in the tube as scored as ++++. This type of coagulation was usually observed with plasma diluted 1:10 or less. Incomplete clotting with the presence of some liquid in the tube was recorded as +++. About 50% of clotting or less was scored as ++ and +, respectively. In parallel we evaluated the size of the fibrin clot formed after adding radiolabeled tumor cells, by measuring the level of radioactivity trapped in the fibrin clot. After 4 h of incubation at 37°C, tubes were centrifuged at 200 × g for 10 min. This procedure allowed to separate tumor cells outside of the fibrin clot from those trapped in the fibrin clot which remained floating in the supernatant.

Since complete removal of the clot without supernatant is difficult to achieve, the level of clot radioactivity was measured indirectly. For this purpose 0.1 ml of supernatant was harvested. The floating fibrin clot and the rest of the supernatant were removed and the level of the radioactivity of the tumor cells in the pellet was determined. The level of coagulation was measured as the percentage of the radioactive tumor cells trapped in the fibrin clot as
cells were able to reduce the time of coagulation when they were incubated with human plasma. In comparison to spleen cells, 3LL and BL6 tumor cells showed higher procoagulant activity in murine plasma, but no differences were found between these cells in human plasma. The differences in the procoagulant activity of murine tumor and spleen cells in homologous versus heterologous plasma make murine plasma more appropriate for studies of the effect of plasma coagulation on the cytotoxic activity of normal or IL-2 activated murine spleen cells.

In the first set of experiments, the cytotoxic activity of spleen cells of nude mice was tested against YAC-1 lymphoma cells preincubated with murine plasma or serum. Our data as well as previously published results (15) indicated that murine plasma or serum at relatively high concentrations (1:5–1:10 dilutions) could be toxic for effector or target cells. Therefore, we utilized in our experiments murine plasma and serum at final dilutions between 1:20 and 1:80. Indeed in such concentrations of murine serum, no significant changes in the cytotoxicity of NK cells were observed (Fig. 1). In contrast, plasma (dilutions, 1:20–1:40) substantially inhibited the cytotoxic effect of NK cells. The cytotoxicity of NK cells in the presence of plasma at a dilution of 1:20 was inhibited by 80% (Fig. 1).

In order to extend these findings to other tumor cell lines we attempted to utilize BL6 melanoma and 3LL cells. However, BL6 and 3LL tumor cells were resistant to cytotoxic action of normal spleen cells in the 4-h 51Cr release assay. After 3 days in culture with IL-2 (1000 units/ml) spleen cells became highly cytotoxic for a variety of tumor cell lines including BL6 and 3LL cells. Therefore, in the next series of experiments, the cytotoxic activity of LAK cells against these cells was tested in the presence of murine plasma or serum.

When BL6 melanoma cells were admixed with LAK cells in the presence of murine serum (final dilutions, 1:20–1:160) no changes in the cytotoxicity of LAK cells were found (Fig. 2). In contrast mixing of the target and effector cells in the presence of plasma (dilutions, 1:20 or 1:40) was associated with significant protection of the tumor cells.

Usually at the higher concentration of plasma (dilution, 1:10) a large fibrin clot was formed and in this case tumor cells were completely protected from the cytotoxic action of the LAK cells. Whereas partial protection of the tumor cells was associated with smaller sizes of fibrin clot. Since the fibrin clot contained radiolabeled tumor cells, the level of clotting could be evaluated by measurement of its radioactivity. Indeed, the size of the fibrin clot was found to parallel the number of the radioactive tumor cells trapped and total clot radioactivity. Plotting the level of the LAK cytotoxicity and fibrin clotting of radiolabeled tumor cells revealed an inverse correlation between these two parameters (Fig. 3A). At plasma dilutions of 1:80 or 1:160, no clotting was detected and the cytotoxicity of LAK cells against BL6 (A) and 3LL cells (B) was tested at effector:target ratios of 50:1 and 20:1, respectively. Bars, SD.
and this was associated with a decrease in the cytotoxicity exerted by LAK cells (Fig. 3A).

Similarly, 3LL tumor cells were first mixed with plasma and incubated for 30 min before LAK cells were added. The cytotoxicity of these LAK cells against 3LL tumor cells decreased with increased concentrations of plasma and increased fibrin clotting (Fig. 3B). The maximum clotting and inhibition of cytotoxicity was found at a plasma dilution of 1:20 (Fig. 3B).

When 3LL, plasma, and LAK cells were mixed simultaneously, fibrin formation was observed at 1:20-1:80 plasma dilutions (Fig. 4). In parallel the cytotoxicity was substantially inhibited. In all of these experiments, serum in contrast to the plasma at dilutions of 1:20-1:80 did not affect the cytotoxicity of the LAK cells.

Since plasma contains various biologically active substances which can be activated by the triggering of the hemostatic cascade and might affect cytotoxicity by LAK cells, it was of interest to evaluate the role of some of the major components of fibrin formation. Therefore, the cytotoxic assay was performed in the presence of fibrinogen and thrombin. Thrombin could convert fibrinogen into fibrin. The fibrinogen was used at a constant concentration (1 mg/ml), whereas different doses of thrombin were tested (Fig. 5). The preventive effect of fibrin deposition on the target tumor cells was studied when the fibrinogen and thrombin were preincubated with BL6 or 3LL tumor cells for 20 min, and then the LAK effector cells were added. It was assumed that fibrin deposition around the effector cells also could prevent them from destroying target tumor cells. To test this hypothesis, fibrinogen and thrombin were incubated first with LAK cells for 20 min before radiolabeled tumor cells were added. In some groups, the target and effector cells were simultaneously mixed with fibrinogen and thrombin (Fig. 5).

Inhibition of cytotoxicity of LAK cells against BL6 or 3LL tumor was observed at low doses of thrombin (0.006-0.012 unit/ml) and persisted with the higher concentrations of thrombin (up to 0.2 unit/ml). All of these doses of thrombin were sufficient to induce clotting 40-60% of tumor cells, as determined by calculation of the level of tumor cell radioactivity trapped in the clot (data not shown). The level of fibrin coagulation could be substantially increased by increasing the concentration of fibrinogen above 1 mg/ml (data not shown).

Inhibition of cytotoxicity was observed in situations where fibrin formation was initiated either around the tumor target cells or around LAK effector cells. Similarly, the cytotoxicity of LAK cells was abrogated when both target and effector cells were trapped in the fibrin clot (Fig. 5). In some experiments more profound inhibition of cytotoxicity was observed when tumor cells were first mixed with fibrinogen and thrombin (Fig. 5A). In other experiments the most efficient protection was found when LAK cells were trapped in the clot and target cells were added later (Fig. 5B). However, from results of repetitive experiments it appeared that the level of tumor cell protection were similar regardless of the sequence of exposure of the target or effector cells to plasma or fibrinogen and thrombin.

These data demonstrate that fibrin deposition can protect tumor cells from destruction by cytotoxic effector cells. One would then predict that by blocking fibrin formation, the cytotoxicity by the effector cells could be completely restored. To investigate this, the effect of heparin on the coagulation and cytotoxicity of LAK cells was studied (Table 2). Tumor cells, fibrinogen, thrombin, and LAK cells were mixed in various orders and the cytotoxic effects of LAK cells in the presence of heparin (2 units/ml) was studied. In the absence of fibrinogen and thrombin, LAK cells had strong cytotoxic effects against BL6 and 3LL tumor cells. Admixing tumor and LAK cells with fibrinogen (1 mg/ml) and thrombin (0.1 unit/ml), in any of the chosen combinations, resulted in the fibrin formation and substantial inhibition of the cytotoxicity by LAK cells (Table 2). However, adding as little as 2 units/ml of heparin was sufficient to prevent fibrin formation and made tumor cells vulnerable to the cytotoxic action of LAK cells. Heparin alone did not show any direct effect on the cytotoxicity of LAK cells. Coagulation and inhibition of cytotoxicity were observed only when fibrinogen and thrombin were mixed together. No coagulation and no effect of tumor cell destruction by LAK cells were found.

![Fig. 4. Cytotoxic activity of LAK cells and fibrin clot formation after simultaneously mixing effector and target cells in plasma. 3LL tumor cells (5 x 10^6) were mixed simultaneously with LAK cells at a cell/tumor ratio of 50:1 in the presence of murine plasma (dilutions, 1:20-1:60). After 4 h of incubation at 37°C, cytotoxic effects of LAK cells and percentage of radiolabeled tumor cells trapped in the clot were evaluated. Bars, SD.](image-url)

![Fig. 5. Effect of fibrinogen and thrombin on cytotoxic activity of LAK cells. In A, BL6 melanoma cells (2.5 x 10^4) were mixed with fibrinogen (1 mg/ml), thrombin, and LAK cells at an effector/target ratio of 50:1. Radiolabeled tumor cells (2.5 x 10^4) were mixed with fibrinogen (1 mg/ml), thrombin, and LAK cells at an effector/target ratio of 50:1. Radiolabeled tumor cells, fibrinogen, thrombin, and LAK cells were mixed simultaneously (O), or tumor cells were incubated with fibrinogen and thrombin for 20 min and then LAK cells were added (△), or LAK cells were preincubated with fibrinogen and thrombin for 20 min and then radiolabeled tumor cells were added (●). Percentage of cytotoxicity was determined in a 4-h 51Cr release assay. Bars, SD.](image-url)
when fibrinogen or thrombin were mixed separately with target and effector cells (Table 2).

Inhibition of LAK cells cytotoxicity by fibrin could be due to the prevention of close contact between target and effector cells or affecting the lytic phase of the cytotoxicity of the effector cells. To clarify these mechanisms the effect of fibrin coagulation on the ability of the effector LAK cells to bind 3LL tumor target cells was investigated in the presence of murine plasma or serum at a final dilution of 1:20 (Table 3). When fluorescein deacetate labeled LAK cells were mixed with nonlabeled 3LL tumor cells in the presence of plasma, fibrin coagulation occurred. Analysis of the cell pellet under UV microscope revealed that very few of effector cells were able to bind target cells (0.7%) and no conjugates between target and effector cells in the fibrin clot were found. However, when fibrin coagulation

\[
\text{Table 2 Prevention of coagulation and restoration of the cytotoxic activity of LAK cells by heparin}
\]

<table>
<thead>
<tr>
<th>Group</th>
<th>Heparin treatment</th>
<th>Fibrin coagulation</th>
<th>% of cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(TC + LAK)</td>
<td>−</td>
<td>−</td>
<td>54.7, 32.3</td>
</tr>
<tr>
<td>(TC + Fb + Thr) + LAK</td>
<td>−</td>
<td>+</td>
<td>8.0, 5.5</td>
</tr>
<tr>
<td>(TC + Fb + Thr + LAK)</td>
<td>+</td>
<td>+</td>
<td>18.9, 5.9</td>
</tr>
<tr>
<td>(LAK + Fb + Thr) + TC</td>
<td>−</td>
<td>+</td>
<td>13.6, 8.4</td>
</tr>
<tr>
<td>(TC + Fb) + LAK</td>
<td>−</td>
<td>+</td>
<td>53.7, 32.9</td>
</tr>
</tbody>
</table>

* Radiolabeled BL6 or 3LL tumor cells (2.5 x 10⁶) and LAK cells (12.5 x 10⁵) were admixed with fibrinogen (1 mg/ml) and thrombin (0.1 unit/ml). In some groups fibrinogen (Fb) and thrombin (Thr) were admixed with tumor cells (TC) and 20 min later LAK cells were added. In some groups the sequence of mixing was opposite or the cell components were mixed simultaneously. The components included in parentheses were mixed first and components outside of the parentheses were added 20 min later. Heparin was added at a final concentration of 2 units/ml. After 4 h of incubation, the level of released radioactivity was measured. Differences in cytotoxicity of greater than 10% were significant (P < 0.05).

*+, no coagulation; +, fibrin coagulation.

\[
\text{Table 3 Effect of fibrin coagulation on the formation of effector-target cell conjugates}
\]

| 3LL tumor cells (2 x 10⁶) were mixed with 2 x 10⁵ LAK cells labeled with fluorescein deacetate in the presence of murine plasma or serum (final dilution, 1:20). In one group heparin (2 units/ml) was added. Cells were incubated 15 min and centrifuged, and the numbers of fluorescing (effector) cells binding to nonfluorescing (target) cells were determined under a UV microscope. |
|---------------------|---------------------|---------------------|
| No. of effector-target conjugates | No. of effector cells | % of conjugates |
| 3LL + LAK + plasma  | 2/202               | 0.7                 |
| 3LL + LAK + plasma + heparin | 58/279              | 20.8                |
| 3LL + LAK + serum   | 54/227              | 19.9                |

\[
\text{Table 4 Cytotoxicity of LAK cells exposed with the target cells before or after fibrin coagulation}
\]

[1°C]-labeled BL6 melanoma cells were mixed with plasma or serum 30 min before or simultaneously with adding of LAK cells. In some groups, plasma or serum were added 15–120 min after exposition of tumor and LAK cells. In all groups final dilutions of plasma or serum was 1:20. The effector/target ratio was 10:1. The cytotoxic effect of LAK cells was evaluated after 4 h of incubation. The differences between groups in more than 10% were found significant (P < 0.05).

| % of cytotoxicity at following times of mixing plasma with target and effector cells |
|---------------------------------|---------------------------------|
| Groups                         | 30 min | 0 min | 15 min | 30 min | 60 min | 120 min |
| BL6 + LAK + plasma             | 10.8    | 31.2   | 59.1   | 59.4   | 58.2   | 61.8     |
| BL6 + LAK + serum              | 59.0    | 60.3   | 60.6   | 59.8   | 58.6   | 63.1     |
| BL6 + LAK                     | 61.0    |        |        |        |        |          |

DISCUSSION

Investigations of mechanisms of interaction of tumor cells with hemostatic factors and the immune system could be important for understanding the process of metastatic spread as well as the mechanisms of the antimetastatic effects of anticoagulant drugs. Numerous experimental data indicate that the formation of thrombotic emboli composed of platelets, fibrin, and tumor cells was observed almost immediately after administration of tumor cells into the vascular system (7–9). Four h later about 84% of tumor cells arrested in the capillaries and arterioles of the lungs were incorporated into platelet-fibrin clot which substantially diminished in 24 h and completely disappeared 48 h after tumor cell inoculation (7–9). Fibrin deposition around tumor cells at this time period could be crucial for their survival. Indeed results of i.v. inoculation of the radiolabeled tumor cells indicate that more than 90–99% of inoculated tumor cells could be eliminated in the first 24 h (1). At the time of disappearance of pulmonary thrombi vast majorities of survived tumor cells accomplished their extravasation (8). The rate of elimination of the radiolabeled tumor cells in the extravascular space substantially reduced and the numbers of surviving radiolabeled cells did not substantially change 2–10 days following tumor cell inoculation (1). Previously we demonstrated that elimination of i.v. inoculated radiolabeled B16 melanoma cells could be significantly increased in mice treated with heparin or warfarin (11, 12). This effect was observed only in the presence of functionally active NK cells since in mice with depressed NK cell function prevention of blood coagulation by anticoagulants did not increase tumor cell destruction in the lungs. Although stimulation of NK cell activity by polyinosinic-polycytidylic acid increased tumor cell elimination and inhibited metastasis formation, the antimetastatic effect of the stimulated NK cells was not optimal. Their efficiency further
increased when blood coagulation was prevented by pretreatment of mice with heparin or warfarin (11, 12). Antimetastatic activity of adoptively transferred spleen NK cells could also be potentiated by prevention of fibrin deposition around tumor cells during their hematogenic spread in the NK reconstituted recipients (12).

These data suggest that fibrin clot could serve as a protective shield which helps tumor cells to escape immune destruction in the blood. Anticoagulant drugs prevent fibrin coating of tumor cells and thus make them more vulnerable to the cytotoxicity of effector cells (11, 12).

In the present study, analysis of the in vitro cytotoxic activity of NK and LAK cells against a variety of murine tumor cells in the presence of whole plasma revealed that fibrin coagulation could protect tumor cells from destruction by NK or LAK cells. The level of inhibition of cytotoxicity paralleled the level of fibrin clotting induced by tumor cells. In contrast, serum did not affect the cytotoxicity of NK or LAK cells. LAK cell cytotoxicity was also prevented when the effector cells were trapped into the fibrin clot. Thus, fibrin deposition around either target or effector cells can increase the chance of tumor cells to escape destruction by the cytotoxic cells. This conclusion was also supported by the experiments in which inhibition of the cytotoxicity was observed when fibrinogen and thrombin instead of whole plasma were used indicating that fibrin formation rather than non-coagulation-related effects were responsible for this inhibition. Tumor cell protection was observed only when fibrinogen was converted by thrombin into fibrin. No effect on tumor cell lysis was found when fibrinogen or thrombin was used separately.

The importance of fibrin clot formation for tumor cell protection was supported by experiments in which heparin prevented fibrin coagulation. Under these conditions, tumor cells were efficiently killed by LAK cells. The direct effect of heparin (2 units/ml) on the effector cells could be excluded by the fact that either in the presence or absence of fibrinogen and thrombin, heparin did not affect the level of LAK cytotoxic activity. Heparin in the dose selected also had no detectable direct cytotoxic effect on target cells.

The protection of tumor cells from the cytotoxic activity of NK or LAK cells by fibrin could be explained by physical segregation and interference with the binding of target and effector cells. Indeed, when fluorescein-labeled LAK cells were used, conjugate formation with tumor cells in the presence of fibrin was prevented.

We have found that tumor cell protection was observed only when fibrin coagulation occurred before formation of target-effector conjugates. Indeed the cytotoxicity of LAK cells was inhibited when plasma was added to tumor cells before LAK cells or simultaneously mixing with target and effector cells. However, when plasma was added to the mixture of tumor and effector cells after 15 min of their contact, plasma coagulation did not affect LAK cell cytotoxicity against BL6 melanoma cells. Failure of fibrin coagulation to inhibit LAK cell cytotoxicity when the tumor-effector conjugates were formed could further support the assumption that fibrin mediated inhibition of LAK cell cytotoxicity was due to the ability of fibrin gel to prevent the intimate contact of target and effector cells rather than by affecting the lytic phase of LAK cytotoxicity.

Thus, the data obtained indicate that fibrin coating of tumor or effector cells could help tumor cells to escape immune destruction in the blood. These findings shed new light on and could help to better understand the biological significance of interaction of the hemostatic and immune system during tumor metastasis formation and the mechanism for the antimetastatic effects of anticoagulant drugs. These data might have broader significance and help to understand the role of fibrin deposition around locally growing primary tumors. It was demonstrated that fibrin gel matrix is a regular stromal component of both autochthonous and transplantable tumors which developed as a result of extravascular leakage of plasma from tumor microvasculature (16, 17). It is considered that fibrin deposition around the tumor could help it to escape immune destruction (16). Our in vitro test of the ability of fibrin to block the cytotoxicity of effector cells could support this possibility.

ACKNOWLEDGMENTS

We would like to thank Drs. R. B. Herberman and S. Shu for the critical review and fruitful suggestions during this work. We are grateful to Dr. G. Tuzchynski for providing us with thrombin and Catherine Fekete for her expertise in typing this manuscript.

REFERENCES

Role of Fibrin Coagulation in Protection of Murine Tumor Cells from Destruction by Cytotoxic Cells

Yoshio Gunji and Elieser Gorelik


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/48/18/5216

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.