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Soluble Fibrin Inhibits Lymphocyte Adherence and Cytotoxicity Against Tumor Cells: Implications for Cancer Metastasis and Immunotherapy

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Circulating soluble fibrin (sFn) is elevated in many cancer patients. It is a marker for ongoing disseminated intravascular coagulation and may have prognostic significance. We have demonstrated that sFn inhibited monocyte adherence and cytotoxicity by a mechanism involving blockade of monocyte αMβ2 and tumor cell CD54. It was, therefore, hypothesized that sFn also inhibits lymphocyte and interleukin-2–activated lymphocyte (LAK) adherence and cytotoxicity against tumor cells. This study sought to identify the lymphocyte subset responsible for adherence and killing of A375 melanoma cells and whether sFn inhibited these parameters. Lymphocyte and LAK cell adherence and cytotoxicity, which was adherence dependent, were inhibited by preincubation with purified or plasma-derived sFn. The lymphocyte and LAK cell activities were primarily a result of CD8+ MHC (major histocompatibility complex) unrestricted cytotoxic T cells. These results suggest that elevated levels of circulating sFn may be immunosuppressive and may reduce the efficacy of adoptive immunotherapies.

Keywords: lymphocyte; cancer; metastasis; fibrin; immunosuppression

Background

Disseminated intravascular coagulation is common in many cancers (reviewed1), and metastasis.2 Prior to overt thrombus formation, disseminated intravascular coagulation is characterized by increased levels of activated coagulation proteins and their products and inhibitors.3 Several of these products, such as tissue factor,4,5 thrombin–antithrombin complex,6 and thrombomodulin,7 are established prognostic markers in cancer. Elevated levels of fibrin clot precursor, soluble fibrin (sFn), are also observed in many cancers,8 and there is some evidence to suggest that circulating sFn may also be prognostically significant.9,10

During normal blood coagulation, fibrin binds to the platelet integrin αIIbβ3, but immobilized fibrinogen and the fibrins (fibrinogen) also have a number of receptor sites for other integrins (e.g., αvβ311 and two of the leukocyte integrins [αmβ212 and αxβ213]). They also adhere to the leukocyte integrin receptor, CD54,14 which is expressed by a wide variety of cells, including many tumor cell lines.

In solid tumors and in experimental systems, the presence of polymerized fibrin has been reported to result in inhibition of the immune response to cancer, particularly using immunotherapies involving lymphokine (interleukin-2 [IL-2])-activated killer (LAK) cells,15 mitogen (phytohemagglutinin) stimulated T-cell blasts, or reinfected tumor infiltrating leukocytes. Furthermore, immunosuppression induced by fibrin degradation products has also been reported.16 In a recent report, regression of melanoma has been...
reported in 2 patients after adoptive transfer of genetically modified CD8+ T cells.17

The authors have previously shown that sFn treatment of melanoma cells enhances metastasis in vivo, in an experimental model,18 and our recent work suggests that sFn inhibits monocyte adherence and killing of tumor cells in vitro.19 The mechanism of inhibition was via its blocking of monocyte αMβ2 and tumor cell CD54. These results suggested that sFn may be an immunosuppressive agent in cancer and that it is involved in the etiology of metastasis. Based on this evidence, it was hypothesized that sFn may similarly inhibit lymphocyte adherence and killing of tumor cells. The aims of this study, therefore, were to characterize the predominant lymphocyte and LAK cell subtype(s) involved in adherence and cytotoxicity of A375 human melanoma cells in vitro and to determine the effect of sFn in these interactions. Inhibition of lymphocyte function by sFn would have important implications in adoptive immunotherapy of cancer in which activated or genetically modified lymphocytes are reintroduced into the circulation of cancer patients to kill circulating metastatic cancer cells and to extravasate and home in on solid tumors.

Methods

Venepuncture
Thirty milliliters of peripheral blood was drawn from normal, healthy volunteers into 3.2% sodium citrate vacutainers (Becton Dickinson VACUTAINER™ Systems, Rutherford, New Jersey). Whole blood was diluted by adding 20 μL of blood into 180 μL 1% crystal violet stain in 0.5% acetic acid. A leukocyte count was performed in an improved Neubauer counting chamber.

Preparation of sFn
For each experiment, sFn was made in the required amount. To produce sFn monomer an excess of GPRP-NH2 is added (4 mM final). To make 1 mL of sFn solution, 25 μL fibrinogen (10 mg/mL; plasminogen–, fibronectin–, and factor-XIII–free; American Diagnostica Inc., Greenwich, Connecticut), 42 μL of the fibrin polymerization inhibitor Gly-Pro-Arg-Pro-amide (24 mM; GPRP-NH2; Sigma Chemical Company, St. Louis, Missouri), followed by 1.25 μL thrombin (100 U/mL; Sigma Chemical Company) were added to 932 μL of RPMI 1640 (RPMI [Roswell Park Institute medium]). For experiments investigating peptide inhibition of sFn binding to lymphocytes and tumor cells, fluorescein isothiocyanate (FITC)–labeled fibrinogen (Molecular Probes, Eugene, Oregon) was used in place of unlabeled fibrinogen above.

For plasma sFn experiments, normal human plasma was diluted to 10% in RPMI containing 4 mM GPRP-NH2.

Culture of Tumor Cells
The A375 human amelanotic malignant melanoma cell line was maintained in continuous cell culture. Cells were detached from plastic using trypsin/EDTA (0.25%; Hyclone, Logan, Utah) and washed in RPMI tissue culture medium containing 10% fetal bovine serum (10% FBS) centrifuged at 200 g, resuspended in 5 mL of 10% FBS, and counted. For static adherence assays, 4 × 10⁴ cells were added to the wells of a 96-well flat bottomed microtiter plate and incubated for 24 to 48 hours or until confluent. For cytotoxicity experiments, cells were incubated with 5 μL of Calcein-AM (Invitrogen, Carlsbad, CA) for 30 minutes, washed, counted, and used at appropriate concentrations in a 96-well, round-bottomed plate, to which effector cells were added.

Isolation of Lymphocytes
Six tubes of citrated blood were diluted 1:1 with RPMI, and 15 mL of it layered over 8 mL AccuPrep™ for lymphocytes (Axis-Shield PoC, Oslo, Norway) in plastic universal containers. These discontinuous density gradients were centrifuged for 25 minutes at 450 g and the mononuclear cell interface removed using a sterile plastic Pasteur pipette. The cells were washed 3 times by resuspension and recentrifugation at 200 g for 10 minutes in 10% FBS so as to remove platelets. The final cell pellet was resuspended in 10 mL RPMI. A cell count was performed by adding 20 μL of blood into 180 μL 0.5% trypan blue vital stain, and cells were counted in an improved Neubauer counting chamber. Mononuclear cells obtained from the AccuPrep™ density gradient were adjusted to 2 × 10⁶ cells/mL and aliquots of 5 × 10⁷ cells added to 75 cm² serum-coated tissue culture flasks. The flasks were incubated at 37°C in 5% CO₂ for 1.5 hours to allow monocyte and natural killer cell adherence to the plastic. The nonadherent cells (lymphocytes) were decanted, washed, and counted.
An aliquot of the final lymphocyte suspension was dried onto a microscope slide for analysis of cell purity. Cell purity was determined by differential counting of lymphocytes and monocytes using the May-Grunwald/Giemsa stain (Sigma, St. Louis, Missouri). In 23 experiments, the mean lymphocyte purity was 95 ± 5%.

**Preparation of IL-2-Activated Lymphocytes**

Nonadherent lymphocytes derived from 60 mL of blood were divided into 2 aliquots containing equal numbers of cells. The cells were centrifuged and the pellets resuspended to 2 × 10^6 cells/mL in either 10% FBS (untreated cells) or 10% FBS containing 2.5 ng/mL IL-2 (LAK cells; R&D Systems, Minneapolis, Minnesota). Lymphocytes were incubated at 37°C upright in 25 cm^2 tissue culture flasks for 72 hours, decanted into universal containers, washed 3 times in RPMI to remove any residual IL-2, and a cell count and viability assessment performed prior to their inclusion in adherence and cytotoxicity experiments.

**Purification of Lymphocyte and LAK Cell Subsets by Fluorescence-Activated Cell Sorting**

Peripheral blood mononuclear cells were incubated for 18 hours in 75 cm^2 tissue culture flasks at a density of 2 × 10^6/mL in 10% FBS. The cells were washed once in azide free-flow cytometry sheath fluid (Facsflow; Becton-Dickinson Immunocytometry Systems, Franklin Lakes, New Jersey) and the cell concentration adjusted to 5 × 10^6/mL in Hanks Balanced Salt Solution (HBSS). The cell preparation was incubated with 2.5 μL of FITC-labeled anti-CD8 monoclonal antibody (Beckman Coulter, Miami, Florida) per 100 μL cells for 20 minutes at room temperature and washed twice in HBSS. An aliquot (100 μL) of unlabeled lymphocytes was also labeled with 2.5 μL isotype IgG1 FITC control (Beckman Coulter, Miami, Florida), incubated for 20 minutes at room temperature, and washed twice in HBSS. All cell preparations were resuspended in 400 μL HBSS per 100 μL cells. The cells were then passed through the cell sorter according to the following procedure.

After preparation of the FACStar Plus (Becton-Dickinson Immunocytometry Systems, San Jose, California) for sterile sorting, the isotype control labeled cells were passed through the flow cytometer to determine the negative to positive delineator for FITC. A “sort gate” was placed around the unlabeled cells, which resulted in the collection of lymphocytes depleted of CD8+ cells. Approximately 5 × 10^6 to 10 × 10^6 cells were collected for inclusion in the cytotoxicity assay. In all experiments, depletion of CD8+ cells was >95% and viability was >97%.

To determine if the passage of the cells through the cell sorter alone had any effect on subsequent lymphocyte activity, preliminary experiments were performed in which unlabeled lymphocytes were passed through the cell sorter and their cytotoxicity compared with that of fresh matched lymphocytes (ie, cells that had not been passed through the cell sorter) that were added directly onto the target cells (untreated A375 melanoma cells). In 3 experiments, no significant difference in tumoricidal activity was observed between unsorted and sorted lymphocytes, demonstrating that passage through the cell sorter did not significantly affect lymphocyte killing of A375 cells (P > .05).

**Cellular Adherence Assay**

Tumor cells were detached from plastic using trypsin (0.25%)/EDTA (0.1 μM) and washed in RPMI. Forty thousand cells (in 200 μL 10% FBS) were added to each well of a 96-well, flat-bottomed tissue culture plate and incubated at 37°C until confluent. Six wells were trypsinized and the cells mixed 1:1 with Trypan blue stain (0.5% in phosphate buffered saline), and counted in a hemocytometer. The mean cell count was recorded. Adherent cells were left untreated (controls) or incubated with sFn, fibrinogen (0.5 mg/mL), GPRP-NH2 (4 mM), or thrombin (0.125 U/mL), in the presence or absence of monoclonal blocking antibody (CD54).

After washing with RPMI, 5 μL of Calcein AM (Invitrogen, Eugene, Oregon) stock solution in DMSO was added to 5 mL of effector cell preparation (lymphocytes; 5 × 10^6 cells/mL) and incubated for 30 minutes at 37°C. Effector cells were left untreated (controls) or incubated with sFn or fibrinogen (0.5 mg/mL), GPRP-NH2 (4 mM), or thrombin (0.125 U/mL). In antibody blocking experiments, cells were also incubated in the presence of specific blocking antibody alone or combined with sFn. The total fluorescence was determined by the addition of 200 μL of fluorescently labeled effector cells to 3 wells, and the fluorescence was measured.
Minimal fluorescence (blank) was measured in 3 wells containing adherent cells to which only RPMI was added. After a further wash, cells were made to $1 \times 10^6$/mL and 200 μL added to appropriate wells, and this was incubated at 37°C for 1 hour. The plates were washed 3 times with RPMI followed by addition of 200 μL of 0.5 M NaOH to lyse the cells. The supernatants were removed into black, 96-well microtiter plates, and fluorescence was measured on a Perkin-Elmer Victor 3 plate reader. Specific adherence was determined using the following expression:

$$\text{(Test – Blank)/Total – Blank) \times 100\%}.$$

Experiments were performed in triplicate wells and the results were expressed as the mean ± SD for 3 separate experiments.

### Measurement of Cellular Cytotoxicity

Tumor cells were detached from plastic using trypsin (0.25%)/EDTA (0.1 μM), washed, and resuspended in 2 mL of culture medium. Five microliters (5 μg) of Calcein AM was added and the cells were incubated at 37°C for 30 minutes, washed, and counted. Cells were left untreated or preincubated as described in the previous section depending on the experiment to be performed. The cells were counted and made to $1 \times 10^5$ cells/mL. One hundred microliters of labeled tumor cells was added to appropriate wells of a 96-well, round-bottomed microtiter plate. To measure spontaneous dye release, 100 μL of 10% FBS was added to 3 wells containing tumor cells. Total dye release was determined by the addition of 100 μL of 6% sodium dodecyl sulfate to 3 wells containing tumor cells.

One hundred microliters of untreated or appropriately preincubated lymphocytes or LAK cells were added to the tumor cells in appropriate wells at effector–target cell ratios of 1.6:1, 3.1:1, 6.3:1, 12.5:1, 25:1, and 50:1, and the plates were incubated at 37°C for 18 hours. The plates were then centrifuged at 250 g for 5 minutes, and 100 μL of supernatant was carefully removed into the appropriate wells of a black, 96-well, flat-bottomed plate. Fluorescence was measured on a Perkin-Elmer Victor 3 fluorescence microplate reader. Specific cytotoxicity was determined by the following expression:

$$(\text{Test release – Spontaneous release}/\text{Total release – Spontaneous release}) \times 100\%.$$
on slides. Cells were then imaged on an Olympus BX61 fluorescence microscope equipped with a long pass 535 nm dichroic filter.

**Statistical Analysis**

In static microplate adherence and cytotoxicity assays, each data point was obtained as the mean ± SD for 3 replicates. Each experiment was performed at least 3 times. Student’s *t* test for independent variables was used to determine significant differences between groups.

One goal of the proposed study was to test the null hypothesis that the population means (sFn pretreatments) are 0.00 compared with the control mean (−/−). The criterion for significance (α) was set to 0.05, and the mean standard deviation value was incorporated into the analysis. The test is two-tailed, which means an effect in either direction would be interpreted. With the proposed sample size of 3 cases, this study has a power of 81.3% to yield a statistically significant result.

**Results**

**Adherence of sFn to Lymphocytes and Tumor Cells**

Lymphocytes or tumor cells were incubated alone or in the presence of fluorescently labeled sFn. Fig. 1 shows considerable binding of sFn to lymphocytes (A) and tumor cells (C), whereas no fluorescence was observed on cells incubated in the absence of sFn.

**Effect of sFn on Lymphocyte and LAK Cell Adherence to Tumor Cells**

Experiments (*n* = 3) were performed to determine the effect of sFn preincubation of either lymphocytes, tumor cells, or both on lymphocyte adherence to A375 melanoma cells in static microtiter plate assays (Fig. 2). In the absence of sFn, lymphocyte adherence was 29.9 ± 8.8%. Pretreatment of tumor cells with sFn significantly (*P* < 0.05) inhibited adherence by 19 ± 11%, compared with untreated controls. Addition of sFn-treated lymphocytes to untreated tumor cells also inhibited adherence by 44 ± 22% (*P* < 0.01 compared with untreated control) and to a significantly greater degree than tumor cell pretreatment with sFn (*P* < 0.05 compared with sFn-treated tumor cells). Maximal inhibition of adherence was observed (78 ± 21%; *n* = 3; *P* < .01 compared with untreated controls and sFn pretreatment of tumor cells; *P* < .05 compared with sFn pretreatment of lymphocytes alone; *n* = 3) when both lymphocytes and tumor cells were pretreated with sFn. LAK cell adherence to untreated A375 cells was 29.6 ± 9.3%. Preincubation of LAK cells and tumor cells with sFn also significantly inhibited LAK cell adherence (*P* < .01 compared with untreated cells) by 70.0 ± 6.2%.

**Effect of sFn on Lymphocyte and LAK Cell Cytotoxicity Against Tumor Cells**

Lymphocyte cytotoxicity against A375 melanoma cells was measured in a static microplate assay (Fig. 3). In the absence of sFn (effector–target cell ratio, 50:1) lymphocyte cytotoxicity was 29.7 ± 7.21% (*n* = 3). Pretreatment of A375 cells or lymphocytes alone with sFn significantly (*P* < 0.05; *n* = 3) inhibited cytotoxicity (29.4 ± 1.6% and 32.8 ± 4.5% inhibition respectively) compared with the untreated control, but maximal inhibition (*P* < 0.01 compared with untreated control; *P* < 0.05 compared with individual sFn cell treatment) was observed when both lymphocytes and tumor cells were sFn treated (52 ± 4.1%; *n* = 3). Pretreatment with individual sFn components (fibrinogen, thrombin, or GPRP-NH2) was not inhibitory. Significant inhibition of cytotoxicity (55.0 ± 4.9%; *P* < .01; *n* = 3) was also observed in the presence of recalcified citrated human plasma in the presence of GPRP-NH2 compared with recalcified normal human serum in the presence of GPRP-NH2 (cytotoxicity in serum was not significantly different from that in 10% FBS). Preincubation of both LAK and tumor cells with sFn significantly (*P* < .01 compared with untreated LAK cells; *n* = 3) inhibited LAK cell killing by 70.5 ± 6.2%, from 67.6 ± 0.8% to 19.9 ± 4.2%.

**Requirement for Cell Contact for Lymphocyte and LAK Cell Cytotoxicity Against Tumor Cells**

To investigate whether cell–cell contact was necessary for lymphocyte cytotoxicity against A375 melanoma cells, effector cells were physically separated from target cells in the culture wells by polycarbonate culture well inserts that were porous to soluble molecules but would not allow the passage of cells. Cytotoxicity observed under these conditions was compared with that in which the membranes
were absent (Fig. 4). In 3 experiments performed in 24-well microplates, lymphocyte cytotoxicity was 22.2 ± 6.2%. Separation of lymphocytes from tumor cells resulted in only minimal killing (2.1 ± 1.8%), which was significantly lower than direct lymphocyte killing (*P* < 0.01; *n* = 3). Similarly, LAK cell cytotoxicity was 67.8 ± 3.6% when cells were in direct contact, but physical separation reduced killing to 1.8 ± 0.9% (*P* < .01 compared with direct contact; *n* = 3). Supernatants taken from cultured lymphocytes and LAK cells were also ineffective in cytotoxicity assays (data not shown), confirming that the reduction of cytotoxicity in membrane-containing wells was not an artifact of the presence of membranes in these wells.

**Subset-Depleted Lymphocyte and LAK Cell Killing of A375 Cells**

Lymphocyte and LAK cells were depleted of CD8+ cells by fluorescence cell sorting, and their cytotoxicity against A375 cells was compared with that of
the whole cell population in each case. Fig. 5 shows the dose–response curves for lymphocyte- and CD8+− depleted lymphocyte cytotoxicity. Maximal killing (20 ± 4.3%; n = 3) was observed at an effector–target cell ratio of 50:1. Depletion of CD8+ cells resulted in a marked decrease in lymphocyte tumoricidal activity by 86 ± 7% to 2.8 ± 2.4% (n = 3) specific cytotoxicity (P < .01 compared with undepleted cells). LAK cell cytotoxicity (Fig. 6) was also maximal (88 ± 3.3%) at the highest effector–target cell ratio tested (50:1). CD8+ depletion reduced cytotoxicity by 68 ± 4% to 29 ± 2.4% (n = 3).

Discussion

The aim of this study was to determine if sFn, which is elevated in the circulation of many cancer patients, affected the ability of lymphocytes to adhere to and kill tumor cells in vitro. Initial experiments demonstrated that sFn bound to both lymphocytes and tumor cells (Fig. 1). Because many T lymphocytes do not express αMβ2, adherence was probably via another integrin such as αxβ2, αxβ1 or αxβ3, although no experiments were performed in this study to determine the exact mechanism of binding. In standard microplate adherence assays, sFn preincubation of either lymphocytes or tumor cells inhibited adherence, but maximal inhibition was observed when both cells were sFn pretreated prior to inclusion in the assay, which compared well with the inhibition observed for monocyte binding to tumor cells, described previously.19 This may be physiologically important because it is likely that in patients in whom sFn is elevated, leukocytes (or adoptively
transferred leukocytes) or tumor cells would be more likely to be coated with sFn prior to encountering each other in the circulation. Because sFn is made by combining fibrinogen with thrombin in the presence of GPRP-NH2, these components were tested individually for their ability to affect lymphocyte adherence to tumor cells. Slight inhibition was observed when both cells were fibrinogen treated, but no effect on adherence was observed using either thrombin or GPRP-NH2 (data not shown). LAK cell adherence was also inhibited by nearly 70% when both cells were sFn treated. Lymphocyte cytotoxicity was inhibited by sFn in a similar pattern to adherence, and maximal inhibition was again observed when both lymphocytes and tumor cells were sFn pretreated. As a more physiologically relevant control, sFn was formed in recalcified citrated human plasma in the presence of GPRP-NH2. Pretreatment of both cells with sFn-containing plasma inhibited cytotoxicity to a similar degree to sFn prepared from pure reagents. LAK cell killing was also inhibited by 70% in the presence of sFn.

Figure 4. Effect of effector–target cell separation on lymphocyte cytotoxicity. Lymphocyte and interleukin-2–activated killer (LAK) cell cytotoxicity of A375 cells was assessed after 18 hours incubation in the presence or absence of 0.8 mm pore size polycarbonate filters. In 3 experiments (means and SDs are shown), both lymphocyte and LAK cell killing was almost completely inhibited by the presence of the membranes between the cells (n = 3).

Figure 5. Lymphocytes cultured in RPMI 1640/fetal bovine serum (RPMI/FBS) for 24 hours were depleted of CD8+ cells (>96% depletion in 3 experiments), and their cytotoxicity against untreated A375 cells was compared with that of the whole lymphocyte population. In 3 experiments (>97% viability of both effector cell populations), lymphocytes were cytotoxic at an effector–target cell ratio (E:T) of 6.3:1 (compared with spontaneous release; P < .05), reaching a maximum at 50:1 (P < .05 compared with 25:1). Depletion of CD8+ cells resulted in almost complete inhibition of cytotoxicity (compared with the whole population; P < .05 at E:T ≥ 6.3:1).

PBL, Peripheral blood lymphocyte.

Separation of lymphocytes by nonadherence to plastic yields a heterogeneous population containing several lymphocyte subsets, including CD4+ and CD8+ T cells, B lymphocytes, and some NK cells. Some lymphocytes require adherence to their targets to be cytotoxic, whereas others can release cytolytic molecules such as interferons and tumor necrosis factors. To determine if one particular subset was predominant in adherence and killing of A375 melanoma cells in our assays, effector and target cells were physically separated by a porous polycarbonate membrane. Under such conditions, both lymphocyte and LAK cell cytotoxicity was almost completely inhibited, demonstrating that cell adherence followed by delivery of the lethal cytotoxic event was required, rather than bystander killing. This was strengthened by the observation that supernatants collected from cultured lymphocytes and LAK cells were not cytotoxic against...
A375 cells (data not shown). In further experiments, lymphocytes and LAK cells were depleted of the CD8\(^+\) T-cell population (Figs. 5 and 6) before their inclusion in cytotoxicity assays. Removal of CD8\(^+\) cells significantly reduced the cytotoxicity by both lymphocytes and LAK cells, indicating that the cytotoxicity observed in sFn inhibition experiments was primarily the result of the action of CD8\(^+\) MHC unrestricted cytotoxic lymphocytes.

The results of this study have clearly demonstrated that sFn binds to lymphocytes, LAK cells, and tumor cells, resulting in a profound inhibition of both cellular adherence and cytotoxicity.

Because elevated levels of sFn are observed in many cancer patients, the observations in this study would suggest that circulating lymphocytes as well as activated (expressing CD54) endothelial cells will bind this fibrin, possibly resulting in a decreased immune response against the tumor. Furthermore, this mechanism may also adversely affect the ability of reintroduced activated lymphocytes during adoptive immunotherapy to home in on and eradicate tumor cells. Further studies are thus needed to test the physiological relevance of this hypothesis in in vivo models.

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