Fibronectin Enhances In Vitro Monocyte-Macrophage-Mediated Tumoricidal Activity

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Control of neoplastic proliferation reflects in part monocye/macrophage destruction of target cells—destruction that evidently requires cell–cell interaction. We herein show it to involve the natural plasma opsonin, fibronectin. With two cultured human tumor lines—Malme melanoma and CAV-1 renal carcinoma cells—addition of fibronectin, purified to homogeneity, enhances macrophage-mediated cytotoxicity 2–4-fold (p < 0.01). Both fresh human monocytes or the U-937 cultured macrophage line become more lethal to tumor cells with added fibronectin. The fibronectin-enhanced monocyte and U-937 tumoricidal activity occurred in a dose-dependent fashion. Specificity of fibronectin’s action was validated by use of affinity-purified rabbit antifibronectin antibody, which completely abated its enhancement of tumoricidal activity. Enhancement of tumoricidal activity did not occur when monocytes or U-937 were exposed to fibronectin-coated plates. However, the addition of soluble fibronectin to fibronectin-coated plates was then capable of enhancing cytotoxic activity. These studies demonstrate that human fibronectin is capable of increasing both fresh and cultured human monocyte tumor-directed cytotoxicity. Fibronectin appears to be a potentially important circulating molecule that may favorably influence human monocyte tumor cell cytotoxicity.

PREVIOUS INVESTIGATIONS have demonstrated the importance of macrophage and monocytes in the control of neoplastic proliferation. Murine studies have shown that activation of macrophages by administration of Bacillus Calmette-Guerin (BCG) increases resistance to transplantable tumors,1 while inhibition of macrophage lysosomal enzyme function by trypan blue administration decreases resistance to transplantable tumors.2 In man, it is known that tumors are often infiltrated with macrophages3,4 and that a given tumor’s metastatic potential may be governed, to some extent, by the number of resident macrophages.1,5,6 For example, patients with breast carcinoma with increased numbers of resident macrophages infiltrating the primary tumor site have a lower incidence of axillary lymph node metastasis5 and patients with involvement of axillary nodes that show sinus histiocytosis have a lower incidence of distant metastasis.7 The potential role of monocyte–tumor-cell interaction has been strengthened by the recent demonstration of human monocyte and macrophage cytotoxicity for tumor cells in vitro.8

The mechanisms of macrophage surveillance function are not completely clear but seem dependent on the presence of plasma recognition factors or opsonins.8–12 That these plasma factors are relevant to malignant growth in vivo is suggested by the finding that plasma from patients with carcinoma is depleted of recognition factors,13,14 which are restored following surgical or radiation treatment of the patients’ primary tumors.15

We have become interested in the possible role of fibronectin as a plasma recognition factor capable of modulating tumor growth. Fibronectin is a major nonimmune opsonin15,16 and is important in various cell–cell interactions and in connective tissue structure.17,18 Fibronectin has been found within the connective tissue stroma of solid tumors.17 Moreover, endogenous production and secretion of fibronectin by human macrophages has been shown,19 and recently, the presence of plasma membrane receptors for fibronectin of human peripheral blood monocytes has been demonstrated.20

The present in vitro studies demonstrate a potent enhancement of both monocyte and macrophage tumor cell cytotoxicity in the presence of highly purified fibronectin.

MATERIALS AND METHODS

Monocytes

After informed consent was obtained, heparinized peripheral blood was obtained from normal volunteer donors. A mononuclear cell interface was obtained by Ficoll-Hypaque centrifugation.21 Interface cells were washed in Seligman’s balanced salt solution (SBSS) three times. Differential and total cell counts were performed. Interface cells contained 25–45% monocytes and 55–75% lymphocytes. Interface cells were suspended to 5 x 10⁶ monocytes/ml in RPMI-1640 with 20% autologous serum. Aliquots of 0.1 ml were placed in individual 6-mm diameter microttes (Limbro, New Haven, Conn.) and were incubated for 1 hr at 37°C with 5% CO₂. Cells were then washed with cold HBSS and 0.1 ml of final
media consisting of RPMI-1640 with 20% pooled heat-inactivated AB sera, 100 μg/ml penicillin, 100 μg/ml streptomycin, 0.3 mg/ml glutamine, and 0.075% NaHCO₃ was added to the monocyte monolayers. Monolayers were >95% monocytes and contained 0.8–1.2 x 10⁶ cells.

**U-937 Cell Line**

The U-937 cell line (gift from Dr. H.S. Koren, Duke University) was derived from a pleural effusion of a patient with histiocytic lymphoma and possesses macrophage-like characteristics. This cell line was adapted to rapid growth and exhibits marked augmentation of ADCC against erythroid and tumor target cells. Under usual conditions, these cells are nonadherent and do not express Ia antigens. These cells are termed macrophages in this report. They are routinely maintained in RPMI-1640 supplemented with 5% fetal calf serum (KC Biological, Lenexa, Kans.). For experiments, 10⁶ cells were plated into 6-mm microwells in 0.1 ml of final media (see above) and incubated at 37°C with 5% CO₂ for 1 hr.

**Tumor Cells**

Two tumor cell lines were studied: Malme-3M, a melanoma cell line, and CAK-1, a renal carcinoma cell line. They were maintained in culture as previously described.

**Cytocidal Assay**

Tumor cells were prelabeled with ³H-thymidine (New England Nuclear, Boston, Mass.) 24 hr prior to addition to microwells. Tumor cells were added to empty control wells or to monocyte/macrophage monolayers and incubated at 37°C with 5% CO₂. Cells were harvested after 48 hr. Wells were emptied, washed 5 times with cold Hank's balanced salt solution (HBSS), and the contents of each well were removed with cotton swabs as previously described. The swabs were allowed to dry for 24 hr and then the cotton tips were counted in liquid scintillation fluid. Macrophages or monocytes did not incorporate thymidine. Percentage cytocidal activity was determined by the formula:

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\text{1 - \frac{\text{cpm target cells + effector cells}}{\text{cpm target cells alone}} \times 100}
\]

**Preparation of Fibronectin**

Fibronectin was isolated from pooled citrated human plasma on a 2.5 x 20 cm gelatin-agarose affinity column prepared according to the method of Cuatrecasas and Anfinsen. The column was equilibrated in phosphate-buffered saline (PBS), pH 7.4, prior to sample application. This same buffer was used to wash nonbound material from the column. Fibronectin was eluted with 0.02 M sodium acetate, 1.0 M sodium bromide, pH 5.

After extensive dialysis against 0.02 M sodium phosphate, 0.075 M NaCl, pH 7.4, fibronectin was further purified on a diethylaminoethylcellulose column (Whatman DE 52). Fibronectin was then eluted with a linear sodium chloride gradient (0.075–0.3 M) and was concentrated by precipitation with 50% ammonium sulfate. The purity of the fibronectin preparations was routinely assessed by reduced sodium dodecyl sulfate polyacrylamide gel electrophoresis.

**Coating of Plates**

Purified fibronectin was diluted to the appropriate concentrations (see below) in carbonate buffer, pH 9.6, and dispensed into plates used for the cytotoxicity assay. After incubation at 37°C for 2 hr, the plates were washed 3 times with HBBS and used for the cytotoxicity assay. Parallel plates were prepared as described, washed, and examined qualitatively for the presence of fibronectin by an enzyme-linked immunosorbant assay procedure (ELISA). Briefly, the washed plates were incubated for 15 min in the presence of rabbit antifibronectin, wells were washed 3 times with phosphate-buffered saline containing 0.5 M NaCl, 0.5% Tween-80 (PBS-Tween), and goat anti-rabbit immunoglobulin coupled to peroxidase (Cappel Lab, Cochranville, Pa.) was added. The plates were then incubated for an additional 15 min. After 3 washes with PBS-Tween, 0.1 ml of 4 M o-phenylenediamine in 0.1 M Na₂HPO₄, 0.05 M citric acid, pH 5.0, with 0.001 ml 30% H₂O₂/ml was added to the wells. The resulting colorimetric reaction was terminated by the addition of 0.05 ml of 2.5 M H₂SO₄ and the plates were inspected visually.

**Affinity Purification of Antibody**

Affinity-purified rabbit antifibronectin was prepared from pooled rabbit antisera. Briefly, pooled antisera were applied to a fibronectin-agarose column equilibrated in PBS (bed volume approximately 20 ml). After washing of the column with PBS to remove nonbound material, the antibody was eluted with 0.2 M glycine, pH 2.3. The eluate was immediately neutralized with addition of 1 M Tris, pH 11, and was subsequently dialyzed against PBS. The reactivity of the purified antibody was verified as described above by ELISA.

**Reagents**

Heparin (1000 U/ml) was obtained from Organon (West Orange, N.J.).

**Statistical Analysis**

The significance of differences between mean values was calculated by Student’s t test.

**RESULTS**

The collective series of monocyte and macrophage cytocidal activities measured at 48 hr is shown in Fig. 1. Macrophage (U-937) cytocidal activity was greater than monocyte cytocidal activity (p < 0.01) against each tumor cell line studied. Moreover, the addition of soluble fibronectin consistently enhanced monocyte and macrophage tumoricidal activity for both tumor cell lines studied (p < 0.01) (Fig. 2), with the macrophage continuing to manifest the greatest tumoricidal activity with similar fibronectin concentrations. Fibronectin enhanced monocyte–macrophage tumoricidal activity in a log-dose dependent manner (Fig. 3). While no enhancement of cytocidal activity was seen at 2 μg/ml fibronectin, cytocidal activity was increased at 20 μg/ml and was striking at levels of fibronectin present in normal human plasma (200 μg/ml). It should be noted that the sera used in these studies contained fibronectin at a concentration less than 80 μg/ml; therefore, the amount present in any of these assays is less than 16 μg/ml, greater than an order of magnitude outside the effect range. Additionally, neither adherence nor viability was affected by the addition of the various fibronectin concentrations.

We next sought to determine if this enhancement of monocyte–macrophage tumoricidal activity noted was the result of the fibronectin providing a better substra-
activity to levels obtained with soluble fibronectin alone. Thus, the enhancement of monocyte–macrophage tumoricidal activity by fibronectin appears likely to be the result of a bridging effect of soluble fibronectin, resulting in increased monocyte–macrophage and tumor cell interaction.

To further define the specificity of the fibronectin enhancement of monocyte–macrophage tumoricidal activity, the effect of affinity-purified antibody to human fibronectin was evaluated. Affinity-purified rabbit antibody to human fibronectin was capable of completely blocking enhancement of monocyte–macrophage tumoricidal activity by fibronectin (Fig. 5). Additionally, antibody-associated blockade of fibronectin-enhanced tumoricidal activity was concentration dependent. Complete blockade of fibronectin-enhanced monocyte–macrophage tumoricidal activity was present both at 1/1 and 1/10 antibody dilutions. No blockade of fibronectin enhancement of monocyte–macrophage tumoricidal activity was present at a 1/1000 antibody dilution.

Fibronectin is known to have binding sites for heparin. In order to determine if these sites were involved in fibronectin-enhanced monocyte–macrophage tumoricidal activity, 10 U of heparin (1000 U/ml) were added to wells containing soluble fibronectin. Tumoricidal activity was decreased to similar levels seen in wells containing no fibronectin in 5 of 6 experiments using monocytes as the effector cell (5.1% ± 6% versus 7.6% ± 3.4%, mean ± SEM). This inhibitory effect by heparin on fibronectin-enhanced tumoricidal activity was not consistently seen in experiments using macrophages.
FIBRONECTIN ENHANCES TUMORICIDAL ACTIVITY

DISCUSSION

Peripheral blood monocytes, macrophages, and other mononuclear phagocytic cells of the reticuloendothelial system perform critical functions in inflammation and immunity. Recently, the role of these cells in the host's reaction to malignant growth has been increasingly recognized. A large body of data now indicates that certain plasma proteins can regulate monocyte-macrophage cell function. In this study, we have shown that one of these plasma proteins, fibronectin, a major nonimmune "recognition factor" or opsonin, enhances monocyte- and macrophage-mediated tumoricidal activity. The fibronectin-enhanced monocyte-macrophage tumoricidal activity was dose dependent with maximal tumoricidal activity at 200 μg/ml, which is approximately a physiologic plasma concentration. This enhancement does not appear to be the result of promoted monocyte and macrophage adhesion to substratum by fibronectin, as demonstrated by the absence of any increased tumoricidal effect when fibronectin-coated plates alone were used. Only when soluble fibronectin was added to the effector-target cell suspension was enhanced tumoricidal activity noted, suggesting that fibronectin is providing a bridging effect accounting for increased effector-target cell interaction. Besides the observation that affinity purified fibronectin produced enhanced monocyte-macrophage tumoricidal activity, we also demonstrated that rabbit antibody to human fibronectin blocked fibronectin-induced enhancement of monocyte-macrophage tumoricidal activity.

The further elucidation of the mechanism remains for future studies, but is suggested by the observation that heparin could block fibronectin-mediated enhancement of monocyte tumoricidal activity. Although heparin will precipitate fibronectin at 4°C, this explanation for the effect of heparin in these experiments seems unlikely, since the assay conditions do not favor heparin precipitation of fibronectin. An alternative explanation is that regions on the fibronectin molecule that are identical with or close to the heparin binding
domains are involved in promoting monocyte-mediated tumoricidal activity.

Recently, the use of peptide mapping and monoclonal antibodies\textsuperscript{32,33} has defined unique heparin binding domains in human fibronectin. One heparin binding domain is at the amino terminus in the region that binds fibrin, staphylococci, and adjacent to the collagen binding region. The second heparin binding domain in human plasma fibronectin is in the carboxyl terminal 25\%–35\% and is near the cell binding region. This proximity of the heparin and cell binding domains could possibly account for the heparin blocking of cytotoxicity by virtue of altering the binding of fibronectin to cells.

Regardless of mechanism, previous studies have shown marked decreases in plasma levels of "recognition factors" or opsonins in malignant disease\textsuperscript{13,14} with a direct relationship noted between the stage of disease and the levels of these plasma proteins.\textsuperscript{34} In vitro studies in rats using Shay chloroleukemic cells have shown that injection of leukemic cells but not normal leukocytes is accompanied by the rapid loss of opsonic or recognition factor activity,\textsuperscript{35} suggesting that the formation of tumor-cell–opsonin complexes may be an early event in neoplastic transformation and proliferation. Macrophages, monocytes, and other mononuclear cells of the reticuloendothelial system function most effectively in the presence of opsonins found in normal human plasma.\textsuperscript{9,12} The formation of these complexes may provide important assistance in the recognition and destruction of tumor cells by monocytes and macrophages and thus may be an important regulatory role of fibronectin for maintaining a normal environment. Administration of a heparin-precipitable recognition factor fraction from normal human plasma that would include fibronectin along with Shay chloroleukemic cells to rats resulted in an 86\% decrease in tumor weight compared with rats receiving only leukemic cells.\textsuperscript{36}

Surface-bound fibronectin has been shown recently to mediate attachment of human peripheral blood monocytes to gelatin-coated surfaces, indicating the presence on human monocytes of membrane receptors for fibronectin.\textsuperscript{29} Macrophages previously have been shown to synthesize and secrete fibronectin.\textsuperscript{19} In our studies, macrophages consistently have greater baseline and fibronectin-enhanced tumoricidal activity than do monocytes. It is interesting to speculate that this increased cytotoxic activity of the macrophage compared to the monocyte might be the result of an increased capability for endogenous fibronectin production.

These studies reemphasize the important role of monocytes and macrophages in the regulation of neoplastic proliferation. The importance of the major nonimmune opsonin fibronectin in enhancing monocyte–macrophage tumoricidal activity is defined. It would be our hope that a better understanding of host regulatory mechanisms for the control of neoplastic proliferation might be utilized in planning future attempts at treating malignant conditions in man.
REFERENCES

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