Concentration of Fibronectin in Plasma of Tumor-bearing Mice and Synthesis by Ehrlich Ascites Tumor Cells

Luciano Zardi, Claudio Ceconi, Ottavia Barbieri, Barbara Carnemolla, Marina Picca, and Leonardo Santi

Instituto Scientifico per lo Studio e l’A Curaper dei Tumori, Instituto di Oncologia Università di Genova, Viale Benedetto XV, 10, 16132 Genova, Italy

ABSTRACT

In the present paper we have studied: (a) the concentration of fibronectin (FN) in plasma and in ascitic fluid of mice at different times after inoculation of Ehrlich ascites tumor cells; (b) the ability of Ehrlich ascites cells to synthesize and release FN; and (c) the localization of FN in Ehrlich ascites cells by immunofluorescence microscopy.

It was found that (a) 4 to 5 days after inoculation of the tumor, the plasma concentration of FN was significantly higher [1.7 ± 0.07% (S.E.) of total plasma protein] than that in the normal control mice (0.8 ± 0.035); (b) FN is present in the ascitic fluid in all phases of tumor growth; (c) Ehrlich ascites cells cultured in vitro synthesize and release large amounts of FN in the culture medium; and (d) only about 1 to 2% of the tumor cells show a very small amount of FN, and this is mostly in the area of cell-cell contact.

INTRODUCTION

Human plasma FN was reported for the first time in 1948 by Morrison et al. (12) and further purified and characterized by the group of Mosesson (13, 14) who referred to this protein as “cold-insoluble globulin.”

FN, a glycoprotein with the electrophoretic mobility of a β,-globulin, is present in human plasma at a concentration of 300 to 400 µg/ml (14). It is a multichain molecule of which the subunits, with a molecular weight of 220,000, are linked by disulfide bridges (13, 15). It has recently been reported that plasma FN may play an important role in the removal from the blood of neoplastic cells, collagenous particles, and other debris via sessile macrophages of the reticuloendothelial system (1, 2).

In recent years, a protein immunologically similar to FN has been found in the periphery of primitive mesenchymal cells, fibroblasts, and astroglia cells (25, 29). The same antigen has been identified by immunofluorescence microscopy in vertebrate basement membranes and loose connective tissue (10, 23). This protein had been previously known as surface fibroblast antigen (10), large external transformation-sensitive protein (8), cell surface protein (28), and galactoprotein “α” (7). One of the most intriguing observations is the absence or decrease of FN on the surface of most transformed cell lines (Refs. 17, 24, 27, and references therein). There is, in fact, a striking correlation between the absence of FN from the cell surface and several characteristics which are associated with transformation.

A protein analogous to human FN seems to be present in other vertebrate species (24, 27). In mice, plasma and certain cell surfaces have revealed a protein with biological and physicochemical characteristics very similar to that of the human FN (4, 31). Most previous studies on the function of FN have been in vitro with cultivated fibroblasts in which FN is a major cell surface-associated glycoprotein. While studies on cultured cells are a rich source of information on malignant phenotypes, they may not reveal all facets of the complex biology of cancer cells in vivo (23).

To contribute to the study of the function of FN in neoplasms in vivo, we have studied the concentration of FN in plasma and ascitic fluid of mice inoculated with Ehrlich ascites tumor cells, the localization of FN in Ehrlich ascites cells, and their ability to synthesize this protein.

MATERIALS AND METHODS

Preparation of Plasma and Ascitic Fluid. Ehrlich ascites tumor cells were maintained in the peritoneal cavities of 20- to 25-week-old female Swiss mice by weekly transfer. The survival time of infected animals was about 15 days.

To study the concentrations of FN in plasma and ascitic fluids, animals were inoculated with 5 × 10⁶ cells. At various times (days) after inoculation, blood was collected by puncture from the abdominal cavity. About 2 IU of heparin per ml were added to the blood and tumor cell suspension; plasma and ascitic fluid were obtained by centrifugation at 2000 × g for 30 min at room temperature. The total protein concentration of plasma and ascitic fluids was determined by the biuret method (26) using Boehringer reagents (Mannheim, W. Germany).

Antiserum to Mouse Fibronectin. Specific rabbit antiserum to mouse FN was prepared as described by Zardi et al. (30).

Mouse plasma FN was purified using gelatin (Difco Laboratories, Inc., Detroit, Mich.) coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) as described by Engvall and Ruoslahti (5).

Twenty ml of mouse plasma were passed through a 2- × 20-cm plain Sepharose 4B chromatography column previously equilibrated with PBS to remove material binding to Sepharose. The proteins eluted were pooled and recycled for 16 to 20 hr through a 1.5 × 20-cm gelatin/Sepharose 4B column, equilibrated with PBS, at a flow rate of 27 ml/hr at room temperature. The column was then washed with 200 ml of PBS and 200 ml
of 4 M NaCl/0.05 M Tris-HCl, pH 7.5, to remove nonspecifically bound proteins. Finally, FN was eluted with 3 M urea in 0.05 M Tris-HCl, pH 7.5, dialyzed extensively against PBS, and used to immunize rabbits.

Four injections, each containing 100 µg of purified FN in PBS, homogenized in an equal volume of complete Freund's adjuvant (Difco Laboratories, Inc.) were given intradermally on the first, eighth, 16th, and 24th days. A s.c. booster injection was given 7 days later. The antiserum was collected 7 days after the booster and absorbed with Sepharose conjugated to FN-free mouse plasma proteins obtained by passing plasma through gelatin-Sepharose 4B as described by Zardi et al. The antiserum obtained was monospecific as determined by immunoelectrophoresis (Fig. 1).

Radial Immunodiffusion, Immunoelectrophoresis, and Electroimmunoassay. Radial immunodiffusion in 1% agarose (Bio-Rad Laboratories, Richmond, Calif.) was carried out as described by Mancini et al. (11).

The procedure of Ouchterlony and Nilsson (18) was used for immunoelectrophoresis, and the procedure described by Laurell (9) was used for electroimmunoassay.

Cells. Ehrlich ascites tumor cells were removed from the abdominal cavity of mice 6 days after cell inoculation and cultivated in vitro as described by Paul (20) in MEM (Flow Laboratories, Irvine, Scotland) supplemented with 10% FCS. Normal mouse embryonic cells were prepared from mouse embryo (19) and grown at 37°C in MEM supplemented with 10% FCS.

Immunofluorescence. For immunofluorescence studies, cells were plated on glass coverslips in 40-mm Petri dishes and were used 4 days after plating. The cultures were washed 3 times with PBS at room temperature and drained and fixed for 30 min with methanol at —20°C. After rinsing with PBS, the first antiserum was added, and the cells were incubated for 30 min in a moist chamber at 37°C. The cells were then thoroughly washed with PBS and incubated for another 30 min with fluorescein-conjugated goat antiserum to rabbit IgG (Behringwerke, Marburg-Lahn, W. Germany) diluted 1/20 with PBS. After rinsing thoroughly in PBS, the cells were gently and briefly washed in water and were mounted in 90% glycerol diluted in PBS. Cells were examined by phase contrast and epifluorescent illumination in a Leitz Orthoplan microscope with an L2 filter block using a ×100 oil immersion lens. Rabbit antiserum to mouse FN was used diluted 1/30. Sera from preimmune rabbits were routinely used as controls.

RESULTS

Fig. 1 shows a typical result of immunoelectrophoresis of mouse serum using rabbit antibodies to mouse FN. The mouse FN migrated toward the cathode as a single precipitin band. Using such antibodies in the radial immunodiffusion assay (Fig. 2), we have measured the concentration of FN in the plasma and in the ascitic fluid of normal control mice and of mice inoculated with Ehrlich ascites tumor cells.

Chart 1 shows the concentrations (µg/ml) of FN and total proteins in the plasma and in the ascitic fluid of normal control mice (Day 0) and of mice at different times after inoculation of Ehrlich ascites tumor cells. Points, mean of the data obtained from 6 animals ± S.E.

Chart 2 shows the concentrations of FN and total proteins in the ascitic fluid at different tumor growth times. Two days after the inoculation of the tumor (the first day in which it was possible to collect sufficient ascitic fluid), the total protein concentration was about 48 mg/ml and increased during the following 24 hr to about 70 mg/ml and then decreased to about 30 mg/ml. On the third and fourth days, the concentration of total protein was slightly higher in the ascitic fluid than it was in the plasma. The concentration of FN was about 470 µg/ml during the first 8 days after cell inoculation and then decreased to about 300 µg/ml at Day 15. Chart 3 shows the ratios between the FN concentration in plasma and ascitic fluid of mice at different times after inoculation of the Ehrlich ascites tumor cells. It can be seen that the concentration of FN in the ascitic fluid is almost identical to that in the plasma on the second and third days, whereas from Day 4 it was about 30 to 50% of the plasma concentration.
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Chart 3. Ratio between the concentrations of FN in the plasma and in ascitic fluid of mice at different times after inoculation of Ehrlich ascites tumor cells.

Chart 4. Concentration of FN relative to total proteins in plasma (○) and ascitic fluid (●) of mice at different times after inoculation of Ehrlich ascites tumor cells. Points, mean of the data obtained from 6 animals ± S.E.

Chart 4 shows the concentrations of FN relative to that of total protein (µg of FN per mg of total protein) in the plasma and ascitic fluid at different times after tumor inoculation. The concentration of FN in plasma on the sixth day after inoculation of the tumor had more than doubled. The relative concentration of FN on Day 2 was higher in the ascitic fluid than it was in the plasma.

To establish whether Ehrlich ascites cells synthesize FN, these cells were grown in vitro. Fig. 4 shows immunofluorescence experiments using antifibronectin antibodies with mouse fibroblasts (a) and Ehrlich ascites cells (b) cultured in vitro. It can be observed that mouse fibroblasts show a large number of fibrillar surface-associated structures containing FN. In contrast, Ehrlich ascites cells were almost devoid of these structures. Only very few fibrillar structures were seen in the areas of cell-cell contact. Immunofluorescence studies of Ehrlich ascites cells grown in vivo were completely negative.

We have also examined the release of FN into the tissue culture medium by normal mouse fibroblasts and by Ehrlich ascites cells grown in vitro using electroimmunoassay (Fig. 3). It was found that a considerable amount of FN is released by Ehrlich ascites cells. In fact, we found that $5 \times 10^5$ Ehrlich ascites cells grown for 24 hr in fresh MEM supplemented with 10% FCS were able to release and accumulate about 60 µg of mouse FN in the medium compared to the 40 µg of mouse FN accumulated in the medium by $5 \times 10^6$ mouse fibroblasts under the same conditions.

DISCUSSION

Using the electroimmunoassay, we observed that Ehrlich ascites cells, originating from a spontaneous carcinoma of the mammary gland (20), when grown in vitro release larger amounts of FN into the medium than do normal mouse fibroblasts. However, in contrast to fibroblasts, in Ehrlich ascites cells only small amounts of surface-associated FN were seen, and these were mostly in the cell-cell contact areas. Experiments are in progress to establish whether the ability to release FN by epithelial tumor cells is a general phenomenon.

FN was also found in the ascitic fluid in all phases of tumor growth at concentrations ranging from 500 to 300 µg/ml. On the second day after inoculation, the concentration of FN in the ascitic fluid, relative to that of the total protein, is close to that in the plasma, but by the fifth day, there is considerably more FN in the plasma.

Since Ehrlich ascites cells cultured in vitro released FN into the medium, it is unlikely that all of the FN in the ascitic fluid originates from the plasma.

In plasma of normal control mice, FN was present at a concentration of about 545 µg/ml; this represents about 0.8% of the total plasma protein. Five days after inoculation of Ehrlich ascites tumor cells, the plasma level of FN increases to about 850 µg/ml which represents about 1.7% of the total plasma proteins, whereas total plasma protein decreases from 65 to 50 mg/ml during the same period.

We cannot at present explain such an increase of FN concentration, but it is not likely to be due to a massive synthesis of FN by epithelial cells, since the concentration of FN in the ascitic fluid is lower than that in the plasma.

The accumulation of FN in the plasma could be due to either increased synthesis or decreased catabolism. It has been reported that plasma FN has an opsonic activity and may be an important host defense factor in a variety of pathological processes including neoplasia (2). Thus, an increased synthesis of FN may be correlated with the organisms needed to increase the activity of sessile macrophages in the reticuloendothelial system due to the presence of neoplastic cells. On the other hand, the accumulation of FN may be a consequence of diminished hepatic degradation (6, 27), since liver function may be reduced by the presence of the tumor. These possibilities are under investigation. However, our preliminary studies on plasma concentration of FN in rats after partial hepatectomy seem to indicate a decrease of the FN level suggesting that the liver is not involved in the degradation of FN.

Recently, it has been reported that the plasma level of a plasma opsonic factor identical to FN (1) is decreased during advanced stages of tumor growth in humans and animals (1, 21, 22). On the other hand, immunological measurements of the concentration of FN in plasma of patients with tumors is increased or unchanged (3, 16).

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* L. Zardi, unpublished observations.
Even if we are unable to generalize and extend our observation on Ehrlich ascites tumor to other kinds of tumors, our preliminary data on the level of FN in the plasma of patients with different types of tumors do not indicate a decrease of FN concentration. This contradiction, therefore, remains unexplained.

In conclusion, the major points are: (a) Ehrlich ascites cells grown in vitro synthesize large amounts of FN but retain little of this substance on the cell surface; (b) FN is present in the Ehrlich ascitic fluid and is immunologically indistinguishable from plasma FN. Therefore, this fluid could be used as a source from which mouse FN may be purified; and (c) the plasma concentration of FN doubles after inoculation of Ehrlich ascites tumor cells. This seems to indicate that the Ehrlich ascites tumor could interfere with the mechanisms which are involved in regulating the accumulation of such a protein in the plasma. We are presently extending our study to other tumors to establish whether this is a general phenomenon.

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REFERENCES

Fig. 1. Immunoelectrophoresis in 1% agarose of mouse serum using antibodies to mouse plasma FN (a) and to total mouse serum proteins (b).

Fig. 2. Radial immunodiffusion assay for determination of the concentration of FN in plasma and ascitic fluid. Plasma (a, b, and c) and ascitic fluid (e, f, and g) of 3 animals inoculated with Ehrlich ascites tumor cells; standards (d).

Fig. 3. Electroimmunoassay for determination of the amount of FN in the culture medium of mouse fibroblasts and Ehrlich ascites cells. To avoid cross-reactions with the FCS present in the medium, the antibodies to mouse FN were previously absorbed with calf FN as described (26). Mouse serum diluted 1/40 (a), 1/20 (b) and 1/10 (c). Tissue culture medium of mouse fibroblasts (d) and of Ehrlich ascites cells (e). The medium was taken from the culture flasks 3 days after cell plating. The number of cells present at this time in both cultures was not significantly different.
Fig. 4. Indirect immunofluorescence of (a) mice fibroblasts and (b) Ehrlich ascites tumor cells cultured in vitro and stained with rabbit antibodies to mouse FN. × 1500. (For the procedure see "Materials and Methods".)
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