PROTEASES AND PROTEASE INHIBITORS in malignant tissues and cells have been reported by many investigators (1-11). The role of these proteases in the growth, vascularization, and metastasizing potential of tumors has been studied, with particular reference to tumor fibrin forming the scaffolding for vasculogenesis (12). Studies with 125I-labeled fibrinogen and fibrin antibodies have confirmed the presence of fibrin in tumors (13). O'Meara (14) initially proposed the concept that fibrin deposition and dissolution were in dynamic balance in neoplastic tissue. Support for this concept was provided by the studies of Wood et al. (15), Rudenstam (16), Thornes (17), and Back et al. (18), who established a direct correlation between plasmin uptake by transplanted rodent tumors and the fibrinogen (or fibrin) concentration within the tumor.

Fibrinolytic and antifibrinolytic agents have been used to study the role of fibrin in tumor growth and spread. Fibrinolytic agents inhibited cell growth in vitro (18, 19) and also decreased pulmonary metastases in rodents and rabbits after intravenous administration of tumor cell suspensions (20, 21). While plasmin decreased the metastasis-promoting effect of trauma in mice inoculated with various tumor cells (22), hypofibrinolysis induced by hyperlipemia (23), pancreatic proteinase inhibitor (20), and epsilon aminocaproic acid (EACA) (22, 24) increased metastases in rodents. However, fibrinolytic agents increased pulmonary metastases in mice with transplanted mammary carcinoma, whereas EACA decreased both lung metastases and tumor growth (25). Back et al. (25) showed that plasmin decreased pulmonary metastases in a spontaneously metastasizing rodent tumor system, whereas EACA and the mammalian protease inhibitor Trasylol had no effect.

The above studies suggest that as yet unexplored forces and mediators may be involved in tumor growth processes, since neither protease inhibitors nor plasmin per se is vasoactive (26). However, plasmin can form vasoactive kinins from an appropriate kininogen substrate (27, 28). The present studies were undertaken to investigate the fibrinolysin system during the growth of the Ehrlich ascites tumor in vivo. In a forthcoming paper, we will report on a kinin-forming protease system in the Ehrlich ascites tumor which interrelates closely with both the fibrinolysin and blood coagulation systems (29). The ascites tumor was selected as the tumor model because capillary permeability changes are reflected by fluid effusion into the peritoneal cavity, and changes in system components can be measured easily.

MATERIALS AND METHODS

Tumor transplantation and harvest.—Ehrlich ascites tumor (provided by Dr. P. Hebborn of our department) was maintained by weekly transplants in male albino Swiss mice, weighing 20-25 g. On day 7 or 8 after tumor transplant into recipient mice, the mice were killed by cervical fracture and placed onto a dissecting board. The abdominal skin was peeled away carefully to expose the abdominal muscles. With the board positioned upright over a funnel, a vertical and two horizontal incisions were made, enabling the peritoneal cavity fluid to drain into the funnel leading to a 50-ml centrifuge tube. The tumor cell number was established by microscopic cell count, and the tumor cell suspension brought to a final concentration of 20 million cells/ml saline. Tumor cell viability was confirmed with lissamine green by the technique of Holmberg (30).

The tumor was transplanted by the injection of 10 million cells (0.5 ml suspension) into the lower quadrant of the peritoneal cavity. With the day of tumor transplant designated as day zero, 5 mice were killed by cervical fracture on days 5, 7, 9, 11, 13, and 15 after transplant, and the undiluted ascitic fluid was harvested and pooled for each time interval. Sodium citrate, 3.8%, was used as the anticoagulant in a ratio of 1 ml/10 ml ascitic fluid. The ascitic fluid decreased during tumor growth. The nuclear and mitochondrial-subcellular fractions of the tumor cell exhibited plasminogen activator activity. No significant changes in the above parameters occurred in the plasma during the tumor growth period we studied.—J Natl Cancer Inst 54: 881-886, 1975.

Summary—Component levels of the fibrinolysin system in the plasma and ascitic fluid of Swiss mice bearing Ehrlich ascites tumors were determined during a 15-day tumor growth time phase. During tumor growth, the concentration of plasminogen in the ascitic fluid decreased inversely to the total packed cell volume. Free plasmin was not present in the ascitic fluid nor was there any measurable plasminogen activator activity. Both antiplasmin activity and fibrinogen levels present in the fluid decreased during tumor growth. The nuclear and mitochondrial-microsomal subcellular fractions of the tumor cell exhibited plasminogen activator activity. No significant changes in the above parameters occurred in the plasma during the tumor growth period we studied.—J Natl Cancer Inst 54: 881-886, 1975.
fluid then was centrifuged at 1,000Xg for 10 minutes at 4°C, the total packed cell volume (TPCV) recorded, and the supernatant assayed for components of the fibrinolysin system.

Whole blood collection.—Whole blood for biochemical assay was obtained by cardiac puncture before the mice were killed. The blood was anticoagulated with sodium citrate as above and placed in a refrigerated centrifuge for preparation of the plasma used in the assays.

Assay for components of the fibrinolysin system. Plasminogen assay.—The euglobulin fraction of plasma (which contains the plasminogen component) or ascitic fluid was precipitated with CO₂ by a modification of the method of von Kaulla (31). A 0.5-ml aliquot was placed into a 25-ml flask containing 7.0 ml cold distilled water, and the CO₂ was passed above the surface of the sample for 3 minutes. Then the flask was placed on ice for 2 minutes and centrifuged at 500Xg for 5 minutes at 4°C. The precipitate was collected and dissolved in 0.48 ml of a 0.1 M phosphate buffer, pH 7.2.

A plasminogen activator solution was prepared by the addition of 1,000 U (0.05 ml) of streptokinase (Bayer, West Germany) to 0.2 ml of the human euglobulin precipitate preparation. Exactly 0.02 ml of this euglobulin-streptokinase preparation was added to 0.48 ml of the mouse euglobulin precipitate and incubated at 28°C for 10 minutes. The plasmin generated was assayed on an azocasein substrate according to the method of Markus and Werkheiser (32). One ml of a 1.5% azocasein solution (33) was added to the activated euglobulin solution and incubated at 37°C for 60 minutes. The reaction was stopped by the addition of 1.0 ml perchoric acid, 1.12 M. The precipitate was separated by centrifugation for 15 minutes at 1,000Xg; the supernatant was collected and then read at 390 nm in a Beckman DV spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). The blank consisted of 0.5 ml of 0.1 M phosphate buffer instead of the euglobulin fraction. The control contained 0.48 ml of buffer and 0.02 ml activator solution. Controls were necessary because the activator preparation contained trace amounts of plasmin activity. The plasmin activity, representing plasminogen content based on a standard curve, was obtained by subtracting the optical density of the control from that of the sample.

Plasmin assay.—Exactly 0.2 ml plasma or ascitic fluid was added to 0.3 ml of a 0.4% purified human fibrinogen solution. That amount of purified human thrombin which gave a 15-second clotting time was added, and the solutions were shaken so as to form a firm clot. The tubes were incubated in a 45°C water bath, and the end point of clot lysis was recorded when the bubbles entrapped within the coagulum during clot formation rose to the surface. The fibrinogen and thrombin reagents were purified according to the method of Hink (34). A control standard urokinase-activated plasmin (1 U of which lysed the clot in 2 min) was used throughout the assay procedure.

Antiplasmin assay.—Antiplasmin activity was determined on the basis of the inhibition of spontaneous plasmin activity by the test fluids. A volume of 0.4 ml spontaneous human plasmin, prepared by the method of Klime (35), was incubated with 0.1 ml plasma or ascitic fluid for 60 minutes at 28°C, and 1.0 ml of a 1.5% azocasein solution added. This was incubated further for 60 minutes at 39°C and the reaction stopped with 1.0 ml of 1.12 M perchloric acid. The tubes were centrifuged, and optical density was read at 390 nm on a Beckman DV spectrophotometer. Control tubes contained 0.4 ml spontaneous plasmin and 0.1 ml imidazole buffer. Percent change in plasmin inhibitory (antiplasmin) activity was calculated on the basis of the optical density of the test sample to that of the control, and the result multiplied by 100.

Activator activity assay.—Activator activity was determined by the fibrin plate method of Astrup and Mullertz (36) and fibrin tube procedure. Fibrin plates were prepared by the addition of 8.0 ml of plasminogen-contaminated fibrinogen (0.16%) dissolved in 0.025 M Tris buffer, pH 7.63, to 0.04 ml of bovine thrombin (Parke, Davis & Co., Detroit, Mich.) dissolved in 50% glycerol. After clot formation, 0.05 ml of plasma or ascitic fluid was placed onto the clot surface, and the plate incubated at 37°C for 21 hours. Similar plates with plasminogen-free fibrin were prepared by the plates being heated as described for 60 minutes at 85°C, which thus destroys the plasminogen. Activator activity also was determined by the fibrin tube lysis time method based on activation of a standard plasminogen solution and the assay of the amount of plasmin generated as described above under “Plasmin assay.” The euglobulin fraction of ascites and the ascitic fluid treated with a-thymotic acid also were tested. To 0.1 ml of human plasminogen (0.2%) was added 0.1 ml of the test fluid and the mixture incubated for 60 minutes at 28°C. Control tubes contained 0.1 ml bicarbonate buffer, 0.1 M at pH 8.3, and test fluid or 0.1 ml of plasminogen solution. Plasmin assays were conducted after incubation for 60 minutes at 28°C.

Euglobulin clot lysis time (ECLT).—The ECLT of plasma or ascites was determined by the dilution of a volume of 0.7 ml of the test fluid with 12.6 ml of 0.0075% acetic acid to a final pH of 5.4. This was kept at 4°C for 30 minutes and then centrifuged at 500Xg for 5 minutes at 4°C. The supernatant was discarded, and the euglobulin precipitate redissolved in 0.7 ml of 0.1 M phosphate buffer at pH 7.2. Five units of purified bovine thrombin (Parke, Davis & Co.) were placed into a tube containing 0.5 ml of the redissolved precipitate. The mixture was swirled gently until the clot formed, and then the tubes were placed in a 37°C water bath for determination of lysis times. Times were recorded as described under “Plasmin assay.”

Fibrinogen assay.—Fibrinogen determinations were made by the method of Andersch (37). To 1.0 ml of ascitic fluid or plasma was added 2.5 ml of alcoholic thrombin (1 mg of thrombin plus 10 ml of alcohol diluted to 10% with physiologic saline). The clot first was collected on a wooden applicator stick twisted for 15-30 seconds, then washed thrice with
distilled water, removed from the stick, and washed again three times with distilled water. The clot was placed into a tube containing 1.0 ml distilled water and 4.0 ml weak biuret solution at 37°C until it was completely dissolved. The test and blank (1.0 ml distilled water and 4.0 ml weak biuret) solutions were read at 550 nm in a Beckman DV spectrophotometer. The fibrin concentration was obtained from a standard curve made with varying concentrations of bovine albumin.

RESULTS

Plasminogen Levels

Text-figure 1 depicts the percent changes in plasminogen levels in ascitic fluid and plasma during 15 days of tumor growth; no significant change in plasma plasminogen levels occurred. Also represented are the changes in TPCV during tumor growth. However, there was a significant steady decrease in the plasminogen content of ascitic fluid, with the lowest levels at 11 days after tumor transplantation. Subsequently, plasminogen levels gradually increased, with a concomitant increase in TPCV. Regression analysis of plasminogen versus TPCV showed a 0.1% level of significance (text-fig. 2).

Plasminogen Activator Activity

No plasminogen activator activity was found in the cell-free ascitic fluid or in the euglobulin fraction of the fluid. Apparently, the labile plasminogen activator in plasma could not be detected by the methods used (38, 39).

Cellular fractions were prepared by an ultracentrifugal technique (40) to determine the plasminogen activator potential of the ascites tumor cell components. A quantity of 0.05 ml cell fraction was placed onto both standard and heated fibrin plates, and the plates were examined for areas of lysis after 20 hours of incubation at 37°C. The results are summarized in table 1. None of the cell fractions had any free plasmin activity as indicated by the absence of lysis of the heated, plasminogen-free fibrin plates. Both the nuclear and mitochondrial-lysosomal fractions produced zones of lysis, which confirms the presence of plasminogen activator; as no attempt was made to quantitate these activities, they represent activator activity in the cell fractions.

<table>
<thead>
<tr>
<th>Cellular fractions</th>
<th>Fraction (ml)</th>
<th>Standard plate</th>
<th>Heated plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear</td>
<td>0.02</td>
<td>1,963</td>
<td>0</td>
</tr>
<tr>
<td>Microsomal</td>
<td>0.05</td>
<td>2,827</td>
<td>0</td>
</tr>
<tr>
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<td>0</td>
</tr>
<tr>
<td>Lysosomal</td>
<td>0.05</td>
<td>500</td>
<td>0</td>
</tr>
<tr>
<td>Soluble</td>
<td>0.05</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

* 10.7X10^9 cells were used for the cell fractionation studies. Cells were washed twice with saline, centrifuged at 800Xg for 10 min, resuspended in 48 ml 0.25 M sucrose (1 to 4 ratio), and blended in a VirTis homogenizer. Nuclear fraction was obtained at 1,000Xg, mitochondrial and lysosomal fraction at 15,000Xg, microsomal and supernatant soluble-cell fractions at 100,000Xg.
Plasmin Activity

Plasma activity was absent in the ascitic fluid studied at various dilutions or in the ascites euglobulin preparation. Thus the ascites euglobulin preparation did not show any clot lysis activity, as measured by the ECLT. Reducing the fibrinogen concentrations in the euglobulin assay so as to detect even traces of plasmin failed to uncover plasmin activity. The clots always were observed for 48 hours.

Antiplasmin Activity

Antiplasmin activity of ascitic fluid, calculated as percent plasmin inhibitory capacity, decreased significantly from the 7th day after transplantation (text-fig. 3). Plasma antiplasmin activity changed little during tumor growth. Ascites antiplasmin activity decreased inversely to the total volume of ascitic fluid. Regression analysis showed this relationship to be significant at the 0.1% level (text-fig. 4).

Fibrinogen Levels

Fibrinogen levels in the ascitic fluid decreased steadily from 150–200 to 20–40 mg/100 ml from the 7th to 15th day after transplantation and then decreased below normal levels by the 13th to 15th day (text-fig. 5). The overall total protein concentration and albumin/globulin ratio in the ascitic fluid did not change significantly during the growth of the tumor.

DISCUSSION

During the growth of Ehrlich ascites tumor in mice, plasminogen levels, antiplasmin activity, and fibrinogen concentration of the ascitic fluid decreased. These decreases occurred in the absence of detectable activator activity and plasmin. The initial source of plasminogen in ascitic fluid may derive from the plasma of the host. Possible synthesis by the tumor cells is not likely, since plasminogen levels decrease in the face of increasing concentrations.
of tumor cells; this decrease in plasminogen may have been due to digestion by cathepsins shown to be present in cell-free ascites (41), although Christensen and Riggs (42) found no evidence of proteolysis or unusual concentrations of intermediate protein breakdown products in ascitic fluid. The most reasonable explanation for the plasminogen decrease is that the plasminogen was converted to plasmin by an activator from the tumor cell. Indeed, Funahara et al. (43) demonstrated the presence of a plasminogen activator in Ehrlich ascites tumor cells extracted with 2 m NaCl. In a series of recent abstracted comments on tumor-associated fibrinolysis, Reich (44) suggested that the association of fibrinolysis and neoplasia is quite general. He showed that neoplastic cells initiate fibrinolysis by releasing a cell activator (an arginine-specific protease) which hydrolyzes a serum factor, the zymogen plasminogen. Björlin et al. (1) and Rikfin et al. (45) also recently reported on the release of plasminogen activators from neoplastic human cell cultures. Davidson et al. (46) described a giant-cell carcinoma of the lung that produced high levels of plasminogen activator.

The decrease in antiplasmin activity of the ascitic fluid probably reflects the complexing of the antiplasmin with the former plasmin. Decreased formation of antiplasmin and increased turnover rate also may represent factors contributing to the decrease. Plasmin, a labile protease, can be demonstrated in plasma when present in higher than normal levels in certain pathologic conditions (47). Furthermore, antiplasmin levels are normally far in excess of plasmin so that only excessive plasminogen activation will yield plasmin levels sufficient to overcome the fibrinolytic inhibitory activity of a biologic fluid (48). The concept that antiplasmin activity is influenced by formed plasmin receives support from the studies in patients whose antiplasmin activity decreased as plasminogen levels were lowered during the course of thrombolytic activator therapy (49).

The changes in antiplasmin activity in the ascitic fluid paralleled plasminogen level changes up to the 13th day post transplant. Plasminogen levels started to increase after the 11th day, concomitant with decreases in TPCV. The increase in plasminogen may have been due to an accelerated movement of the molecule into the peritoneal cavity as a result of the oncologic process, or to decreased plasminogen utilization because the source of activator was diminished. The continued lower level of antiplasmin activity may represent altered filtration or lymphatic absorption from the peritoneal cavity at that time period.

The decrease in fibrinogen concentration may be attributed to the fibrinogenolytic activity of the plasmin generated as well as to a filtration phenomenon. While the ability of an antiplasmin—plasmin complex to destroy fibrinogen may be questioned, this complex has been shown to act as a reservoir of potential fibrinolytic activity (50). In the presence of an appropriate substrate as fibrin or fibrinogen, plasmin is yielded by the inhibitor preferentially to the substrate.

Thus the data show that during the growth of the Ehrlich ascites tumor, plasminogen is consumed, free antiplasmin activity decreases, and fibrinogen levels decrease. The presence of a plasminogen activator activity in the nuclear and mitochondrial-lysosomal fractions of the tumor cell would support the hypothesis that plasminogen is converted to plasmin which, while complexing with the antiplasmin, consumes fibrinogen. Alternative interpretations of the changes in component levels include possible production and destruction of factors by the growing cell population, or transudation of the factors with destruction and consumption by accompanying factors in the fluid. Additional experiments would be required to discriminate between these alternatives.

Possible functions suggested for the fibrinolysis system in neoplastic processes include a) influencing the invasiveness of tumors, b) altering frequency and distribution pattern of metastases, c) releasing nutritional factors for tumor growth, and d) releasing vasopeptides that may influence tumor vascularity (51). While Reich (44) states that there is no basis for assuming a link between fibrinolysis and tumor growth, invasiveness, or metastases, he does suggest that these are subjects for further study. Goetz et al. (52), on the basis of observations showing that proteases take part in the initiation of cell division in culture, demonstrated that the beef pancreas protease inhibitor alters some aspects of the morphology and social behavior of hamster tumor cells in culture. Subsequent studies to be reported will attempt to elucidate some of these possible roles.

REFERENCES


