Pathogenesis of Ascites Tumor Growth: Fibrinogen Influx and Fibrin Accumulation in Tissues Lining the Peritoneal Cavity

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ABSTRACT

In the immediately preceding paper, we demonstrated that the microvasculature supplying peritoneal lining tissues of mice bearing either of two transplantable ascites carcinomas was hyperpermeable to circulating macromolecules. Solid tumors have been shown to exhibit similar levels of microvascular hyperpermeability, leading to extravasation of plasma proteins, including fibrinogen which clots on extravasation to form an extravascular fibrin gel. To determine whether similar extravasation and clotting of plasma fibrinogen occurred in ascites tumors, we used 125I-labeled fibrinogen (125I-F) as a tracer to measure inflow of fibrinogen into the peritoneal cavities, and influx and accumulation of fibrinogen/fibrin in the peritoneal lining tissues (peritoneal wall, mesentery, and diaphragm) of mice bearing syngeneic TA3/St or MOT ascites tumors. The percentage of circulating 125I-F that extravasated into the peritoneal cavity was increased from 10- to 50-fold in mice bearing either ascites tumor. Influx into the peritoneal walls of ascites tumor-bearing mice was 3-7 times that of control mice and became maximal on day 8 (TA3/St) and day 15 (MOT). Accumulation of 125I-F in ascites fluid and peritoneal lining tissues was also increased substantially in mice bearing these ascites tumors, reaching maximal values on days 7-8 (TA3/St) and 19-29 (MOT) at levels 2- to 3-fold (peritoneal wall) and 33- to 148-fold (ascites fluid) above control levels. Significant amounts of the 125I-F that accumulated in the peritoneal lining tissues of ascites tumors-bearing animals were insoluble in 3 M urea, consistent with clotting of 125I-F to cross-linked fibrin. Autoradiographs of SDS-PAGE gels performed on extracts of peritoneal lining tissues of both ascites tumors revealed the characteristic signature of cross-linked fibrin, i.e., γ-γ dimers and α-polymers. Fibrin was also identified in peritoneal lining tissues of both ascites tumors by immunohistochemistry. Taken together, these data indicate that fibrinogen, like other circulating macromolecules, extravasates into the peritoneal cavity and peritoneal lining tissues of ascites tumor-bearing mice and does so with kinetics similar to those of other macromolecular tracers we have studied. Moreover, a portion of the fibrinogen that extravasated into peritoneal lining tissues clotted to form a cross-linked fibrin meshwork which trapped tumor cells and favored their attachment to the peritoneal surface. By analogy with solid tumors, such fibrin deposits may also be expected to have a role in initiating angiogenesis and the generation of mature tumor stroma.

INTRODUCTION

Fibrinogen and fibrin are regular components of the stroma of solid tumors, both autochthonous and transplantable (reviewed in Refs. 1–7). These deposits result from the characteristic hyperpermeability of the tumor microvasculature, which leads to local extravasation of plasma fibrinogen; much of the fibrinogen extravasated is rapidly clotted to fibrin by tumor cell-associated and perhaps other tissue procoagulants. The fibrin gel matrix so deposited organizes solid tumors into discrete nests of malignant cells and serves as a provisional matrix that facilitates the ingrowth of macrophages, new blood vessels, and fibroblasts and the generation of mature stroma.

In contrast to solid tumors, ascites tumor cells grow primarily as a cell suspension in body cavities (8–10), although tumor cells may also implant on peritoneal surfaces (11–13). Like the vasculature supplying solid tumors, that lining the peritoneal cavities of ascites tumor-bearing animals is hyperpermeable to macromolecules (14–16). As a result, animals bearing ascites tumors accumulate proteinaceous fluid in their peritoneal cavities, sometimes in enormous quantity (15–22). However, unlike the plasma exudate extravasated into solid tumors, that which accumulates in tumor ascites generally remains fluid in vivo, i.e., it does not form an insoluble fibrin gel. Seemingly, therefore, extravasated fibrinogen is processed differently in solid and ascites tumors. The reasons for these differences have not been determined but apparently are related primarily to differences in the sites of tumor implantation rather than to intrinsic differences in tumor cells. In support of this interpretation, many transplantable syngeneic tumors have the capacity to grow in either solid or ascites form, depending only upon whether they are injected s.c. (to form solid tumors with obvious fibrin deposits) or i.p. (to form ascites tumors growing in fluid suspension).

Previous studies of solid tumors (reviewed in Refs. 1–6) and our more recent studies of the hyperpermeability of peritoneal lining vessels in ascites tumor-bearing mice (14–16) prompted us to examine another possibility, namely, that, although tumor ascites fluid did not clot, fibrin might nonetheless be deposited in tissues lining the peritoneal cavities of ascites tumor-bearing mice, i.e., in those tissues which house the hyperpermeable vessels. To test this possibility, we followed the influx, accumulation, and urea solubility of i.v.-injected tracer 125I-F in syngeneic mice bearing either the TA3/St or MOT transplantable ascites tumors that we have studied previously; both tumors grow in either solid or ascites form, depending only on the inoculation site. We found that fibrinogen extravasated from the hyperpermeable blood vessels lining the peritoneal cavities of mice bearing either ascites tumor. Much of the extravasated fibrinogen passed through the peritoneal lining tissues and entered the peritoneal cavity, where it contributed to ascites fluid. However, a smaller fraction remained within peritoneal lining tissues; and a portion of this clotted to form cross-linked fibrin comparable to that which, in solid tumors, serves as a provisional matrix that supports angiogenesis and the generation of mature tumor stroma.

MATERIALS AND METHODS

Tumors. Handling and growth properties of TA3/St and MOT tumor cells in syngeneic mice were as described (14).

Fibrinogen. Human fibrinogen, obtained from Kabi Diagnostics (Franklin, OH), was further purified by ion-exchange HPLC (23). Fibrinogen was iodinated with Na125I (Dupont-NESS, Boston, MA) using iodogen (Pierce Chemical Co., Rockford, IL; Ref. 24) to a specific activity of 0.032 mole of 125I per mole of fibrinogen. The resultant 125I-F was more than 95% precipitable in 10% TCA, and its clottability ranged from 85 to 90%; after i.v. injection, the...
clottability of circulating $^{125I}$-F increased to 94 to 97% within 5 min as nonclottable $^{125I}$-F was rapidly cleared by the reticuloendothelial system.

**Inflow Studies using $^{125I}$-F as Tracer.** Inflow measurements were performed by injecting 2.5 $\times$ 10$^5$ cpm of $^{125I}$-F (in 0.2 ml saline) i.v. At various times thereafter, ascites tumor-bearing or control animals were sacrificed by CO$_2$ narcosis, and the amount of $^{125I}$-F in the plasma, peritoneal wall, and ascites fluid was determined. The amounts of $^{125I}$-F present in each compartment were expressed as a percentage of injected $^{125I}$-F (e.g., for peritoneal wall, calculated as the total cpm present in the entire parietal peritoneum/total cpm injected i.v.).

**Influx and Accumulation Experiments.** $^{125I}$-F influx and accumulation in peritoneal lining tissues were measured at various intervals after i.p. injection of 1 $\times$ 10$^6$ tumor cells. For influx experiments, mice were injected i.v. with 2.5 $\times$ 10$^5$ cpm of $^{125I}$-F. At intervals from 5 to 360 min thereafter, mice were injected i.v. with 0.2 ml of an anticoagulant-antifibrinolytic mixture, HET (2). Blood samples were collected by retroorbital puncture to a known volume of heparin for preparation of platelet-poor plasma (Microfuge; 10 min at 4°C). Immediately thereafter, animals were sacrificed by CO$_2$ narcosis. The peritoneal cavities were opened by a small ventral incision, and 2 ml of HEET buffer was installed i.p. (2); the contents of the peritoneal cavity were recovered and the fullest extent possible; then the total volume was recorded, and the tumor cells were counted. The recovered fluid was then centrifuged (160 x g for 10 min at 4°C), and the cell-free supernatant was removed and its volume recorded. The entire parietal peritoneal wall was excised and cut into four pieces. Each section was weighed and placed in a tube containing 2 ml of HEET buffer. The entire diaphragm, a portion of mesentery, and roughly equivalent amounts of leg muscle (as a control tissue) were harvested and treated similarly. Radioactivity was measured in a gamma spectrometer.

Accumulation experiments were performed in identical fashion to influx experiments except that 10 times as much $^{125I}$-F (2.5 $\times$ 10$^5$ cpm) was injected 18 h prior to animal sacrifice. $^{125I}$-F influx and accumulation experiments were also performed in normal control animals.

**Extraction of Peritoneal Walls and Control Tissues.** The peritoneal wall, diaphragm, a portion of mesentery, as well as a control tissue distant from the peritoneal tumor (leg muscle), were each minced in cold HEET buffer and subsequently extracted in this buffer and in 3 M urea as described previously (2). The aqueous and urea extracts and the urea-insoluble pellets were separately counted for radioactivity. Total $^{125I}$-F detected in tissues may consist of a mixture of fibrinogen, fibrin, and fibrinogen/fibrin degradation products. We have shown that fibrinogen and certain fibrin degradation products appear in the aqueous extract; that noncross-linked fibrin and some partly degraded fragments of cross-linked fibrin appear in the urea extract; and that cross-linked fibrin as well as some of its early degradation products remain in the urea-insoluble fraction (2).

**Calculation of $^{125I}$-F Influx and Accumulation.** We calculated total $^{125I}$-F influx into the peritoneal wall, diaphragm, mesentery, and control tissue (leg muscle) by dividing cpm/g of tissue by cpm/µl of plasma, giving a value whose units are microliters of plasma/g wet tissue; i.e., the number of microliters of plasma $^{125I}$-F present in each gram of tissue at each interval after i.v. injection of radioactive tracer. $^{125I}$-F was determined as the amount of extravasated $^{125I}$-F that was insoluble in 3 M urea (2).

Accumulation of $^{125I}$-F in tumor or control tissues was similarly calculated by dividing the specific radioactivity of the tissue (cpm/g wet weight) by the specific radioactivity present in the plasma at the time of animal killing (cpm/µl) 18 h later. At this interval, tissue radioactivity reflects a balance between tracer influx and efflux. Because plasma levels of labeled fibrinogen were falling exponentially throughout the 18-h experiment, this calculation does not provide a satisfactory estimate of absolute fibrinogen accumulation (2). However, it does provide a useful measure of relative accumulation that allows valid comparisons between different tissues within the same animal or the same inbred strain in which plasma clearance rates of radioactive fibrinogen are identical. We also determined the amount of accumulated $^{125I}$-F that was insoluble in 3 M urea.

4 The microliter plasma/gram wet tissue may be converted to the microgram of fibrinogen/gram wet tissue by multiplying the microliter plasma/gram wet tissue by the concentration of fibrinogen in plasma, i.e., $\sim 3$ µg/µl.

**SDS-PAGE and Autoradiography of Tissue Extracts.** Ascites tumor-bearing mice were injected i.v. with 5.0 $\times$ 10$^5$ cpm $^{125I}$-F. Eighteen h later, animals received HEET i.v. and were sacrificed by CO$_2$ narcosis and exanguinated; sections of parietal peritoneal wall, diaphragm, and mesentery were collected, minced, and extracted with buffer and urea as described above. Urea-extracted tissue was assessed for radioactivity and was frozen. For gel electrophoresis, urea-insoluble tissue was dissolved for 1 h at 90°C in a mixture composed of 4% SDS, 8 M urea, 4 mM EDTA, 20% glyceral, and 0.1 M DTT, all in 0.06 M Tris buffer, pH 6 (25). All samples were analyzed by 4–20% SDS-PAGE (26), followed by protein electrotransfer (27) to nitrocellulose (Schleicher & Schuell). Autoradiographs were then prepared by exposing the dried nitrocellulose membranes to Kodak XAR-2 film at $-70°C$ for intervals of 1 to 7 days. Protein molecular weight markers (Amersham Corp., Arlington Heights, IL) and $^{125I}$-F were always included as standards.

**Immunohistochemistry.** To detect cross-linked fibrin and to distinguish it from fibrinogen and other fibrinogen- or fibrin-related proteins, we perfuse-fixed ascites tumor-bearing and control mice with 4% formalin-2% acetic acid at room temperature. Because cross-linked fibrin is insoluble in 2% acetic acid (28), it remains in situ, whereas fibrinogen and other fibrinogen- and fibrin-related proteins are largely extracted from tissues by this fluid before the formalin present renders them insoluble (29). Following perfusion, portions of peritoneal wall, diaphragm, and mesentery were excised, immersed in acetic acid-formalin for an additional 2–4 h, and processed for paraffin embedding. Immunoperoxidase staining was performed using a peroxidase-antiperoxidase technique (29) with primary rabbit antibodies to fibrinogen (Dako Corp., Carpinteria, CA). After reaction and color development with 3,3-diaminobenzidine tetrahydrochloride, sections were counterstained with hematoxylin.

**Statistics.** Because our data did not conform to bell-shaped distributions with equal SDs, comparisons were made with nonparametric tests, the Kruskall-Wallis test, and Dunn’s variation of the Bonferroni test (30).

**RESULTS**

**Extravasation of Circulating $^{125I}$-F Tracer Into the Peritoneal Cavities and Peritoneal Walls of TA3/St and MOT Ascites Tumor-Bearing Mice.** As we have reported for other macromolecular tracers (14–16), substantial amounts of circulating $^{125I}$-F entered the peritoneal cavities of mice bearing either ascites tumor (Fig. 1). Extravasation became statistically significant by day 7 after i.p. injection of TA3/St tumor cells, and maximum inflow was observed on day 8 when as much as 25% of tracer was found in ascites fluid 120 min after i.v. injection (versus 0.5% in control mice; P from <0.05 to <0.001). In MOT-bearing mice, significantly increased tracer inflow was observed from days 10–20 after i.p. tumor cell injection (P from...
<0.05 to <0.01); on day 20, ~50% of the i.v.-injected dose of $^{125}$I-F had collected in ascites fluid within 180 min (versus 0.5% in control mice). These differences in inflow were not biased by differences in plasma clearance rates of $^{125}$I-F, which were not statistically different between ascites tumor-bearing and control mice, at least during the first 3 h after i.v. tracer injection (data not shown).

Extravasated $^{125}$I-F was also found in the peritoneal lining tissues of ascites tumor-bearing mice, although in much smaller amounts than were found in ascites fluid. In normal control A/J or C3Hb/FeJ mice, ~0.4% (range, 0.3 to 0.7%) of injected tracer was detected in the peritoneal walls at intervals from 5 to 180 min after i.v. tracer injection (Fig. 1, d0). Thereafter, inflow of $^{125}$I-F into the peritoneal walls increased substantially above control levels in TA3/St ascites tumor-bearing mice, reaching a maximum of 1.7% of the injected dose at 120 min on day 8 ($P < 0.05$; Fig. 1). In MOT ascites tumor-bearing mice, more than 2% of i.v.-injected $^{125}$I-F was found in peritoneal walls at 60 min ($P < 0.05$) on day 15 after tumor cell inoculation (Fig. 1). Overall, $^{125}$I-F inflow into the peritoneal walls of MOT and TA3/St ascites tumor-bearing mice increased by as much as 3.3- to 4.7-fold, respectively.

**Inflow of $^{125}$I-F into the Peritoneal Wall, Mesentery, and a Distant Control Tissue (Leg Muscle) of Ascites Tumor-bearing Mice.** When the amount of tracer entering tissues was small relative to the amount injected i.v., calculation of the percentage of injected dose became subject to considerable error; this reflected unavoidable imprecision in determining the exact amount of tracer that was injected i.v. at time zero. To avoid such error, tracer influx was better expressed as microliters of plasma $^{125}$I-F measured per gram of tissue (Fig. 2). Using this measure, $^{125}$I-F influx into the peritoneal walls of TA3/St ascites tumor-bearing mice increased >3-fold from ~25 μl/g in control animals (day 0) to 81 μl/g in 8-day ascites animals (at 180 min; $P < 0.001$). In MOT ascites tumor-bearing mice, $^{125}$I-F influx into the peritoneal walls increased nearly 7-fold above control levels (15 μl/g), peaking at ~103 μl/g in 15-day animals ($P < 0.001$); $^{125}$I-F influx was also significantly increased in MOT ascites tumor-bearing mice at 10, 20, and 27 days (Fig. 2, shown through day 20).

Accompanying increased influx of total $^{125}$I-F tracer into the peritoneal walls of ascites tumor-bearing mice was an increase in the amount of $^{125}$I-F tracer that had become insoluble in 3 M urea. For example, at 180 min after i.v. tracer injection, 10 and 7 μl/g urea-insoluble fibrin was found in 7- and 8-day TA3/St ascites tumor-bearing mice, respectively (Fig. 2). Significantly increased amounts of the $^{125}$I-F that entered the peritoneal walls of 10-, 15-, and 20-day MOT ascites tumor-bearing mice were also insoluble in 3 M urea (6–12 μl/g at 60 to 180 min, compared with values in control mice of 2–4 μl/g).

A similar increase in $^{125}$I-F influx, both total and urea-insoluble, was also observed in the mesenteries of mice bearing either ascites tumor, achieving statistical significance on day 7 (TA3/St) and on day 10 (MOT) and persisting thereafter (Fig. 2). In comparison, influx of $^{125}$I-F into leg muscle, a control tissue distant from the peritoneal cavity, remained low in animals bearing either tumor. Nonetheless, a small but statistically significant increase in total $^{125}$I-F influx was observed into leg muscle on days 15 and 20 in MOT-bearing mice; significantly increased urea-insoluble $^{125}$I-F was not detected in skeletal muscle (Fig. 2).

**Accumulation of $^{125}$I-F in Peritoneal Cavity Fluid of TA3/St and MOT Ascites Tumor-bearing Mice.** The percentage of i.v.-injected radioactivity that was found in the peritoneal fluid of control and ascites tumor-bearing mice 18 h after $^{125}$I-F tracer injection is presented in Table 1. In TA3/St animals, a maximum of ~23% of the injected dose of $^{125}$I-F accumulated in the ascites of 7–8-day ascites tumor-bearing animals, a ~32-fold increase above the pooled values from days 0 and 2 ($P < 0.001$). In MOT-bearing mice, peritoneal fluid accumulation of i.v.-injected $^{125}$I-F tracer also increased significantly,
beginning at 19–22 days and reaching a maximum value of >59% by days 29–33 (P < 0.01).

Accumulation of 125I-F in Peritoneal Lining Tissues and Leg Skeletal Muscle of TA3/St and MOT Ascites Tumor-bearing Mice. The percentage of i.v.-injected 125I-F that accumulated at 18 h in the peritoneal walls of control and ascites tumor-bearing animals is also presented in Table 1. In TA3/St animals, a maximum of 0.62% of the injected dose accumulated on days 7–8, a value statistically greater than that of pooled values from 0- and 2-day mice. In MOT-bearing mice, peritoneal wall accumulation of the i.v.-injected 125I-F tracer had increased significantly (P < 0.01) at 19–22 days, amounting at that time to 0.87% of the injected dose. Furthermore, the amount of urea-insoluble 125I-F that accumulated in peritoneal walls was significantly increased in MOT animals from 14–26 days after i.v. tracer injection (0.11–0.35%). In contrast, the amount of urea-insoluble 125I-F tracer that accumulated in TA3/St-bearing mice (0.09%) remained unchanged as compared with control levels (Table 1).

As with influx measurements, accumulation of 125I-F tracer in peritoneal lining tissues was more reliably expressed as microliters plasma 125I-F/gram wet tissue (Fig. 3). In TA3/St-bearing mice, significantly increased amounts of 125I-F accumulated in the peritoneal wall as early as day 5 after i.p. tumor cell injection and in both diaphragm and mesentery by day 7. Maximal accumulation was 5–10-fold higher than in control, nontumor-bearing mice. Furthermore, typically 19–28 μl/g of urea-insoluble 125I-F accumulated in 7- and 8-day TA3/St ascites tumor-bearing mice. Of interest, these values were only 2.4–3.7-fold greater than those of control animals; hence, the percentage of total accumulated 125I-F that was urea-insoluble (12–14%) was actually significantly lower (P < 0.001) than the 21–24% measured in the peritoneal lining tissues of control mice not harboring tumor (Table 1).

In MOT-bearing mice, significantly increased 125I-F accumulation was observed by day 14 in the peritoneal wall, diaphragm, and mesentery and persisted thereafter, reaching a peak between days 15 and 19 (Fig. 3). Maximal accumulation was 3–7-fold higher in MOT ascites tumor-bearing than in control mice. A substantial amount of the 125I-F that accumulated in peritoneal lining tissues was urea-insoluble; i.e., 24–98 μl/g on days 15–19, 5–21 times the amounts found in these tissues in control mice. As a result, and in contrast to TA3/St-bearing mice, the percentage of 125I-F that accumulated in MOT peritoneal lining tissues (21–49%) was significantly (P < 0.001) higher than that found in control mice (13–17%; Table 1).

Biochemical Characterization of Ascites Tumor-associated 125I-F. Mice bearing TA3/St and MOT ascites tumors were injected i.v. with 125I-F, and 18 h later, the peritoneal walls, diaphragms, and mesenteries were recovered and extracted for SDS-PAGE. Autoradiographs of reduced SDS-PAGE gels revealed clear γ-γ dimers and polymerized α chains (Fig. 4), characteristic features of cross-linked fibrin (25, 31, 32).

Immunohistochemical Detection of Fibrin in Peritoneal Lining Tissues of Ascites Tumor-bearing Mice. By using an acetic acid-formalin fixation procedure, non-cross-linked fibrin species were extracted from peritoneal tissues prior to tissue embedding; therefore, the remaining deposits observed by immunohistochemistry to be reactive with anti-fibrinogen antibodies largely represented cross-linked fibrin (29). Fibrin staining was not observed in the peritoneal wall (Fig. 5A), diaphragm, mesentery, or bowel serosa of normal mice.

Table 1 Accumulation (18 h) of 125I-F in peritoneal fluid and peritoneal walls of TA3/St and MOT ascites tumor-bearing and control mice

<table>
<thead>
<tr>
<th>Mouse Strain (tumor)</th>
<th>Days after i.p. tumor cell injection</th>
<th>Total 125I-F Accumulation peritoneal fluid (% ID)</th>
<th>Total 125I-F Accumulation peritoneal wall (% ID)</th>
<th>Accumulation of Urea-Insoluble 125I-F in peritoneal wall (% ID)</th>
<th>% of Accumulated 125I-F in peritoneal wall that is Urea-Insoluble</th>
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</thead>
<tbody>
<tr>
<td>A/Jax (TA3/St)</td>
<td>0, 2</td>
<td>0.71 ± 0.12</td>
<td>0.36 ± 0.04</td>
<td>0.09 ± 0.01</td>
<td>21.9 ± 1.05</td>
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<td></td>
<td>5</td>
<td>7.25 ± 1.27</td>
<td>0.44 ± 0.05</td>
<td>0.09 ± 0.01</td>
<td>21.1 ± 1.17</td>
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<tr>
<td></td>
<td>7-8</td>
<td>22.96 ± 2.90***</td>
<td>0.62 ± 0.06*</td>
<td>0.09 ± 0.01</td>
<td>12.8 ± 0.77***</td>
</tr>
<tr>
<td>C3Heb/FvJ (MOT)</td>
<td>0</td>
<td>0.40 ± 0.05</td>
<td>0.34 ± 0.11</td>
<td>0.05 ± 0.01</td>
<td>18.2 ± 1.51</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2.42 ± 0.65</td>
<td>0.49 ± 0.10</td>
<td>0.09 ± 0.01</td>
<td>19.6 ± 1.43</td>
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<td>8</td>
<td>3.58 ± 0.55</td>
<td>0.33 ± 0.04</td>
<td>0.07 ± 0.01</td>
<td>20.8 ± 1.50</td>
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<tr>
<td></td>
<td>14–15</td>
<td>24.99 ± 2.4</td>
<td>0.49 ± 0.04</td>
<td>0.11 ± 0.01**</td>
<td>21.3 ± 0.61</td>
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<tr>
<td></td>
<td>19–22</td>
<td>35.27 ± 2.99***</td>
<td>0.87 ± 0.07**</td>
<td>0.35 ± 0.02***</td>
<td>38.7 ± 1.52***</td>
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<tr>
<td></td>
<td>26</td>
<td>51.78 ± 6.27***</td>
<td>0.80 ± 0.08</td>
<td>0.19 ± 0.01**</td>
<td>24.9 ± 5.05</td>
</tr>
<tr>
<td></td>
<td>29–33</td>
<td>59.42 ± 15.63***</td>
<td>0.49 ± 0.03</td>
<td>0.08 ± 0.01</td>
<td>20.4 ± 1.40</td>
</tr>
</tbody>
</table>

*From three to eight mice were studied at each time interval. Data are expressed as mean % ± SE. Significant Ps were calculated by the Kruskall-Wallis test (see “Materials and Methods”).
and Methods”) prepared from the peritoneal wall (Lane 1), diaphragm (Lane 2), and representing @I-F prior to iv. injection (Lane 4) and of the urea residue (see “Materials and Methods”) prepared from the peritoneal wall (Lane 1), diaphragm (Lane 2), and mesentery (Lane 3) of a 7-day TA3/St ascites tumor-bearing mouse 18 h after i.v. 125I-F injection. Distinct γ-γ dimer (M, ~98,000) and α-polymers (M, >200,000) bands, indicative of cross-linked fibrin, are present in the tissue extracts.

Control animals. However, in ascites tumor-bearing animals, fibrin deposits were detected as early as 4–5 days (TA3/St) or 5–7 days (MOT) as fibrillar strands on the surface of the peritoneal wall, independent of adherent tumor cells, and extending into the submesothelial space; also, fibrin was deposited in the fascia separating the longitudinal and circular muscle layers (Fig. 5). Fibrin deposits became progressively more intense and extensive thereafter, reaching a maximum at 7–8 days (TA3/St) and 10–20 days (MOT) (Fig. 5). Not uncommonly, clumps of tumor cells were held together and apparently attached to the peritoneal surface by a fibrin meshwork (Fig. 5C). After ~20 days in MOT ascites tumor-bearing mice, fibrin deposited between muscle bundles of the peritoneal wall was gradually replaced by fibrous connective tissue, whereas that on the surface persisted for a longer time (Fig. 5, E and F) before it too was replaced by fibrous connective tissue (Fig. 5, G and H). Fibrin deposits similar to those found on and within the peritoneal wall were also found in the mesentery and sometimes also on the surface of the large and small bowel (Fig. 5, I–K).

DISCUSSION

Our data call attention to an apparently paradoxical difference in the processing of extravasated plasma fibrinogen in solid and ascites tumors. In solid tumors, circulating fibrinogen extravasates from hyperpermeable tumor-associated blood vessels, and a large fraction of extravasated fibrinogen clots to form extravascular deposits of fibrin gel (1–6). The microvessels lining the peritoneal cavities of ascites tumor-bearing animals are also hyperpermeable to circulating macromolecules (14–16), but the ascites fluid that accumulates does not coagulate to form a peritoneal cavity-filling fibrin gel. Several possible explanations could individually or collectively account for the failure of tumor ascites fluid to gel in vivo: (a) peritoneal wall blood vessels, although abnormally permeable to other macromolecules, are not hyperpermeable to fibrinogen; (b) fibrinogen leaks from peritoneal vessels but is clotted in fibrin in the peritoneal lining tissues and never reaches the peritoneal cavity; (c) clottable fibrinogen leaks into the peritoneal cavity but does not clot in ascites fluid; and (d) fibrinogen leaks from peritoneal vessels, reaches the peritoneal cavity, and is degraded there, either before clotting or immediately thereafter, and before significant fibrin accumulation can occur.

The experiments reported here have begun to distinguish among these several possibilities. First, we have shown that the tissues lining the peritoneal cavity of ascites tumor-bearing mice are indeed hyperpermeable to 125I-F, as they are to other macromolecular tracers such as fluoresceinated dextrans and radiolabeled albumin (14–16). Influx of 125I-F into the peritoneal cavity, parietal peritoneum, diaphragm, and mesentery of mice bearing either tumor greatly exceeded the influx of this tracer into the same tissues in normal animals. Moreover, the permeability of peritoneal lining vessels of ascites tumor-bearing mice was increased to essentially the same extent for 125I-F as for the other tracers we have studied, i.e., 3–10-fold (14–16). However, as with other macromolecular tracers that we have studied, increased permeability of peritoneal lining tissues to 125I-F did not become detectable for some days after i.p. injection of tumor cells; i.e., much later than when the same tumor cells were injected s.c. to form solid tumors (2, 4, 33, 34). Likely reasons for the delayed hyperpermeability of peritoneal vessels in ascites tumors were discussed in the preceding paper (14).

We have also shown that a significant percentage of the 125I-F that extravasated from leaky peritoneal vessels accumulated in peritoneal wall, mesentery and diaphragm. In fact, the amounts of 125I-F that accumulated in peritoneal lining tissues, although somewhat less on a per gram basis, were nonetheless of the same order of magnitude as accumulated in these same tumors grown in solid form (33). Thus, solid TA3/St tumors growing in the s.c. space accumulated 470 ± 30 µl/g 125I-F (33), whereas ascites TA3/St tumors accumulated 229 ± 11, 202 ± 17, and 188 ± 20 µl/g 125I-F in the peritoneal wall, diaphragm, and mesentery, respectively (Fig. 3). Similarly, solid MOT tumors accumulated 302 ± 20 µl/g 125I-F (33), whereas 125I-F deposits in the peritoneal lining tissues of ascites MOT tumors were: peritoneal wall, 202 ± 10 µl/g; diaphragm, 147 ± 6 µl/g; and mesentery, 285 ± 24 µl/g. Of course, when total tracer accumulation in peritoneal lining tissues was considered, rather than microliters tracer per gram tissue, the extent of 125I-F accumulation was substantial. For example, in an 8-day MOT ascites tumor, 125I-F accumulation was 50 µl/g, and the total weight of parietal peritoneal wall was 1 g; hence, the peritoneal wall of a mouse bearing an 8-day MOT ascites tumor contained a total of 50 µl plasma 125I-F; an amount 8–9 times greater than that found in a comparably aged MOT solid tumor (typically weighing 20–30 mg). Similar calculations showed that more than 200 µl of plasma 125I-F accumulated in the parietal peritoneal walls of mice bearing 19-day MOT ascites tumors.

As with solid tumors, a fraction of the 125I-F that accumulated in peritoneal lining tissues of ascites tumor-bearing mice was insoluble in 3% urea and, therefore, presumably represented cross-linked fibrin. The presence of fibrin was confirmed by immunohistochemistry, which permitted identification of fibrin deposits both within and on the peritoneal lining surfaces of ascites tumor-bearing mice (Fig. 5). Incontrovertible evidence for the presence of cross-linked fibrin in peritoneal lining tissues came from autoradiographs of reduced SDS-PAGE gels (Fig. 4), which demonstrated its characteristic signature, namely, γ-γ dimers and polymerized α chains. Thus, peritoneal lining tissues, like skin, the s.c. space, etc., were able to support extravascular clotting (2, 31, 33, 35). Nonetheless, the percentage of accumulated 125I-F that was urea-insoluble was generally lower in ascites than in solid tumors. Thus, whereas 46% of the 125I-F that accumulated in solid TA3/St tumors was urea insoluble (33), comparable percentages for peritoneal lining tissues of TA3/St ascites tumor-bearing mice were only 11–14% (Table 1; Fig. 3). In solid MOT tumors, 44% of accumulated 125I-F was urea insoluble (33), whereas 21–49% (Fig. 3) of the 125I-F that accumulated in peritoneal lining

Fig. 4. Autoradiographs of a reduced 4–20% Tris-glycine SDS-polyacrylamide gel representing 125I-F prior to i.v. injection (Lane 4) and of the urea residue (see “Materials and Methods”) prepared from the peritoneal wall (Lane 1), diaphragm (Lane 2), and mesentery (Lane 3) of a 7-day TA3/St ascites tumor-bearing mouse 18 h after i.v. 125I-F injection. Distinct γ-γ dimer (M, ~98,000) and α-polymers (M, >200,000) bands, indicative of cross-linked fibrin, are present in the tissue extracts.
tissues of 14–15-day MOT ascites tumor-bearing mice was insoluble in 3 M urea. The reasons for the observed differences in processing of extravasated 125I-F in the vicinity of solid versus ascites tumors are presently unknown.

Although some of the 125I-F that extravasated from leaky peritoneal lining vessels clotted and remained in peritoneal tissues, the great majority (Fig. 1) of extravasated 125I-F passed through these tissues and entered the peritoneal cavity. Others have reported fibrinogen and also soluble fibrin and fibrinogen/fibrin degradation products in ascites fluid and have suggested that both clotting and fibrinolytic mechanisms had been activated (36–39). However, these reports did not determine whether clotting and fibrinolysis actually took place within ascites fluid or whether, instead, the products identified were generated in the surrounding peritoneal tissues and subsequently diffused into ascites fluid (40). Our data are at least consistent with the latter possibility.

It is likely that the fibrin deposited on peritoneal surfaces has an important role in tumor cell implantation. Immunohistochemistry revealed that adherent tumor cells were enmeshed in an acetic acid-insoluble fibrin meshwork which bound them to each other and to the peritoneal surfaces (Fig. 5). That fibrin or proteins associated with it (such as fibronectin) might play a role in tumor cell attachment to peritoneal surfaces is not unexpected in that these matrix proteins have been commonly linked with attachment of cells to a variety of substrates by RGD-integrin and other interactions (41, 42). Furthermore, embedding free tumor cells within a fibrin clot favors tumor implantation in the serosa (43), and heparin inhibits tumor cell implantation (44). Previous authors have postulated a role for fibrin in the attachment of tumor cells to denuded peritoneal basement membranes, although they lacked methods that could identify fibrin definitively (13, 45–50). As one example, Sampson (45) proposed more than 60 years ago that, "Next to the cancer cells themselves, fibrin is the most important factor in the life history of peritoneal implantation. It is fibrin which holds the cancer cells in place by splitting them against the denuded peritoneum. Fibrin is both the temporary framework and the scaffolding of organized implants." Our experiments in MOT and TA3/St tumors provide definitive support for Sampson's hypothesis, and it is likely that our findings can be generalized to other tumors. Thus, although the time course of fibrin accumulation was not investigated, prominent fibrin deposits have also been found by immunofluorescence within and on the surfaces of the parietal peritoneum of guinea pigs bearing line 10 ascites tumor (51).

Finally, our data provide the first evidence that, in animals with a heavy tumor burden, blood vessels at a distance from tumor cells may also become hyperpermeable. Thus, 125I-F influx was consistently increased by a small but statistically significant amount in leg skeletal muscle of mice bearing 7–8-day TA3/St and 14–22-day MOT ascites tumors (Fig. 3); 125I-F influx was also increased in mice.
bearing 15- and 20-day MOT ascites tumors (Fig. 2). The mechanisms of this distant hyperpermeability have not been investigated, but it may be attributable to systemic dispersion of tumor cell-secreted VPF, which is known to be present in tumor ascites fluid in extremely high concentrations (14, 52).

In summary, although the kinetics of fibrin deposition in ascites tumor-bearing mice differed from those observed when these same tumors were grown in solid form, the observation that cross-linked fibrin is deposited in the peritoneal walls of ascites tumor-bearing animals indicates that ascites tumor growth is accompanied by a sequence of events that closely parallels those reported for solid tumors (4–6). Moreover, this sequence recapitulates many of the events of normal wound healing, including microvascular hyperpermeability to circulating macromolecules, extravasation of plasma fibrinogen, extravascular clotting, and deposition of cross-linked fibrin. Thus, although it remains uncertain as to why tumor ascites fluid fails to clot in vivo, we have demonstrated fibrin deposition in and on the surface of peritoneal walls, mesenteries, and diaphragms. By analogy with solid tumors (4), such deposits might be expected to trigger a cascade of events leading to angiogenesis and fibroblasia in peritoneal lining tissues, a possibility that we tested in the following paper (53).

REFERENCES

Pathogenesis of Ascites Tumor Growth: Fibrinogen Influx and Fibrin Accumulation in Tissues Lining the Peritoneal Cavity
