Pathogenesis of Ascites Tumor Growth: Angiogenesis, Vascular Remodeling, and Stroma Formation in the Peritoneal Lining

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ABSTRACT

In the accompanying papers, we demonstrated that two murine ascites tumors (MOT and TA3/St) induced peritoneal lining blood vessels to become hyperpermeable to plasma proteins, leading to extravasation of fibrinogen and its clotting to cross-linked fibrin in peritoneal lining tissues (peritoneal wall, mesentery, and diaphragm). In solid tumors, vascular hyperpermeability and fibrin deposition lead to the generation of vascularized connective tissue. In order to determine whether fibrin had similar consequences in ascites tumors, the vasculature and stroma of peritoneal lining tissues were analyzed at successive intervals after i.p. tumor cell injection. In both MOT and TA3/St ascites tumors, the size and number of peritoneal lining microvessels increased significantly by 5–8 days. Subsequently, peritoneal lining vessels increased in cross-sectional area by as much as 15-fold and peritoneal vascular frequency increased by up to 11-fold. Incorporation of $^3$H(thymidine by mesenteric blood vessels was negligible in control animals but came to involve 20 and 40% of endothelial cells lining mesenteric vessels in MOT and TA3/St ascites tumor-bearing mice, respectively. After an early dramatic increase in cross-sectional area, peritoneal lining microvessels subsequently underwent a novel form of remodeling to smaller average size as the result of transvascular bridging by endothelial cell cytoplasmic processes. Thus, both of the ascites tumors studied here induced angiogenesis and stroma similar to that elicited when these same tumors were grown in solid form. However, stroma developed more slowly in ascites than in solid tumors and was entirely confined to a compartment (peritoneal lining tissues) that was distinct from that (peritoneal cavity) containing the majority of tumor cells and ascites fluid. These findings are consistent with the hypothesis that vascular hyperpermeability, induced in both solid and ascites tumors by tumor cell-secreted vascular permeability factor, is a common early step in tumor angiogenesis, resulting in fibrinogen extravasation, fibrin deposition, and likely other alterations of the extracellular matrix that together stimulate new vessel and fibroblast ingrowth.

INTRODUCTION

Experimental tumors growing in solid form commonly initiate the generation of vascularized stroma by rendering local blood vessels hyperpermeable to plasma proteins (1–5). As a consequence of vascular hyperpermeability, plasma fibrinogen, normally confined to vascular hyperpermeability and fibrin deposition lead to the generation of vascularized connective tissue. In order to determine whether fibrin had similar consequences in ascites tumors, the vasculature and stroma of peritoneal lining tissues were analyzed at successive intervals after i.p. tumor cell injection. In both MOT and TA3/St ascites tumors, the size and number of peritoneal lining microvessels increased significantly by 5–8 days. Subsequently, peritoneal lining vessels increased in cross-sectional area by as much as 15-fold and peritoneal vascular frequency increased by up to 11-fold. Incorporation of $^3$H(thymidine by mesenteric blood vessels was negligible in control animals but came to involve 20 and 40% of endothelial cells lining mesenteric vessels in MOT and TA3/St ascites tumor-bearing mice, respectively. After an early dramatic increase in cross-sectional area, peritoneal lining microvessels subsequently underwent a novel form of remodeling to smaller average size as the result of transvascular bridging by endothelial cell cytoplasmic processes. Thus, both of the ascites tumors studied here induced angiogenesis and stroma similar to that elicited when these same tumors were grown in solid form. However, stroma developed more slowly in ascites than in solid tumors and was entirely confined to a compartment (peritoneal lining tissues) that was distinct from that (peritoneal cavity) containing the majority of tumor cells and ascites fluid. These findings are consistent with the hypothesis that vascular hyperpermeability, induced in both solid and ascites tumors by tumor cell-secreted vascular permeability factor, is a common early step in tumor angiogenesis, resulting in fibrinogen extravasation, fibrin deposition, and likely other alterations of the extracellular matrix that together stimulate new vessel and fibroblast ingrowth.

In the two accompanying papers (18, 19), we demonstrated that two transplantable murine ascites carcinomas, MOT and TA3/St,(13–17) shared several important properties with these same and many other transplantable and autochthonous tumors growing in solid form (1–4, 7, 20–27). Shared properties included: (a) secretion by tumor cells of a potent cytokine, VPF; (b) hyperpermeability of tumor blood vessels; (c) extravasation of plasma proteins, including fibrinogen; and (d) clotting of a fraction of extravasated fibrinogen to form cross-linked fibrin deposits in peritoneal lining tissues.

Whether resulting from solid tumors (4, 7, 26), wound healing (21, 23), or from direct implantation of fibrin-filled porous chambers (28), deposition of fibrin in tissues has been shown to stimulate the ingrowth of new blood vessels and the subsequent development of fibrous connective tissue stroma. The present study was undertaken to determine whether fibrin deposited in the peritoneal lining tissues of ascites tumor-bearing animals also induced angiogenesis and the laying down of new connective tissue stroma.

MATERIALS AND METHODS

Tumors. TA3/St and MOT tumor cells ($1 \times 10^6$) were passaged weekly in the peritoneal cavities of syngeneic, 5- to 6-week-old A/Jax and C3H/HeJ mice, respectively (18). Sequential study of peritoneal lining tissues was conducted through day 8 after i.p. injection of $10^6$ TA3/St tumor cells; most animals died by day 9 or 10. In contrast, the majority of animals injected i.p. with $10^6$ MOT tumor cells survived for >1 month and were followed at intervals for up to 28 days.

Vascular Mapping with Microfil. To map the microvasculature of the tissues lining the peritoneal cavity, animals were perfused with Microfil White (MV-112; Canton Bio-Medical products, Inc., Boulder, CO), a silicone rubber compound (27). After the Microfil had hardened, peritoneal lining tissues were dissected free, fixed in formalin, dehydrated in a sequence of ethyl alcohols, and cleared in methyl salicylate for examination in a Wild microscope.

Tissue Processing for Microscopy. Tissues lining the peritoneal cavity (peritoneal walls, diaphragm, and mesentery) were fixed and processed for 1-µm-thick, Epon-embedded and Giemsa-stained light microscopic sections (18, 27). To evaluate blood vessel size and frequency at various stages of ascites tumor growth, 1-µm-thick Epon sections of randomly selected portions of mesentery were photographed at constant magnification (×675). Color prints (4 x 6 inches; Ektar 25; Eastman Kodak) were prepared, and all microvessels (defined as vessels lacking muscle in their walls) were identified, and their lumens were outlined with a photo marking pen. Individual prints were then imaged with a video camera into a computer equipped with IM-series version 3.46p software (Analytical Imaging Concepts). Once digitized, measurements of vessel size (diameter and cross-sectional area) and density (number of vessels per unit area) were calculated.

Identification of Hyperpermeable Blood Vessels and Endothelial Cells That Had Incorporated $^3$HThymidine. To identify leaky tumor blood vessels, mice received colloidal carbon i.v. 1 h prior to harvest (18). In some experiments, animals were also injected i.v. with $^3$HThymidine (25 µCi/mouse; Du Pont New England Nuclear, Boston, MA) 2 h prior to harvest. Mesenteries were fixed and processed for 1-µm Epon sections as above. Epon sections were coated with Kodak NTB-2 emulsion for autoradiography. Sections from randomly selected blocks were examined in their entirety at a magnification of ×1000; all blood vessels were identified, and carbon-labeled (hyperpermeable) blood vessels and $^3$HThymidine incorporating (proliferat-
ing) endothelial cell nuclei were separately counted. More than 5000 individual vessels and a somewhat larger number of endothelial cell nuclei were counted in the course of these studies.

**Statistics.** Data did not fit a Gaussian distribution, and there were significant differences in the SDs of the various test groups; therefore, data were analyzed with nonparametric tests (Kruskal-Wallis and Dunn’s multiple comparisons test) as described previously (18, 29).

**RESULTS**

**Microvascular Changes in the Peritoneal Lining Tissues of Mice Bearing Syngeneic MOT and TA3/St Ascites Tumors.** In both animal models, the vasculature supplying the peritoneal wall and diaphragm underwent important changes within a few days of i.p. tumor cell injection. As noted previously (18, 19), the initial change was microvascular hyperpermeability to circulating macromolecules, followed by extravasation of plasma fibrinogen and its clotting to cross-linked fibrin. Leaky microvessels were often distended with erythrocytes, suggesting sludging of blood flow (Fig. 1). Thereafter, microvessels underwent a dramatic increase in cross-sectional area, becoming large, thin-walled channels with persistent hyperpermeability to colloidal carbon (Fig. 1, B-D). These vessels became clustered superficially in the muscular layer of the peritoneal wall and diaphragm and for the most part were oriented parallel to the surface; lining endothelial cells appeared activated with enlarged nuclei, increased basophilic cytoplasm (Fig. 1B), and frequent mitotic figures. Overall vascular proliferation and vessel orientation parallel to the peritoneal surface were readily appreciated by en face macroscopic examination of peritoneal walls following perfusion with Microfil (Fig. 2).

The mesentery underwent changes similar to those of the peritoneal wall and diaphragm, but the process evolved with a faster tempo. In normal mesentery, capillaries were relatively inconspicuous and of normally small size (Fig. 3A). However, within a few days of i.p. injection of tumor cells, preexisting microvessels became greatly enlarged and became grouped together in prominent clusters (Fig. 3, B and C). Like their counterparts in the peritoneal wall (Fig. 1) and diaphragm, these vessels were congested with erythrocytes and were hyperpermeable to colloidal carbon. By 7 days (TA3/St) or 10–15
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Fig. 2. En face macroscopic view of Microfil White-perfused peritoneal walls of a control C3Heb/FeJ mouse (A) and of mice 12 (B) and 16 (C) days after i.p. injection of 1 × 10^6 MOT tumor cells. Note large increase in the number of perfused vessels in ascites tumor-bearing (B and C) as compared with control animals (A). In all instances, most vessels are oriented parallel to the peritoneal surface. All × 8.

days (MOT), clusters of enlarged, thin-walled microvessels had increased in prominence and had become concentrated at the mesenteric surface (Figs. 3, D-G). Individual vessels bulged into the peritoneal cavity (Fig. 3, D-G; Fig. 4). Projecting vessels commonly retained broad-based attachments to the underlying mesentery (Fig. 3, D-G; Fig. 4A); sometimes, however, projecting vessels with associated connective tissue formed long stalks that extended for significantly greater distances (i.e., 125 μm) into the peritoneal lumen (Fig. 4B). Bulging and protruding peritoneal surface vessels were fragile and highly susceptible to injury and bleeding (Fig. 4B). This was especially true in TA3/St-bearing animals whose ascites fluid was commonly bloody by day 8; i.p. hemorrhage was often sufficient to cause serious anemia or death. Even at the height of the response, vascular and interstitial changes were confined to fatty-vascular portions of the mesentery; portions of mesentery that lacked adipose tissue remained avascular.

As also observed in the peritoneal wall and diaphragm, tumor cells commonly adhered to the mesenteric surface, individually and in clumps; attachments were distributed over both fatty and nonfatty portions of mesentery, in all cases TA3/St >> MOT (Fig. 3H). As noted previously (18), attached TA3/St tumor cells often invaded the mesentery, whereas MOT tumor cells only did so rarely. However, mesenteries of MOT-bearing mice showed striking, progressive loss of fat cell lipid content and reversion of fat cells to a more undifferentiated phenotype with prominent basophilic cytoplasm and activated nuclei (Figs. 3, D-G). Individual vessels bulged into the peritoneal cavity (Fig. 3, D-G). A modest mononuclear cell and/or plasma cell infiltrate was sometimes also present.

Kinetics of Microvascular Hyperpermeability vis-à-vis Endothelial Cell DNA Synthesis. In control mice, hyperpermeable (carbon-labeled) mesenteric vessels were not observed and only rare mesenteric vascular endothelial cells incorporated [3H]thymidine (Fig. 5). However, as early as 2 days after i.p. injection of 10^6 TA3/St tumor cells, 2.4% of mesenteric vessels were carbon-labeled, and this frequency increased to a maximum of 43% by day 6, falling slightly thereafter until animal death after day 8 (Fig. 5A). Incorporation of [3H]thymidine by vascular endothelial cells was insignificant in control animals. In TA3/St-injected mice, [3H]thymidine incorporation was slower to develop than carbon labeling, becoming statistically significant only on day 6 when 42.1% of endothelial cells were labeled. Thereafter, [3H]thymidine labeling declined somewhat but remained significantly elevated to the time of animal death.

Similar patterns of carbon and [3H]thymidine labeling were obtained in MOT ascites tumor-bearing mice, but the process developed more slowly (Fig. 5B). Four days after i.p. injection of 1 × 10^6 MOT tumor cells, 5.6% of 387 mesenteric vessels counted were carbon-labeled, and this percentage increased progressively to a peak on day 12 of 59% (Fig. 5B). Thereafter, the frequency of carbon-labeled vessels declined but remained significantly elevated above control levels as late as day 28. As with the TA3/St tumor, labeling of mesenteric microvascular endothelial cell nuclei with [3H]thymidine developed more slowly than mesenteric microvascular carbon labeling in MOT ascites tumor-bearing mice; statistically significant thymidine incorporation did not occur before day 8 when 22% of endothelial cells counted were labeled (Fig. 5B). Thymidine labeling declined progressively thereafter, although remaining significantly above control levels at least through day 20.

An additional feature of the mesenteric microvasculature in ascites tumor-bearing animals was a change in the ratio of endothelial cell nuclei encountered per vascular cross section (Fig. 6). This ratio provides a cumulative measure of the number and (to a lesser extent) the size of endothelial cell nuclei. In normal control A/Jax and C3Heb/FeJ mice, the value of this ratio was 0.4 ± 0.1 and 0.65 ± 0.04, respectively. In TA3/St ascites tumor-bearing mice, the ratio increased more than 3-fold to a peak on day 8 (just prior to animal death) of 1.31 ± 0.11. In MOT-bearing mice, the ratio increased more than 4-fold to a maximum on day 15 of 1.82 ± 0.11; thereafter, it declined slightly but remained significantly elevated through day 28.

Morphometric Analysis of the Size and Frequency of Mesenteric Blood Vessels as a Function of Ascites Tumor Growth. To test our impression that the blood vessels present in peritoneal lining tissues underwent a striking early increase in cross-sectional (luminal) area and frequency, we performed morphometric analysis on mesenteries harvested at successive intervals after i.p. injection of either MOT or TA3/St tumor cells. The data in Table 1 indicate that, in fact, the mean luminal cross-sectional areas of blood vessels increased significantly within 6—7 days of tumor cell injection. In TA3/St-bearing animals, mean mesenteric vessel area increased substantially, rising from control levels of 28 ± 5.3 μm² to a value of 121 ± 15 μm² at 8 days after i.p. tumor cell injection, a 4.3-fold increase; later values could not be obtained because most animals died shortly after day 8. In MOT tumor-injected animals, mean cross-sectional vessel area increased from 32 ± 5 μm² in control animals to 139 ± 26 μm² by day 6 (a 4.3-fold increase) and to 489 ± 48 μm² by 8.5 days (a 15.3-fold increase). Thereafter, mean cross-sectional vessel area declined but remained significantly above control values as late as day 23.

The increase in average vessel size that accompanied ascites tumor growth was followed by an equally dramatic increase in the heterogeneity of vessel cross-sectional area (Figs. 7 and 8). Thus, 2 days after i.p. injection of TA3/St tumor cells into A/Jax mice, almost 90% of mesenteric vessels had cross-sectional areas of ±50 μm² and only
Fig. 3. Mesenteries of mice bearing MOT (A–F and H) or TA3/St (G) ascites tumors. A, at 0–2 days after i.p. injection of $1 \times 10^6$ MOT tumor cells, the fatty, vascular mesentery appeared normal, consisting of numerous lipid-filled adipocytes (fat cells, f) separated by loose connective tissue stroma and small, inconspicuous blood vessels (arrowheads). B, at 6 days, scattered blood vessels (arrowheads) had enlarged in cross-sectional area and were hyperpermeable (carbon label not well visualized in these black and white photomicrographs). There was also slight interstitial edema and a mild mononuclear cell infiltrate. C, at 8 days, vascular changes were more pronounced. Enlarged, thin-walled, hyperpermeable blood vessels were prominently clustered in the fatty mesentery. D, at 12 days, enlarged vessels persisted within the fatty mesentery, but many vessels (v) were situated at or just beneath the mesenteric surface, bulging into the peritoneal cavity (pc). E and F, at 15 days, vascular hyperplasia persisted at the mesenteric surface, but vessels (v) were now of smaller size. Occasional enlarged, thin-walled vessels (v) remained more centrally located. Adipocytes (f) exhibited a striking reduction of lipid content and appeared smaller with prominent basophilic cytoplasm. G, mesentery from an A/Jax mouse 7 days after i.p. injection of TA3/St tumor cells. Enlarged, thin-walled, carbon-labeled (arrowheads) blood vessels (v) were prominent at the mesenteric surface and bulged into the peritoneal cavity (pc); adherent tumor cells commonly exhibited mitotic figures (m). H, portion of normally thin, fat-free, avascular mesentery (M) with attached tumor cells, 28 days after i.p. injection of MOT tumor cells. A–C and E, $\times$ 220; D, F, and H, $\times$ 430; G, $\times$ 525.
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Fig. 4. Projection of mesenteric blood vessels into the peritoneal cavities of A/Jax mice 8 days after i.p. injection of 1 × 10⁶ TA3/St tumor cells. A, enlarged, engorged vessel (V), still enveloped by activated mesothelial cells (meso), bulges into the peritoneal cavity (pc). Some of the carbon that had been injected i.v. 1 h prior to harvest decorates the basement membrane (arrowheads), whereas other clumps of carbon particles persist in the vessel lumen between erythrocytes, indicative of sludged blood flow because normally carbon is entirely cleared from the circulation within 15 min after i.v. injection. Several smaller vessels (two of which are labeled, v) also exhibit basement membrane labeling with carbon (arrowheads), indicating hyperpermeability. Occasional macrophages (mac) within the peritoneal cavity have ingested carbon. f, fat-laden adipocyte; pc, peritoneal cavity. B, projection of a thin-walled, carbon-labeled microvessel into the peritoneal cavity (pc); arrowheads, focal leakage of erythrocytes. Tumor cells and macrophages are attached to the vascular stalk. Other small vessels (v) are located at the mesenteric surface. A, X 675; B, X 460 (bar, 17 μm).

Fig. 5. Hyperpermeability of mesenteric microvessels to carbon and incorporation of [³H]thymidine by endothelial cells lining these vessels as a function of time after i.p. injection of 1 × 10⁶ TA3/St (A) or MOT (B) tumor cells into A/Jax and C3Heb/FeJ mice, respectively. Counts were performed on 1-μm Epon sections. Data are presented as a percentage of carbon-labeled mesenteric vessels and a percentage of mesenteric small vessel endothelial cells that incorporated [³H]thymidine (mean). a, P < 0.05; b, P < 0.01. n = 3 animals at each time point; bars, SE.

Microvessel density was also expressed as a percentage of total tissue area, a measure that encompasses both microvessel size (cross-sectional area) and frequency (Table 1). In TA3/St ascites tumor-bearing mice, the percentage of mesentery occupied by vessels increased from 0.4 ± 0.1% in control animals to 4.9 ± 1.1% at 8 days, a 12-fold increment. In MOT tumor-bearing mice, the percentage of mesentery occupied by vessels rose from control levels of 0.7 ± 0.4% to a maximum of 18.2 ± 1.8% on day 15.5, a 26-fold increase.

Microvascular Remodeling. The new large-diameter blood vessels that formed in the mesentery, peritoneal wall, and diaphragm during the course of ascites tumor growth subsequently underwent an unusual form of remodeling, beginning about days 7–8 in mice bearing either ascites tumor. Remodeling was characterized by the formation of “bridges” of endothelial cell cytoplasmic processes that

1.6% of vessels had areas >200 μm²; in contrast, by day 8, more than one-half of the mesenteric microvessels had cross-sectional areas >50 μm², and 11% had luminal areas >200 μm². The vascular size heterogeneity that developed in MOT ascites tumor-bearing animals was even more striking (Fig. 8). Thus, whereas nearly 90% of vessels in control C3Heb/FeJ mice had cross sectional areas of ≤50 μm² and fewer than 2% had areas >200 μm², by day 8.5 after injection of MOT tumor cells almost 49% of vessels had areas >200 μm², and 32% had luminal areas >400 μm².

The number of microvessels per unit area also increased progressively in the mesenteries of animals bearing ascites tumors (Table 1). In TA3/St-bearing mice, vessel frequency increased from control levels of 131 ± 11 vessels/mm² to 404 ± 52 vessels/mm² on day 8, a 3.1-fold increase. Vessel frequency also increased in the mesenteries of MOT ascites tumor-bearing mice, rising from 110 ± 13 vessels/mm² in control mice to 282 ± 26 vessels/mm² at 8.5 days, a 2.6-fold increase. Vessel frequency continued to rise thereafter, achieving a peak at 20 days (1223 ± 94 vessels/mm², an 11.1-fold increase) before falling off slightly on day 23.

Fig. 6. Ratio of numbers of endothelial cell (EC) nuclei to number of microvessel profiles in the mesenteries of ascites tumor-bearing mice at various intervals after i.p. injection of 1 × 10⁶ TA3/St (A) or MOT (B) tumor cells. Counts were performed on 1-μm Epon sections. Data are presented as the mean; bars, SE. b, P < 0.01. n = 3 animals at each time point.
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Table 1. Morphometric analysis of vessel size (cross-sectional area), frequency (numbers of vessels/mm²), and percentage of total tissue area occupied by vessels in the mesenteries of control and TA3/St or MOT ascites tumor-bearing A/Jax or C3Heb/FeJ mice.

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Days</th>
<th>No. vessels</th>
<th>Total tissue area (μm²)</th>
<th>Vessel size (cross-sectional area)²</th>
<th>Vessel frequency (no. vessels/mm²)²</th>
<th>Percentage of vessel area²</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mean ± SEM (μm²)</td>
<td>Median</td>
<td>Mean ± SEM (%)</td>
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<tr>
<td>TA3/St</td>
<td>0 (control)</td>
<td>158</td>
<td>1,199,549</td>
<td>12</td>
<td>131 ± 11</td>
<td>0.36 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>88</td>
<td>477,474</td>
<td>16</td>
<td>242 ± 28*</td>
<td>1.0 ± 0.2*</td>
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<td></td>
<td>7</td>
<td>175</td>
<td>598,649</td>
<td>28</td>
<td>292 ± 25**</td>
<td>2.2 ± 0.4***</td>
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<tr>
<td></td>
<td>8</td>
<td>246</td>
<td>589,120</td>
<td>49</td>
<td>404 ± 52***</td>
<td>4.9 ± 1.1***</td>
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<tr>
<td>MOT</td>
<td>0 (control)</td>
<td>119</td>
<td>1,078,134</td>
<td>19</td>
<td>110 ± 13</td>
<td>0.72 ± 0.36</td>
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<td>4</td>
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<td>549,625</td>
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<td>155 ± 7.0</td>
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<td>6</td>
<td>168</td>
<td>1,062,018</td>
<td>44</td>
<td>158 ± 28</td>
<td>2.14 ± 0.64</td>
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<tr>
<td></td>
<td>8.5</td>
<td>315</td>
<td>1,114,222</td>
<td>191</td>
<td>282 ± 26**</td>
<td>11.8 ± 2.00***</td>
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<tr>
<td></td>
<td>12</td>
<td>329</td>
<td>443,398</td>
<td>44</td>
<td>742 ± 62**</td>
<td>12.3 ± 1.3***</td>
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<tr>
<td></td>
<td>15.5</td>
<td>843</td>
<td>1,083,395</td>
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<td>775 ± 58**</td>
<td>18.2 ± 1.8***</td>
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<tr>
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<td>20</td>
<td>624</td>
<td>510,413</td>
<td>30</td>
<td>1223 ± 94***</td>
<td>9.2 ± 1.9***</td>
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<td>23</td>
<td>385</td>
<td>471,588</td>
<td>23</td>
<td>816 ± 50**</td>
<td>4.9 ± 0.6***</td>
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</table>

a, *P < 0.05; **, P < 0.01; ***, P < 0.001.

crossed the lumens of enlarged vessels; as a result, single large-bore vascular channels became divided into two, three, or more channels of smaller diameter (Fig. 9). The vessels that underwent remodeling remained hyperpermeable, as judged by continued deposition of colloidal carbon in their basement membranes (Fig. 9, arrowheads); however, they remained patent, and intravascular clotting or thrombosis was never observed.

**DISCUSSION**

The data presented here demonstrate that TA3/St and MOT ascites tumors elicited a prominent vascularized connective tissue stroma in the tissues lining the peritoneal cavity. This connective tissue stroma was qualitatively similar to that generated in solid tumors but developed more slowly and formed a thin superficial rim in peritoneal lining tissues (peritoneal wall, mesentery, and diaphragm), i.e., stroma developed in a compartment separate from the vast majority of tumor cells which were suspended in ascites fluid. That ascites tumors induce angiogenesis and generate connective tissue stroma has not been generally appreciated; a literature search revealed only six previous citations describing stromal changes of the peritoneal lining in ascites tumors (30–36). Two of these references are abstracts, and none provided a comprehensive description of ascites tumor stroma or attempted to elucidate pathogenesis.

At their respective maxima, new blood vessels occupied 4.9—18.2% of total mesenteric area in TA3/St and MOT ascites tumors, respectively; these values are comparable to those (3–18%) reported for several murine tumors growing in solid form (reviewed in Ref. 37). Therefore, the angiogenesis that developed in ascites and solid tumors appeared to be of similar magnitude. Furthermore, angiogenesis and stroma formation in TA3/St and MOT ascites tumors were preceded by events similar to those that preceded angiogenesis and stroma formation in these same tumors grown in solid form (21). In both tumors, secreted VPF rendered blood vessels hyperpermeable, leading to extravasation of plasma fibrinogen and its clotting to fibrin; extravascular fibrin deposits, in turn, provided a provisional stroma that supported the ingrowth of new blood vessels and of fibroblasts which synthesized and deposited structural proteins and proteoglycans (3, 7,
Fig. 9. Remodeling of microvessels in mesenteries of mice bearing 12-day MOT (A and B) or 8-day TA3/St (C-F) ascites tumors. A and B, thin walled, dilated vessels are concentrated at the mesenteric surface and bulge into the peritoneal cavity (pc). Note multiple thin projections of endothelial cell cytoplasm that cross the vascular lumen (v), subdividing the lumens into multiple smaller channels. The large vessel in B (labeled twice with v) is subdivided into at least 20 subchannels; there are, in addition, several cytoplasmic projections that, at least in this section plane, terminate blindly and do not bridge across the lumen (arrowheads). f, adherent tumor cells; f, adipocyte. C and D, similar mesenteric vessels undergoing remodeling by transluminal bridging in 8-day TA3/St ascites tumor-bearing mice. E and F, tracings of vascular endothelial cells in C and D, respectively, to illustrate more clearly transluminal bridging by endothelial cell processes. Endothelial cell cytoplasm is stippled; colloidal carbon deposits are indicated as slightly larger black densities. A and B, × 670; C and E, × 790; D and F, × 970.

26, 38. VPF is also a selective endothelial cell mitogen (hence, its alternate name, vascular endothelial growth factor or VEGF) and, therefore, likely also promoted angiogenesis by stimulating endothelial cell division. Taken together, it would seem that similar mechanisms are involved in the induction of angiogenesis in solid and ascites tumors.

Despite these similarities, angiogenesis and stroma generation developed with different kinetics in solid and ascites tumors. When TA3/St or MOT cells were injected s.c. or intradermally to form solid tumors, angiogenesis was evident within 48–72 h (21, 27). By contrast, following i.p. injection of the same number of tumor cells, angiogenesis, as measured by Microfil perfusion (Fig. 2), vascular endothelial cell [3H]thymidine incorporation (Fig. 5), or by morphometric analysis (Table 1), did not become statistically significant until day 6 (TA3/St) or day 8 (MOT) and did not become maximal until day 8 (TA3/St) or day 20 (MOT). The likeliest explanation for the delayed kinetics of stroma formation in ascites tumors is that increased microvascular permeability, the earliest step we have been able to identify in the cascade of events leading to angiogenesis, is similarly delayed; i.e., microvascular hyperpermeability began days after i.p. tumor cell injection in contrast to hours after s.c. injection of the same number of tumor cells (18). Once microvascular permeability had increased, subsequent events such as fibrin deposition and increased vessel number proceeded rapidly as in solid tumors.

Our studies uncovered an additional novel property of ascites tumor growth, namely, that it is accompanied by an early and dramatic...
endothelial cells proliferated (A, endothelial cell mitosis). Subsequently, vessels underwent remodeling by transluminal bridging of endothelial cell processes (C); bridges eventually subdivided enlarged vessels into multiple smaller vessels (D and E) comparable in size to those in (A) prior to i.p. tumor cell injection.

enlargement in the cross-sectional areas of peritoneal microvessels (Figs. 1, 3, 4, 7, 9; Table 1). In both normal A/Jax and C3Heb/FeJ mice, the mean cross-sectional area of mesenteric microvessels measured ~30 μm², corresponding to a mean vessel diameter of 6.2 μm (assuming a circular cross-sectional profile). However, by 8 days after i.p. injection of TA3/St or MOT ascites tumor cells, mean mesenteric cross-sectional areas had increased enormously to 121 μm² and 489 μm², respectively, in TA3/St and MOT ascites tumor-bearing animals (corresponding to vessel diameters of ~12.4 and 25 μm, respectively). Increased microvascular size was accompanied by a parallel increase in vessel size heterogeneity (Figs. 7 and 8). In normal mice of both strains, microvascular cross-sectional area was relatively homogeneous; i.e., ≥90% of microvessels had cross-sectional areas of ≤50 μm² (mean diameter of ≤7.8 μm). However, 8.0—8.5 days following i.p. tumor cell injection, only 50% of TA3/St and <20% of MOT mesenteric microvessels fell into any single 50-μm² size bin, and cross-sectional areas of individual vessels varied from <50 to >2000 μm². With the further passage of time, mean vascular cross-sectional area returned toward control levels in MOT-bearing animals, and vascular cross-sectional areas became less heterogeneous (Fig. 8).

Others have reported that the blood vessels of solid tumors may also be increased in size (27, 37, 39—44). More particularly, Less et al. (41) reported a mean diameter of 10 μm for capillary meshwork vessels in the rat R3230AC mammary adenocarcinoma, a value significantly larger than that for capillaries in most normal tissues. Similarly, Solestik et al. (37, 43) found that mean vessel diameters ranged from 14.1 to 29.3 μm in three solid syngeneic mouse tumors and in five melanoma xenografts. Therefore, increased average cross-sectional area is likely a general property of tumor microvessels, both solid and ascites.

The mechanisms responsible for microvessel enlargement in ascites (or solid) tumors have not been carefully investigated. Increased mean vascular cross-sectional area was accompanied by vascular endothelial cell proliferation. Therefore, increased microvascular size cannot simply be explained by dilation of pre-existing, normal-sized vessels as might have resulted, for example, from a vascular response to smooth muscle-relaxing metabolites (e.g., adenosine) released from hypoxic tissues (45).

Several groups have suggested that partial proteolytic breakdown of vascular basal lamina occurs as an early step in the cascade of events leading to angiogenesis (46, 47). In support of this hypothesis, Paku and Pawelez (48) reported “expansion” of individual vessels surrounding the rat BSp73 AS adenocarcinoma within 1—2 days of transplant. These enlarged vessels, which they termed “mother” vessels because they subsequently served as the source of the more familiar tumor vessel sprouts, appeared by electron microscopy to have lost much or all of their basal lamina; however, although basal lamina ultrastructure was markedly altered, immunocytochemistry revealed that some laminin and type IV collagen persisted. Taken together, these data are consistent with the possibility that basal lamina proteolysis is among the earliest events in angiogenesis, leading to reduction in basal lamina mass and integrity. Such changes, accompanied by maintenance of a positive pressure gradient across the vascular wall (intravascular hydrostatic + colloid osmotic pressure > extravascular hydrostatic + colloid osmotic pressure) would be expected to cause vessels to dilate by stretching their walls and thinning and flattening their lining endothelium. This physical effect could itself have important biological significance. Others have shown, for example, that prolonged “stretching” of the blood vessel wall leads to increased vessel size (45) and that flattening of endothelial cells stimulates endothelial cell growth (49—51). It seems likely, therefore, that partial proteolysis of vascular basal lamina and consequent mechanical stretching are responsible for the observed increase in ascites tumor microvessel cross-section and may simultaneously stimulate vascular endothelium to divide.

The formation of enlarged microvessels early in the course of ascites tumor angiogenesis may also relate to the fibrin stroma in which these vessels develop. In an accompanying paper, we showed that fibrinogen extravasates from leaky blood vessels to form a fibrin gel matrix in the peritoneal lining tissues of mice bearing TA3/St or MOT ascites tumors (19). Moreover, the diameters of newly formed vessels were maximal in temporal association with maximum fibrin deposition. Relevant to these findings are studies of Nicosia and Ottinetti (52), who used a three-dimensional culture system to study vessel development in vitro. They related the size of newly forming vascular sprouts to the extracellular matrix in which their cultures were embedded. Sprouts that formed in plasma clots or fibrin gels had significantly larger mean luminal areas than vessels that formed in either collagen or Matrigel matrices. Taken together, these data suggest that the extracellular matrix influences microvessel morphogenesis and that fibrin matrices favor the generation of larger-sized vessels. The underlying mechanisms that might be responsible for determining this relationship are presently unknown.

An additional novelty of the enlarged vessels that form in ascites tumor-bearing animals was their subsequent return toward normal size by a peculiar form of vascular remodeling. Remodeling was accomplished by the formation of endothelial cell cytoplasmic bridges which crossed vascular lumens; as a consequence of such bridging, individual larger vessels were divided into two, three, and often multiple smaller components (Figs. 9 and 10). To our knowledge, such changes have not been reported previously in other tumors. However, somewhat analogous events have been described during primary vascularization of fetal lung (53), in cultured visceral capillary endothelial cells (54), and, more recently, in the chorioallantoic membrane of the developing chick, where the process has been referred to as “intussusceptive microvascular growth” (55). To the extent that this analogy.

Fig. 10. Schematic diagram illustrating the proposed sequence of vascular changes involving peritoneal lining microvessels during ascites tumor growth. Initially, microvessels of small calibre (A) became leaky (carbon deposits indicated between endothelial cell ablumens and underlying basement membrane) and increased in cross-sectional area (B); lining endothelial cells proliferated (M, endothelial cell mitosis). Subsequently, vessels underwent remodeling by transluminal bridging of endothelial cell processes (C); bridges eventually subdivided enlarged vessels into multiple smaller vessels (D and E) comparable in size to those in (A) prior to i.p. tumor cell injection.
holds, it represents yet another example of malignancy mimicking ontogeny.

An instructive feature of ascites tumor angiogenesis is the failed attempt of new blood vessel sprouts to extend any significant distance from the peritoneal walls and mesentery into the lumen of the peritoneal cavity. At the height of the angiogenic response in both TA3/St and MOT tumors, the developing new blood vessels became concentrated at the peritoneal cavity interface. Thin-walled vessels often bulged into the peritoneal cavity, and occasional vessels projected for short distances into the cavity lumen on stalks (Figs. 3, 4, and 9). However, such vessels were at great risk of injury, and i.p. hemorrhage commonly led to the death of TA3/St-bearing mice, a finding others have also observed in other tumor systems (32, 34). The frustrated attempts of vessels to grow into the peritoneal cavity lumen emphasize that endothelial and other connective tissue cells require a matrix on which to migrate. In solid tumors, and within the peritoneal lining tissues of ascites tumor-bearing animals, extravascular fibrin and likely other stromal elements provide such a matrix; however, no such solid matrix is to be found within body cavities. In this regard, it is of interest that peritoneal fluid can be clotted in vitro to form a gel within the peritoneal cavity, did not clot in vivo to form a gel within the peritoneal cavity that could support vascular ingrowth.

In summary, TA3/St and MOT tumor cells growing in ascites form a vascularized connective tissue stroma in the peritoneal wall, diaphragm, and mesentery that, although largely two-dimensional and slower to develop, was similar in other respects to that induced when tumor cells secrete a vascular permeability factor that promotes accumulation of fibrin clot. Science (Washington DC), 219: 983—985, 1983.


In summary, TM/St and MOT tumor cells growing in ascites form a vascularized connective tissue stroma in the peritoneal wall, diaphragm, and mesentery that, although largely two-dimensional and slower to develop, was similar in other respects to that induced when these same tumor cells were plated s.c. or intradermally and grew in solid form. Moreover, similar connective tissue stroma was induced, and apparently by similar mechanisms, in both instances. Thus, the differences in angiogenesis and stroma generation between solid and ascites tumors relate primarily to differences in kinetics and to the finding that, in ascites tumors, stromal elements accumulate in two separate compartments: ascites fluid in the peritoneal cavity and vascularized connective tissue in the tissues lining the peritoneal cavity; by contrast, in solid tumors, extravasated plasma exudate and newly formed connective tissue form a single stromal compartment that is interfingered with nests of tumor cells.

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4. Unpublished data.


Pathogenesis of Ascites Tumor Growth: Angiogenesis, Vascular Remodeling, and Stroma Formation in the Peritoneal Lining


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