Pathogenesis of Ascites Tumor Growth: Vascular Permeability Factor, Vascular Hyperpermeability, and Ascites Fluid Accumulation

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ABSTRACT

Previous studies have shown that accumulation of tumor ascites fluid results in large part from increased permeability of peritoneal lining vessels (Nagy et al., Cancer Res., 49: 5449–5458, 1989; Nagy et al., Cancer Res., 53: 2631–2643, 1993). However, the specific microvessels rendered hyperpermeable have not been identified nor has the basis of peritoneal vascular hyperpermeability been established. To address these questions, TA3/St and MOT carcinomas, well-characterized transplantable murine tumors that grow in both solid and ascites form, were studied as model systems. Ascites tumor cells of either type were injected i.p. into syngeneic A/Jax and C3HeB/FeJ mice, and ascites fluid and plasma were collected at intervals thereafter up to 8 and 28 days, respectively. Beginning several days after tumor cell injection, small blood vessels located in tissues lining the peritoneal cavity (mesentery, peritoneal wall, and diaphragm) became hyperpermeable to several macromolecular tracers (125I-human serum albumin, FITC-dextran, colloidal carbon, and Monastral Blue B). Increased microvascular permeability correlated with the appearance in ascites fluid of vascular permeability factor (VPF), a tumor cell-secreted mediator that potently enhances vascular permeability to circulating macromolecules. VPF was measured in peritoneal fluid by both a functional bioassay and a sensitive immunofluorometric assay. The VPF concentration, total peritoneal VPF, ascites fluid volume, tumor cell number, and hyperpermeability of peritoneal lining microvessels were found to increase in parallel over time. The close correlation of peritoneal fluid VPF concentration with the development of hyperpermeable peritoneal microvessels in these two well-defined ascites tumors suggests that VPF secretion by tumor cells is responsible, in whole or in part, for initiating and maintaining the ascites pattern of tumor growth.

INTRODUCTION

Injection or metastasis of tumor cells into body cavities often leads to an “ascites” pattern of growth in which malignant cells grow as a cell suspension in peritoneal fluid in the apparent absence of connective tissue stroma of the type that characterizes solid tumors (1–8). Earlier studies from our laboratory have been concerned with the mechanisms responsible for the ascites tumor phenotype and particularly for the fluid accumulation, often massive, that characterizes this pattern of tumor growth (9, 10). Studies with two syngeneic transplantable murine sarcomas, MOT mouse ovarian tumor and the TA3/St mammary carcinoma, demonstrated that outflow (i.e., the disappearance rate from the peritoneal cavity of i.p.-injected 125I-HSA tracer, µl/min) was markedly (5-fold) impeded within 24 h of i.p. injection of either tumor cell line. Reduced outflow preceded any increase in tumor cell number but was not by itself sufficient to provoke net peritoneal fluid accumulation; apparently, the remaining drainage capacity of the peritoneal cavity (primarily via diaphragmatic lymphatics) was sufficient to counterbalance normal fluid inflow. In contrast to the rapid decrease in tracer outflow that followed i.p. tumor cell injection, inflow (i.e., the rate of appearance in the peritoneal cavity of i.v.-injected 125I-HSA tracer, µl/min) remained unchanged for some days. However, at 5–7 days, at a time when tumor cell number had increased >500-fold, 125I-HSA tracer inflow increased dramatically (13- to 25-fold above control values). Only after the inflow rate of tracer from plasma to peritoneal cavity had increased so that it exceeded the outflow rate was there net peritoneal fluid accumulation. The fluid that accumulated in the peritoneal cavities of ascites tumor-bearing animals had a protein concentration severalfold greater than that of normal peritoneal fluid and came to approximate 85% of the protein level of plasma, including albumin in a proportion similar to that found in plasma (9). Thus, the fluid accumulating in the peritoneal cavities of ascites tumor-bearing mice was a plasma exudate, a finding others have also reported (11–18).

While the mechanism(s) by which peritoneal tumor cells induced an early and dramatic decline in peritoneal outflow remains obscure, we hypothesized a likely explanation for the increased tracer inflow and high protein content of the peritoneal fluid that accumulated subsequently. Studies from our laboratory have shown that a wide variety of tumor cells produce and secrete in vitro a cytokine, VPF; VPF is a Mr 34,000–42,000 disulfide-bonded dimeric protein (12, 19–22). In low nanomolar to picomolar concentrations, VPF (also known as VEGF or vascular endothelial growth factor) increases the permeability of venules and small veins to plasma proteins with a potency some 50,000 times that of histamine on a molar basis (12). Abundant VPF activity has been found in guinea pig (12) and, more recently, in human tumor ascites fluids (23).

Taken together, these findings suggest that tumor ascites fluid accumulation might result from increased permeability of the blood vessels lining the peritoneal cavity and that the hyperpermeability of such vessels might be mediated by VPF secreted by ascites tumor cells. To test this hypothesis, we assessed the permeability of the blood vessels of the peritoneal lining to macromolecules at successive intervals after i.p. injection of either MOT or TA3/St tumor cells and correlated these findings with measurements of ascites fluid volumes, VPF concentrations, and numbers of peritoneal tumor cells.

MATERIALS AND METHODS

Tumor Cells. TA3/St and MOT tumor cells were the kind gifts of Drs. Barbara Sanford and Gerald Kolodny, respectively. The TA3/St ascites tumor is an ascites subtype originally derived from a spontaneous mouse mammary adenocarcinoma found in the A/HeHa strain of mice (24–28). The MOT (mouse ovarian tumor) ascites cell line, an ovarian embryonal cell carcinoma, originated spontaneously in a C57BL/6 female mouse (29). It was originally propagated by s.c. transplantation, where MOT grows in solid form. However, MOT can also be maintained in ascites form following i.p. injection, exhibiting a behavior much like that of ascites human ovarian cancers with lack of hematogenous dissemination (30, 31).
TA3/St and MOT tumor cells (1 x 10^6) were passaged weekly in the peritoneal cavities of syngeneic, 5- to 6-week-old female A/Jax and male C3Heb/FeJ mice, respectively. The MOT tumor grows equally well in both male and female mice (29), and males were chosen for our experiments because of their greater availability from the supplier (The Jackson Laboratory, Bar Harbor, ME).

**Collection of Malignant Ascites Fluid.** At various intervals after i.p. injection of 1.0 x 10^6 TA3/St or MOT tumor cells, tumor-bearing or control animals were anesthetized with ether, and blood samples were collected by retroorbital puncture into a known volume of heparin for preparation of platelet-poor plasma (centrifugation at 15,600 x g for 10 min at 4°C). Animals were sacrificed by CO2 narcosis. For collection of ascites fluid, 2 ml of Hanks’ balanced salt solution were injected i.p., and the contents of the peritoneal cavity were mixed by kneading. Peritoneal fluid was then recovered, its volume recorded, and tumor cells were counted (9). Prior to centrifugation (160 x g for 20 min at 4°C), protease inhibitors were added to the following final concentrations: iodoacetamide, 0.37 mg/ml; N-ethylmaleimide, 0.25 mg/ml; PMSF, 0.35 mg/ml; and aprotinin, 210 KIU/ml. Cell-free ascites fluid and plasma were aliquoted and stored at —80°C for subsequent assay. The 2 ml peritoneal fluid were determined by the indicator-dilution method as described previously (9).

**Infow Studies Using 125I-Albumin as Tracer.** HSA (Sigma Chemical Co., St. Louis, MO) was iodinated (Iodogen; Pierce Chemical Co., Rockford, IL) to a specific activity of 0.02–0.05 mCi/10 ^6 mol for mouse HSA; >97% of 125I-HSA was precipitated by 10% trichloroacetic acid. Infow measurements were performed by injecting 2.5 x 10^5 cpm of 125I-HSA (in 0.2 ml saline) i.v. At various times thereafter, ascites tumor-bearing or control animals were sacrificed by CO2 narcosis; then the amount of 125I-HSA in the plasma and ascites fluid was determined, and the transcompartmental tracer infow rates were calculated as described previously (9). In other experiments, cell-free ascites fluid and partially purified VPF were tested for their ability to increase the influx of circulating 125I-HSA into the peritoneal cavity. Immediately following i.v. injection of 125I-HSA tracer (2.5 x 10^6 cpm) into normal nontumor-bearing mice, 1.0 ml of either MOT 14-day cell-free ascites fluid (1:10 dilution in 5% BSA; Sigma), heparin-Sepharose-purified murine VPF (diluted in 5% BSA; Ref. 21), or a control solution of 5% BSA was injected i.p. At various time points thereafter, animals were sacrificed by CO2 narcosis; then peritoneal fluid and blood samples were collected as described above, and the amounts of 125I-HSA in the plasma and peritoneal fluid were determined by gamma spectrometry for calculation of inflow rates (9).

**Vessel Hyperpermeability Studies with Macromolecular FITC-D, Colloidal Carbon, and MBB as Tracers.** FITC-D of average molecular weight of 150,000 (mean diameter, 17.4 nm) was obtained from Sigma Chemical Co. FITC-D of M, 5,000,000 (mean diameter, 40 nm) was prepared as described (10). Ascites tumor-bearing or normal mice were injected i.v. with ~0.2 µmol FITC-D in PBS. After various time periods (5 to 30 min) following the 2-ml saline injection, animals were sacrificed, and the peritoneal fluid was collected. For microscopic visualization of FITC-D, the entire peritoneal wall, as well as the diaphragm and mesentery, were rapidly excised and immersed in a 70:30 mixture of ethanol:10% formalin (32). Fixation proceeded for 4 h at room temperature. Subsequently, tissues were dehydrated in ascending grades of ethanol (70 to 100%) over 24 h and were cleared in xylene (10, 33). Full-thickness sections of the parietal peritoneal wall (up to 1 cm x 1 cm), diaphragm, and mesentery were mounted under coverslips on glass slides with immersion oil and viewed in a Wild macroscope or in a fluorescence microscope.

**Statistics.** Because our data did not conform to bell-shaped distributions with equal SDs, comparisons were made with the nonparametric Kruskal-Wallis test and with Dunn’s variation of the Bonferroni test (40).
RESULTS

Growth Patterns of MOT and TA3/St Tumors Injected into the Peritoneal Cavity. After a short lag period, MOT and TA3/St tumor cells grew for a time in logarithmic phase in the peritoneal cavity; thereafter, the growth rate plateaued (Fig. 1, A and B; Ref. 9). Significant net fluid accumulation did not begin for 3–4 days (TA3/St) or 6–8 days (MOT) but thereafter increased exponentially (Fig. 1, A and B). Fluid accumulation peaked in TA3/St animals at 2–3 ml by day 7, a day or two prior to animal death; in contrast, fluid accumulation increased progressively in MOT-bearing mice for nearly a month and to a volume of ～25 ml. At all times, and in both tumor systems, tumor cells accounted for ≥99% of nucleated cells in the peritoneal fluid. However, TA3/St and MOT ascites fluids typically became bloody, containing 0.5 to 2.0 × 10⁸ RBC by days 7 and 27, respectively; blood loss into the peritoneal cavity was, therefore, significant and contributed importantly to animal death.

Quantitative Measurement of Peritoneal Vessel Hyperpermeability Using 125I-HSA as Tracer. Consistent with our earlier findings with other macromolecular tracers (9, 10), the inflow rate (µl/min) of 125I-HSA increased dramatically (～10-fold) between days 3 and 7 in TA3/St ascites-bearing mice and between days 5 and 10 in MOT ascites tumor-bearing mice (Fig. 1, C and D).

Macroscopic Identification of Hyperpermeable Peritoneal Lining Blood Vessels Using Colloidal Carbon and MBB as Tracers. Within a few days of i.p. injection of either MOT or TA3/St tumor cells, the peritoneal lining tissues became thickened and hyperemic with loss of their normal glistening appearance. Macroscopic examination revealed carbon- or MBB-labeled blood vessels beginning by 3–4 days; at first, labeling was focal, but over the course of the next several days, it came to involve the entire peritoneal surface (Fig. 2; Table 1).

Vessel labeling appeared first and most intensely in the mesentery, where it was confined to fatty portions that carry the major blood vessels to the intestines and that are themselves normally well vascularized (Fig. 2, A–F; Table 1). The intervening, fat-free mesentery remained normally thin, relatively clear, and avascular. Vessel labeling developed slightly later in the peritoneal wall and diaphragm (Fig. 2, I–L; Table 1). The blood vessels that labeled initially with carbon or MBB were of relatively large caliber (Fig. 2, B and E; Ref. 41). However, at later intervals (e.g., after day 10 in MOT-bearing mice), tracer deposits were noticeably finer and more numerous and appeared predominantly to involve vessels of smaller size (Fig. 2, C, D, and F).

In addition to forming a suspension of tumor cells in the peritoneal cavity, both MOT and TA3/St tumor cells commonly formed solitary macroscopic nodules of solid tumor in the peritoneal wall at the site of the needle track through which tumor cells had been injected i.p.; the blood vessels supplying these solid "needle track" tumors labeled earlier (33) and more intensively with carbon than did the surrounding peritoneal wall (Fig. 2G). At later stages, TA3/St tumor cells also formed nodular solid tumor implants in the mesentery and in the parietal peritoneum away from tumor injection sites; these also became vascularized and labeled more intensively with carbon than the surrounding mesentery (Fig. 2H).

The large and small intestines also contained small blood vessels that labeled with carbon (Fig. 2, A–D), but the labeling pattern was very different from that observed in the free mesentery, parietal peritoneum, and diaphragm. Carbon labeling of bowel wall vessels was evident, even in normal mice; in tumor-injected mice, the pattern of bowel vessel labeling with carbon was irregular and did not evolve in parallel with that of ascites tumor growth as did labeling in the other tissues lining the peritoneal cavity (Table 1). The relative hyperpermeability (e.g., ～10 times that of skeletal muscle vessels) of normal alimentary tract vessels to circulating macromolecules is well known and is thought to reflect the presence of open endothelial cell fenestr (42, 43).

Microscopic Identification of Hyperpermeable Blood Vessels of the Peritoneal Lining with FITC-D. Within minutes of i.v. injection, FITC-D was detected leaking from scattered blood vessels that supplied the mesentery, peritoneal wall, and diaphragm of ascites tumor-bearing animals. FITC-D extravasation was observed as early as 2 days after i.p. tumor cell injection and became much more extensive at later intervals (Fig. 3, B and C). In contrast, FITC-D did not leak detectably from comparable vessels in normal control animals over a period of hours (Fig. 3A).

Microscopic Identification of Hyperpermeable Peritoneal Lining Blood Vessels with Colloidal Carbon. Microscopic study of 1-µm Epon sections taken from animals injected i.v. with colloidal carbon confirmed and extended these observations. Rare carbon-labeled vessels were identified microscopically in the mesentery, parietal peritoneum and diaphragm as early as 2 days after i.p. tumor cell injection and thereafter increased dramatically in both number and labeling intensity (Fig. 4, A–C; Fig. 5, A–D). Accompanying these microscopic changes was significant interstitial edema, particularly evident in the peritoneal wall (Fig. 5B). Surface mesothelial cells became activated (Fig. 5B) and sometimes displayed mitotic figures. At later times, tumor cells attached to the peritoneal and diaphragmatic surfaces as individual cells or as cell clumps (Fig. 5D). Individual carbon-labeled blood vessels were also observed in the bowel musculature and submucosa (Fig. 4D); however, as noted by microscopy (see above), labeling did not differ significantly in number or appearance in tumor-bearing versus control animals.

Immunoperoxidase Staining for VPF. Blood vessels lining the peritoneal walls stained intensely for VPF by day 14 in MOT ascites tumor-bearing mice; however, staining was not evident at earlier intervals up to day 7 (Fig. 5, E and F). Peritoneal wall tumor vessel staining also became evident in TA3/St-bearing mice on day 8 (data not shown). VPF staining was not observed in peritoneal lining vessels of control mice.
Fig. 2. Low power macrographs of leaky blood vessels of mesentery (A-F and H), peritoneal wall (G and I-K) and diaphragm (L) in mice injected i.p. with either MOT (A-C, E, F, and I-K) or TA3/St (D, G, H, and L) ascites tumor cells. Colloidal carbon (A-K) or MBB (L) were used as tracers. A-C, mesentery with attached bowel in a normal control C3Heb/FeJ mouse (A) as compared with the mesenteries of other mice sacrificed on days 6 (B) and 12 (C) after i.p. injection of $1 \times 10^6$ MOT tumor cells. Note lack of labeling of mesenteric vessels in (A) but progressively intense vessel labeling at subsequent stages of ascites tumor growth. D, mesentery of an A/Jax mouse 8 days after i.p. injection of $1 \times 10^6$ TA3/St tumor cells showing extensive vessel labeling, comparable in appearance to that of a 12-day MOT-bearing animal (C). E and F, higher magnification views of carbon-labeled blood vessels in 6- and 15-day MOT ascites tumor-bearing mice, respectively. At 6 days, relatively large caliber vessels are labeled, whereas at 15 days, many more vessels are labeled, but they are of smaller size. G, small solid TA3/St tumor which developed in the peritoneal wall at the tumor cell injection site, 6 days. Extensive vessel labeling of this solid tumor nodule with relative lack of labeling in surrounding peritoneal wall vessels. H, three small solid tumor implants in the mesentery 5 days after i.p. injection of TA3/St tumor cells. Such implants were fairly common in TA3/St-bearing mice and always displayed more numerous leaky blood vessels than the surrounding mesentery. I-K, peritoneal wall vessel labeling in a control animal (I) and in other mice harvested at 9 and 12 days, respectively, after i.p. injection of MOT tumor cells. Vessel labeling was not observed in control animals (I) but had become intense by 9 and 12 days (J and K). L, MBB labeling of the diaphragm of a TA3/ST ascites tumor-bearing animal, 7 days. A-D, $6 \times 14$; E, $27$; F and G, $15$; H, $30$; I-K, $13-20$; L, $13$. 

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Table 1 Macroscopic scoring of carbon-labeled blood vessels in the peritoneal lining at various intervals after i.p. injection into syngeneic mice of MOT or TA3/St ascites tumor cells

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Each data point represents a separate individual animal. Semiquantitative scale of 0 to 4+; f, focal ±.

Vascular Permeability Enhancing Activity of MOT and TA3/St Cell-free Ascites Fluid. Experiments were undertaken to determine whether the ascites fluid induced by peritoneal tumor cells contained microvessel permeability enhancing activity, and, more particularly, VPF. We had shown previously that both TA3/St and MOT tumor cells synthesized and secreted VPF in vitro and that, like the soluble VPFs secreted by guinea pig, rat, and human tumor cells (12, 21, 44, 45), the VPF present in mouse tumor culture medium was completely absorbed by heparin-Sepharose.4

Preliminary experiments demonstrated that cell-free peritoneal fluids from 14-day MOT ascites exhibited substantial vascular permeability-enhancing activity when tested in normal mice by the Miles assay (Refs. 12 and 46; data not shown). Cell-free tumor ascites fluid was also tested for its ability to induce increased permeability in the peritoneal lining vessels of normal syngeneic mice (Fig. 6). When 14-day cell-free MOT ascites fluid was injected i.p. into normal mice, circulating [125I]-HSA extravasated into the peritoneal cavity 10-times more rapidly than in mice that had been injected i.p. with a volume- and protein-matched control solution (CBSA group). Furthermore, 2 h after i.p. injection, more than 15% of i.v. tracer had accumulated in the peritoneal cavities of mice injected i.p. with MOT cell-free tumor ascites fluid as compared with ~2% in the CBSA group (P < 0.001; unpublished data).

Fig. 6. All of the permeability-enhancing activity present in both TA3/St and MOT tumor ascites fluid bound to heparin-Sepharose (data not shown). Mice injected i.p. with partially purified (21) mouse VPF accumulated [125I]-HSA tracer in the peritoneal cavity in amounts and with kinetics similar to those of mice injected with cell-free ascites fluid (data not shown). Finally, when MOT cell-free ascites fluid was injected i.p., circulating macromolecular FITC-D tracer was observed to extravasate from mesenteric and peritoneal wall microvessels by fluorescence microscopy (Fig. 3D).

Immunofluorometric Assay of VPF in Cell-free Ascites Fluid as a Function of Time after i.p. Injection of MOT or TA3/St Tumor Cells. VPF was not detectable with our sandwich immunoassay in the small (<0.5 ml) amounts of fluid that could be recovered from the peritoneal cavities of normal mice (assay sensitivity, <5 pm). However, VPF was readily measured in cell-free tumor ascites fluid as early as 2 or 5 days after injection of 10⁶ TA3/St or MOT tumor cells (Fig. 7). Total VPF (pmol) within the peritoneal cavity increased progressively through day 7–8 (TA3/St) or day 20 (MOT), eventually achieving peak levels equivalent to 14–32 pmol (Fig. 7, A and B). The concentration of VPF, expressed as nmol/liter ascites fluid (or as nmol/tumor cell), reached peak levels on day 3 (or day 8) in TA3/St-bearing mice at ~10–12 pmol (or ~4 × 10⁻¹¹ nmol/tumor cell) and on day 7 (or day 16) in MOT-bearing animals at ~4–5 nmol (or ~1.0 × 10⁻¹¹ nmol/tumor cell; Fig. 7, C and D).
Fig. 4. Giemsa-stained, 1-µm-thick Epon sections illustrating the microscopic appearance of colloidal carbon labeling of mesenteric (A-C) and large bowel (D) blood vessels in control (A) or in TA3/St (B-D) ascites tumor-bearing mice. A, control vessels are neither prominent nor carbon-labeled. B and C, hyperpermeable vessels in mesenteries of A/Jax mice 7 days after i.p. injection of TA3/St ascites tumor cells. In (B), several carbon-labeled vessels are indicated with arrows. In (C), a carbon-labeled vessel (V) and two nearby unlabeled vessels (v) are illustrated. D, wall of large intestine of an A/Jax mouse injected i.p. 2 days previously with TA3/St tumor cells. Note carbon-labeled vessel (arrow) in submucosa. Identical vascular labeling was common in control mice, indicating that the hyperpermeability of this vessel subset is not tumor-dependent. A and B, × 270; C and D, × 540.

Fig. 5. A-D, 1-µm-thick, Giemsa-stained Epon sections illustrating microvascular hyperpermeability in the peritoneal wall (A-C) and diaphragm (D) of mice bearing MOT (A) or TA3/St (B-D) ascites tumors. A, normal peritoneal wall with muscle bundles tightly apposed. This normal appearance persisted for up to 6 days in C3Heb/FeJ mice injected with MOT ascites tumor cells. B, peritoneal wall of a 7-day TA3/St ascites tumor-bearing mouse exhibits mesothelial cell activation, interstitial edema with wide separation of individual muscle bundles, and microvascular hyperpermeability (arrowheads, carbon leakage). Vertical distance in (B) corresponds to that portion of (A) indicated by double-headed arrow. C, higher magnification micrograph of a leaky vessel deeper in the peritoneal wall of another 7-day TA3/St ascites tumor-bearing mouse. D, diaphragm of a TA3/St ascites tumor-bearing mouse at 8 days. Note suspended tumor cells, monolayer of adherent tumor cells (some displaying mitotic figures), and numerous carbon-labeled (arrowheads) superficial blood vessels. E and F, peritoneal walls of MOT ascites tumor-bearing mice stained for VPF by immunohistochemistry. VPF was undetectable at 7 days (E) but was strongly positive by 14 days (F) and thereafter. A, × 220; B, × 430 (bar, 16 µm); C, × 630; D, × 520; E and F, × 275.
of an activity that enhanced the permeability of normal skin and peritoneal fluid and also with the appearance in cell-free ascites fluid with ascites tumor growth correlated closely with the accumulation of anying paper (41), new blood vessels were generated in pentoneal diaphragm (Table 1). Subsequently, as will be discussed in an accom TM/St tumors and up to day 10 in the case of MOT tumors (9, 41); i.e., at times well after our measurements of tracer extravasation (up to day 7 in the case of MOT tumors and in amounts that were readily detected by immunohistochemistry (Fig. 5F).

Vascular hyperpermeability developed more slowly in ascites tumors than when these same and other tumors grew in solid form (49). Hyperpermeable blood vessels have been documented within a few hours of tumor cell transplant to the s.c. or intradermal spaces, sites at which they form solid tumors (38). In contrast, when MOT or TA3/St cells were injected i.p. to generate ascites tumors, peritoneal lining vessels did not become detectably leaky for 2–3 days. Moreover, for an additional several days, the density of hyperpermeable vessels remained significantly lower in peritoneal vessels associated with ascites tumors than was found in their solid tumor counterparts, whether growing in the s.c. space, in the peritoneal wall at tumor cell injection sites, or in mesentery as tumor implants (Fig. 2, G and H; Refs. 33 and 38).

Several explanations could account for the slower kinetics with which vascular hyperpermeability developed in ascites as compared with solid tumors. One possibility is that the mesothelial cell layer that lines the peritoneal cavity and separates ascites tumor cells from the underlying peritoneal lining blood vessels might serve as a barrier to VPF, preventing it from reaching blood vessels of the peritoneal lining. However, this explanation is unlikely because i.p. injection of VPF into normal mice5 or guinea pigs (12) elicited a prompt (within

### DISCUSSION

Extravasation of circulating molecules from blood vessels is a function of both local blood flow and microvascular permeability. Microvessels lined by a continuous endothelium, such as those that supply the mouse peritoneum (42), normally retain high-molecular-weight proteins almost quantitatively. Therefore, whereas extravasation of water and other small circulating molecules into tissues may be significantly affected by blood flow, extravasation of proteins and other macromolecules is, within limits, almost entirely dependent on microvascular pore size. Like the normal peritoneal microvasculature, that supplying the peritoneal walls of mice bearing either TA3/St or MOT tumors is largely of the continuous type;5 however, these tumor-supplying vessels extravasated substantial amounts of circulating 125I-HSA, FITC-D, colloidal carbon, and MBB. This increased extravasation cannot be attributed to vessel disruption. Vessels were intact by electron microscopy,2 and significant leakage of erythrocytes did not occur until after day 7 in the case of TA3/St tumors and after day 28 in the case of MOT tumors (9, 41); i.e., at times well after our measurements of tracer extravasation (up to day 7 in the case of TA3/St tumors and up to day 10 in the case of MOT tumors). Therefore, the extensive extravasation of macromolecular tracers described here in ascites tumor-bearing mice is largely attributable to alterations in peritoneal microvessel permeability (9, 10).

Vascular hyperpermeability was quantified by measurement of the increase in inflow rate of 125I-HSA into the peritoneal cavity (Fig. 1). Extravasation of FITC-D, colloidal carbon, and MBB was visualized directly by macroscopy and/or microscopy (Figs. 2–5). Small vessels of the free mesentery were the first to become leaky to these tracers, followed shortly thereafter by vessels in the peritoneal wall and diaphragm (Table 1). Subsequently, as will be discussed in an accompanying paper (41), new blood vessels were generated in peritoneal lining tissues, and these also were found to be hyperpermeable to macromolecular tracers.

The hyperpermeability of peritoneal lining microvessels associated with ascites tumor growth correlated closely with the accumulation of peritoneal fluid and also with the appearance in cell-free ascites fluid of an activity that enhanced the permeability of normal skin and peritoneal lining vessels (Fig. 6). Several independent pieces of evidence indicate that most, and perhaps all, of this vascular permealizing activity is attributable to VPF: (a) immuno-fluorometric assay revealed that VPF accumulated in substantial concentrations in both MOT and TA3/St cell-free ascites fluids (Fig. 7); these concentrations are well in excess of those needed to induce increased microvascular permeability in vivo or to promote the division of cultured endothelial cells in vitro (12, 19, 47, 48); (b) all of the vascular permealizing activity present in MOT ascites fluid was absorbed by heparin-Sepharose, a property of guinea pig and human VPFs (12, 44). Although the possibility of additional heparin-binding, vascular permealizing factors in tumor ascites fluid has not been excluded, our data indicate that tumor-secreted VPF is present in amounts more than sufficient to account for accumulation of tumor ascites fluid; and (c) as previously described in solid and ascites guinea pig tumors (38), VPF protein accumulated in the leaky blood vessels that line the peritoneal cavities of mice bearing both MOT and TA3/St ascites tumors and in amounts that were readily detected by immunohistochemistry (Fig. 5F).

#### Fig. 6. Inflow of circulating 125I-HSA from the plasma into the peritoneal cavities of otherwise normal mice at various intervals after i.p. injection of 1.0 ml of 1:10 diluted 14-day MOT cell-free ascites fluid (●) or a 5% solution of BSA (○). Fourteen-day MOT cell-free ascites fluid elicited a striking and statistically significant (a, P < 0.05; b, P < 0.001) increase in 125I-HSA inflow into the peritoneal cavity, whereas the volume and protein-matched solution of BSA did not. Data expressed as mean ± SEM. n = 3 mice at each time point.

#### Fig. 7. Kinetics of appearance of immunofluorometric assay-detectable VPF in the peritoneal cavities of mice injected i.p. with TA3/St (A and C) or MOT (B and D) ascites tumor cells. A and B, VPF is expressed as total pmol of VPF present within the peritoneal cavity at various intervals after i.p. tumor cell injection (A). C and D, concentration of VPF expressed as 1011 × nmol/tumor cell (C) or as nmol/liter of ascites fluid (●) at successive intervals after i.p. tumor cell injection.

5 A. M. Dvorak, unpublished data.
a few minutes) increase in the permeability of peritoneal lining vessels, a response with kinetics similar to that when VPF was injected intradermally in the Miles assay. A second possibility is that tumor cells expressed little VPF immediately after i.p. injection but, after a time, began to express more. Against this possibility, however, are the results of Northern analyses performed on both TA3/St and M07 tumor cells that were recovered from the peritoneum at successive intervals after i.p. transplant; these analyses showed no significant changes in VPF mRNA levels over time (data not shown).

Perhaps the most likely explanation for differences in the kinetics of vascular hyperpermeability relates to the very different microenvironments that tumor cells experience in solid and ascites tumors. At early intervals after injection of equivalent numbers of cells, tumor cell density is considerably higher in the s.c. space than in the peritoneal cavity. When injected s.c., tumor cells are largely confined to the peritoneal cavity itself. With respect to vascular hyperpermeability factor (VPF, VEGF) in tumor biology. Cancer Metastasis Rev., 12: 303—324, 1993.

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VPF AND ASCITES TUMOR GROWTH

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