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Significant Expression of Vascular Endothelial Growth Factor/Vascular Permeability Factor in Mouse Ascites Tumors[1]

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ABSTRACT

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is believed to be a potent mediator of peritoneal fluid accumulation and angiogenesis and of tumor growth in ascites tumor. Such roles, however, have not been generally established because of insufficient quantitative and systemic analyses. To address this, we examined the expression of VEGF in 13 mouse ascites tumors (5 sarcomas, 3 carcinomas, 2 lymphomas, 1 leukemia, 1 mastocytoma, and 1 plasmacytoma). Using a newly developed sensitive and specific radioreceptor binding assay and functional assays, we found that active VEGF was significantly accumulated (6–850 ng/ml) in the ascites fluids of all 13 tumors. VEGF concentrations are higher in the tumors of sarcoma and carcinoma origin (430.4 ± 234.2 ng/ml) than in those of lymphoma and hematological tumor origin (19.2 ± 10.45 ng/ml). VEGF that accumulated in the peritoneal fluids or expressed in the ascites tumor cells was easily visualized with immunoprecipitation Western blot analysis with a rough correlation to the expression levels of VEGF gene in these tumor cells, suggesting that the tumor cells, at least in part, contributed to the production of the VEGF that accumulated in the ascites fluid. Most ascites tumors expressed VEGF; the 164-amino acid isoform was predominant, the 120-amino acid isoform was less abundant, and the 188-amino acid isoform was least abundant. Several representative ascites tumors expressed similar, if not higher, levels of VEGF when they were cultured at normoxic states, suggesting that they expressed VEGF at high levels in a constitutive manner. The microvessel densities in the peritoneal walls of tumor-bearing mice, which are significantly higher than those in normal mice, basically correlated to but did not parallel the VEGF concentrations in their respective ascites fluids. Thus, a complicated relationship may exist between the VEGF production and angiogenesis associated with ascites tumor *in vivo*. Taken together, our observations suggest that VEGF plays a fundamental role in ascites tumor formation; however, its importance may vary according to tumor origin.

INTRODUCTION

VEGF[3] is a glycoprotein with two important functions: one is to increase microvessel permeability, approximately 50,000 times more than histamine on a molar basis, and the other is to stimulate the growth of vascular endothelial cells (1–4). VEGF is thought to perform its functions by interacting with two high affinity tyrosine kinase receptors (Flt-1 and KDR/Flk-1), which are selectively expressed in vascular endothelium (5). Recently, several independent findings suggest that VEGF functions in the maintenance and repair of the vascular endothelium (2, 6).

The genomic analysis of mouse VEGF gene indicates that alternative splicing generates the 120-, 164-, and 188-amino acid isoforms, but does not predict a fourth VEGF isoform corresponding to human (7). There exist differences between VEGF isoforms in the affinity for heparin: $VEGF_{120}$ is a freely soluble protein; $VEGF_{164}$ is secreted, although a significant fraction remains cell-associated; and $VEGF_{188}$ is tightly trapped on the cell surface and in the extracellular matrix (8). Native $VEGF_{164}$ has a molecular weight of 45,000–46,000 (2, 4, 9) and displays a single band of M_r 23,000 under reducing conditions, whereas a recombinant $VEGF_{120}$ secreted by COS cells is composed of two subunits of M_r 20,000 (8). $VEGF_{164}$ is shown to be the most abundant isoform in a variety of tissues, with a few exceptions (5, 10). At present, little is known about the differences in biological potency among the isoforms of VEGF *in vivo*.

The overexpression of VEGF has been shown to be induced by several pathophysiological factors, such as the transformed phenotype (11–13), the stimulation of certain cytokines (14), some physiological states (5), and so forth. Among these, hypoxic conditions, commonly seen in solid tumors, as well as in myocardial ischemia and retinal diseases, may play a major role both *in vitro* and *in vivo* (14). In fact, the regulatory sequence in VEGF gene shares elements with another hypoxia-sensitive gene, erythropoietin (15).

The expression of VEGF has been widely demonstrated in various normal tissues (1) and most human and animal tumors (1, 16), including ascites tumors (17). VEGF was easily detected in the peritoneal fluid of two well-characterized transplantable mouse tumors, as early as 2 or 5 days after the injection of 10^6 tumor cells, by both a functional bioassay and an immunofluorometric assay (18). Therefore, it has been proposed that VEGF secretion by tumor cells is responsible for initiating and maintaining the ascites pattern of tumor growth (18), although this remains to be confirmed *in vivo*.

In fact, the expression of VEGF in ascites tumors has not been widely and systematically investigated. To address this question, using a newly developed radioreceptor binding assay and functional assays, we examined the expression of VEGF in 13 mouse ascites tumors. We found that the ascites fluids of all 13 tumors contained significant amounts of VEGF, especially those of sarcoma and carcinoma origins, which are the source of most origins for human malignant ascites. In contrast, VEGF concentrations are relatively low in the ascites tumors originated from lymphoma and hematological tumor. These results suggest that the role of VEGF varies according to the histological type of ascites tumor.

MATERIALS AND METHODS

Animals, Tumors, and Other Reagents. BP-8 sarcoma (19), MH134 hepatoma, MM2 mammary tumor (20), OG/Gardner lymphoma 6C3HED (21), SR-C3H/He sarcoma (22), and X5563 plasmacytoma (23) transplantable ascites-producing tumors were passaged at 4×10^6 cells/mouse weekly in the peritoneal cavities of syngeneic C3H/He mice; Ehrlich mammary tumor (20), S37 (24), and SR-DDD sarcoma (25) ascites tumors in those of DDD mice (kindly provided by Dr. Matsuzawa, University of Tokyo); L1210 leukemia (26), P815 mastocytoma (27) in those of DBA/2; and S180 (20) and RG/Gardner Lymphoma 6C3HED (21) in those of BALB/c and CBA mice, respectively. All mice used were 6-week-old males, which, except where otherwise specified, were purchased from SLC (Hamamatsu, Japan).

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[3] The abbreviations used are: VEGF, vascular endothelial growth factor; r.m-VEGF, recombinant mouse VEGF; IP, immunoprecipitation; RT-PCR, reverse transcription PCR; pN2 and pC2, affinity-purified antibodies to NH_2- and COOH-terminal peptides of mouse VEGF, respectively; RLSEC, rat liver sinusoidal endothelial cell.

r.m-$VEGF_{164}$, platelet-derived growth factor, human placenta growth factor, and an goat antimouse neutralizing antibody, AF-493-NA, were purchased from R&D Systems (Minneapolis, MN). The goat antimouse VEGF polyclonal antibody p-20 used in this study was from Santa Cruz Technology (Santa Cruz, CA). The rabbit antihuman vWF antibody used, which cross-reacts with vWF from mouse,[4] was purchased from DAKO (DAKO A/S, Copenhagen, Denmark).

Collection of Ascites Fluids and Preparation of Peritoneal Tissues. Mice were sacrificed by dislocation of the cervical joint, and the peritoneal fluid was harvested. For the detection of VEGF, the ascites fluids were obtained at two stages, on significant abdominal swelling, while tumor-bearing mice were still vigorous, and when ascites accumulation reached a peak, while tumor-bearing mice were quite weak or dying. After the addition of protease inhibitors (final concentrations: PMSF, 0.35 mg/ml; aprotinin, 200 kallidinogenase-inactivating units/ml) to the ascites fluid, the cell-free ascites fluid was obtained by centrifuging at 180 × *g* for 20 min at 4°C. One-ml aliquots of the cell-free ascites fluid were centrifuged at 15,000 × *g* for 5 min at 4°C and stored at −80°C.

After being washed with cold PBS (pH 7.4), the peritoneal tissues were embedded in OCT compound (Miles Inc., Elkhart, IN) and immediately snap frozen in a mixture of dry ice and acetone. The frozen tissues were cut into 5-μm serial sections in a cryostat at −20°C and placed on poly-lysine-coated slides. The peritoneal tissues for immunochemical staining were derived from tumor-bearing mice in which ascites accumulation had reached a peak.

Fractionation and Cultivation of Ascites Tumor Cells *in Vitro*. The recovered ascites tumor cells were collected by centrifugation at 150 × *g* for 5 min and then resuspended in cold PBS (pH 7.4) and centrifuged at 75 × *g* to remove blood cells. The cell pellet was suspended in MEM containing 10% FCS and plated at 4×10^6 cells/ml in a MS-21250 flask (Sumilon, Tokyo, Japan). After a 3-h adherence period, the media containing tumor cells were collected and centrifuged at 150 × *g* for 10 min. The resultant cells, which contained tumor cells more than 94% pure according to morphological characteristics, were washed once and precipitated in cold PBS (pH 7.4) for RNA extraction or the preparation of cell extracts. For further culture, if necessary, the fractionated cells were washed once, resuspended, and incubated at 4×10^6 cells/ml in MEM containing 2% FCS at 37°C under 5% CO_2 in air. Cells were passaged every two days. The cell-free media were prepared by centrifugation at 1000 × *g* for 10 min, then aliquoted and stored at −80°C.

Preparation of Affinity-purified Antibodies against Mouse VEGF. Polyclonal antisera against mouse VEGF were raised in rabbits with two synthetic peptides corresponding to the termini of mouse VEGF (the one corresponding to the NH_2 terminus is APTTEGEQKSHEVIKFMDVYQRSYC, and the other, corresponding to the COOH terminus, is CKARQLELNERTCRCDKPRR) as antigens. The peptides were covalently conjugated to a carrier protein keyhole limpet hemocyanin (Sigma Chemical Co., St. Louis, MO; Ref. 28) for immunizing rabbits. The antisera against mouse VEGF were affinity-purified on columns prepared by coupling 1 mg of respective peptide to 1 g of formyl-cellulofine-activated Sepharose (Biochemicals, Tokyo, Japan). The affinity-purified antibody against the NH_2 terminus of VEGF was designated pN2, and that against the COOH terminus of VEGF was designated pC2.

Radioreceptor Binding Assay. The assay was carried out in 96-well immunoplates (Immunol-2, Dynatech, Chantilly, VA). Each well was coated with 100 μl of a solution containing 10 μg/ml affinity-purified anti-VEGF antibody pN2 in 50 mM carbonate buffer (pH 9.6), and the plates were incubated for 16 h at 4°C. The plates were washed twice with PBS (pH 7.4), blocked with 300 μl/well 10% FBS in PBS (pH 7.4) for 1 h at 26°C, and washed once with the same blocking solution. One hundred-μl aliquots of the serial 2-fold diluted standard solution of r.m-VEGF (ranging from 0 to 3000 pg/ml, six measurements/each) or the diluted samples of ascites fluid (in triplicate measurements of three serial dilutions) were added to each well, the plates were incubated for 3.5 h at 26°C with gentle agitation, and the supernatants were discarded and washed with PBS (pH 7.4) three times. To each well was added 100 μl of detector solution containing 2×10^5 cpm of ^{125}I-labeled human 7N Flt-1, and incubation was for 2 h at 26°C with gentle agitation. 7N Flt-1, a recombinant protein of the extracellular domain of human fms-like tyrosine (Flt-1) kinase receptor containing seven immunoglobulin-like domains (29), has high affinity for mouse VEGF similar to that for human VEGF.[4] 7N Flt-1 was labeled with ^{125}I as described previously (30), and a specific activity of 1.2×10^5 cpm/ng was obtained. After the supernatant was discarded, the plates were washed with 10% FCS in PBS (pH 7.4), and finally, the activity in the wells was measured with gamma counter.

IP-Western Blot. The fractionated tumor cell pellets or tissues were mixed with cold 1% Triton X-100 lysis buffer as described previously (30) and homogenized. The extracts were centrifuged for 15 min at 15,000 × *g*, and the concentrations of protein in the supernatants were determined with a Bio-Rad (Richmond, CA) protein assay. The extracts or the aliquots of ascites fluid were then immunoprecipitated with pN2 antibody after which protein A-Sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden) were added. The beads were washed three times with Triton X-100 lysis buffer. Western blot analysis was performed according to a standard procedure (31). Samples were separated by 15% SDS-PAGE and blotted with pN2 antibody.

Partial Purification of VEGF. Twenty ml of ascites diluted 2-fold with cold PBS (pH 7.4) were applied to a 5-ml heparin-Sepharose column (Amersham Pharmacia Biotech), washed with 30 ml of 20 mM Tris-HCl (pH 7.4) buffer containing 0.3 M NaCl, and eluted with the same buffer containing 1.2 M NaCl. The eluted fractions were size fractionated with PD-10 column (Amersham Pharmacia Biotech) and dialyzed against PBS (pH 7.4).

Endothelial Cell Growth Assay. RLSECs were isolated from rat liver as described previously (32) and grown in EGM-UV medium (Kurabo, Osaka, Japan) in the presence of r.m-VEGF, the samples of diluted ascites, or VEGF partially purified from ascites fluid by heparin column, of which the amounts of VEGF were determined by the radioreceptor binding assay.

Vascular Permeability Assay. Miles assay (33) was used to determine the vascular permeability. The back of an anesthetized guinea pig was shaved, and the animal was injected intracardiacally with 1 ml of 0.5% EVANS BLUE (Sigma). Thirty min later, 200 μl of samples or control PBS (pH7.4) were injected s.c. into the shaved back, and leakage of dyes was detected by the existence of blue spots surrounding the injected site after another 30 min.

Northern and Southern Blot Analyses and RT-PCR. Preparation of total RNA, Northern blot, and Southern analyses were performed as described (34). A ^{32}P-labeled 0.7-kb *Eco*RI-*Hin*cII fragment of the coding region of mouse VEGF cDNA isolated from mouse placenta cDNA library was used as probe. Two hundred ng of total RNA were used for the synthesis of the first-strand cDNA with a Superscript Kit (Life Technologies, Inc., Gaithersburg, MD), and random hexamers were used as primers following the manufacturer's instructions. The PCR was performed in a 50-μl reaction mixture containing 40 pmol of each primer, 0.2 mM dNTPs, and 0.5 units of Taq polymerase (Takara Shuzo, Kyoto, Japan). Gene-specific primers were designed to span the coding region, ensuring amplification of all of the isoforms of mouse VEGF. The sense primer was 5′-CCCGAATTCCCTCCGAAACCATGAACTTTC-3′, and antisense was 5′-CGGCTGCAGCGGTGAGAGGCTGGTTCCC-3′. RT-PCR of mRNA encoding the $VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$ resulted in PCR products 729, 638, and 509 bp long (7). The PCR profile consisted of 5-min initial denaturation at 95°C, followed by 30 cycles (94°C for 1 min, 62°C for 1 min, and 72°C for 2 min). Each PCR amplification included a negative control containing all of the reaction products except for the reverse transcriptase. Ten μl of the PCR products were separated in 2% Nusieve agarose (FMC Bioproducts, Rockland, ME), stained by ethidium bromide, and then transferred to nylon membrane filter (Hybond-N, Amersham Pharmacia Biotech, Buckinghamshire, England). Filter was hybridized with 0.7 kb ^{32}P-labeled mouse VEGF cDNA probe.

Immunohistochemistry. Immunohistochemistry staining for VEGF or vWF was performed on 4% paraformaldehyde-fixed sections at 4°C, using an avidin-biotin immunoperoxidase technique (Vector Laboratories, Burlingame, CA). Briefly, the procedure for VEGF staining was as follows: 4% paraformaldehyde-fixed sections were treated with 0.05% Triton X-100 (Sigma) in PBS, incubated with 0.6% H_2O_2, and blocked with 5% goat serum (Vector Laboratories) in PBS (pH 7.4) for 60 min. Anti-VEGF antibody pN2 was used at 4 μg/ml in PBS (pH 7.4) containing 2% goat serum at 4°C overnight. Biotinylated antirabbit IgG (Vector Laboratories) was used at a dilution of 1:500 in PBS (pH 7.4) containing 2% goat serum at room temperature for 30 min. After 30-min incubation with avidin-biotin-horseradish peroxidase complex (Vector Laboratories) in PBS (pH 7.4), slides were incubated with peroxide substrate aminoethyl carbazol solution (Vector Laboratories) for

[4] J. C. Luo and M. Shibuya, unpublished data.

about 3 min. Substitution of normal rabbit IgG for anti-VEGF antibody pN2 (Sigma) at the same protein concentration, preabsorption of pN2 antibody with its corresponding peptide, and omission of one reagent (such as pN2, biotinylated antirabbit antibody, and so forth) were used as controls. The vWF staining was performed according to the manufacturer's instructions (Vector Laboratories).

Vascular Density. Vascular density was scored using a masked protocol of the microscopic angiogenesis grading system (35) with some adjustments. Because we observed that new microvessel "hot spots" (*i.e.*, microscopic areas containing the most dense collections of microvessels) were mostly localized in the region at a distance of not more than 200 μm from the peritoneal surface (see Fig. 6), we counted the number of microvessels in such hot spots within an area of 0.6 mm^2 under $\times$200 power magnification with an Olympus microscope BX50 (Olympus, Tokyo, Japan) on vWF-staining sections.

Statistics. An unpaired Wilcoxon Mann-Whitney test was used to compare the vascular densities in the peritoneal walls of tumor-bearing mice and those of normal mice. A value of $P < 0.05$ was considered significant.

RESULTS

Establishment of Radioreceptor Binding Assay for Quantitating Mouse VEGF

When this work was begun, no sensitive mouse VEGF-detecting assay was available. To establish such an assay, an affinity-purified antimouse VEGF polyclonal antibody pN2 was prepared, which specifically identified mouse VEGF (Fig. 1*A*). A single immunoreactive polypeptide of M_r 21,000 was observed in the precipitates from r.m-$VEGF_{164}$ sample, consistent with the manufacturer's descriptions, whereas those of M_r 23,000 and M_r 18,500 were observed in the precipitates from several randomly selected ascites fluids. In contrast, preimmune serum or pN2 antibody preincubated with the corresponding VEGF peptide antigen showed no ability to immunoprecipitate these polypeptides, suggesting that these polypeptides are VEGF-related proteins. Another affinity-purified antimouse VEGF polyclonal antibody pC2 or a commercial affinity-purified goat antimouse VEGF polyclonal antibody P-20 also immunoprecipitated, although relatively weakly, the polypeptides of the same sizes from ascites fluids (data not shown), confirming pN2 antibody as a specific and powerful anti-VEGF antibody.

We therefore coated pN2 antibody on microtiter wells as the capture antibody, used ^{125}I-labeled 7N Flt-1 as the detector reagent, and finally established a mouse VEGF-measuring assay. To test the sensitivity of the assay, serial dilutions of r.m-VEGF ranging from 0 to 3000 pg/ml were detected, and the mean of 6 determinations was plotted for each dilution (Fig. 1*B*). The minimally detectable amount (defined as + 2 SD above the zero standard was 6 pg/ml. The intra- and interassay coefficients of variation were less than 6%. Neither platelet-derived growth factor B/B nor human placenta growth factor-1, sharing approximately 36 and 53% amino acid sequence similarities with VEGF, respectively, displayed any signal in this assay in the range between 0.3 and 3 μg/ml (data not shown).

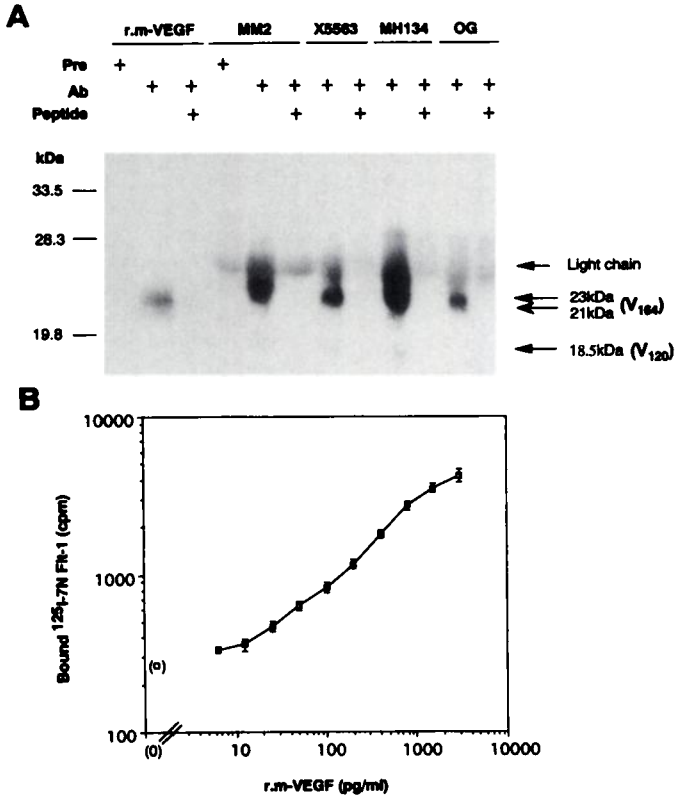

Fig. 1. Development of affinity-purified antibody pN2 against mouse VEGF and standard curve of radioreceptor binding assay. *A*, specific identification of mouse VEGF in the ascites fluid by antibody pN2. Samples of either 10 ng of r.m-VEGF or 0.5 ml of ascites fluids of tumors MM2, X5563, MH134, and OG were immunoprecipitated with rabbit preimmune serum, pN2 antibody, or pN2 antibody preincubated with its corresponding peptide. They were then resolved by 15% SDS-PAGE under reducing conditions and subjected to Western blotting. *Arrows*, the proteins of VEGF. *B*, standard curve of radioreceptor binding assay. 96-well microtiter stripes were precoated with pN2 (1 μg/well), incubated with serial dilutions of r.m-VEGF (range, 0–3200 pg/ml), and finally detected with ^{125}I-labeled human 7N Flt-1 (2×10^5 cpm/well) by gamma counter. *Data points*, average of six determinations; *bars*, SD.

Detection of VEGF in Mouse Tumor Ascites Fluids

Because our preliminary results (36) and the observations of others (17) indicate that the variation in VEGF level in ascites fluid is time-dependent after i.p. injection of tumor cells, we used ascites fluid at two stages (see "Materials and Methods"). The average concentrations in the ascites fluids of 13 tumors, measured by the binding assay, are shown in Table 1. VEGF was readily detected in all 13 tumor ascites, ranging from 6 to 850 ng/ml. Of these, high VEGF values (168–850 ng/ml) were found in seven: five sarcomas and two carcinomas; moderate values (38–83 ng/ml) were found in two: one carcinoma and one plasmacytoma; and low values (6–23 ng/ml) were found in four: two lymphoma, one leukemia, and one mastocytoma. The VEGF levels in the ascites fluids of sarcoma and carcinoma origin (430.4 $\pm$ 234.2 ng/ml) were higher than those of lymphoma and hematological tumor origin (19.2 $\pm$ 10.45 ng/ml). The presence of VEGF in the tumor ascites was easily visualized by IP-Western blot (Fig. 2*A*).

Biological Activities of VEGF in Mouse Tumor Ascites

An important activity of VEGF is to stimulate the growth of vascular endothelial cells. The growth of primary endothelial cells obtained from RLSECs was stimulated *in vitro* after exposure to r.m-VEGF, whereas an antimouse VEGF-neutralizing antibody AF-493-NA blocked this stimulatory effect (Fig. 3*A*). To determine whether the VEGF in ascites fluid was biologically active, the proliferative activity on RLSECs of the partially purified VEGF from MM2 ascites fluid with heparin-affinity column was examined. The partially purified VEGF, the amounts of which were measured by binding assay, stimulated the growth of RLSECs nearly as efficiently as the r.m-VEGF, whereas the addition of the VEGF-neutralizing antibody completely inhibited the response of RLSECs to these samples (Fig. 3*A*). These results indicate that the growth-stimulatory activity of RLSECs can be completely attributed to the heparin-bound VEGF. Similar results were obtained by using the partially purified VEGF derived from X5563 tumor ascites fluid.

Another important biological function of VEGF is vascular

Table 1 *VEGF concentrations in ascites fluid, vascular densities in peritoneal wall, and some characteristics of ascites tumors*
Ascites tumors are listed in descending order of VEGF values.

Tumor	Histology	Invasiveness[a]	Life span (days)	VEGF (ng/ml)	Ascites fluid volume (ml/mouse)	Bloody[b]	Vascular density
Sarcoma/carcinoma							
BP-8	Sarcoma	−	14 ± 2.5[c]	850 ± 22.5	4.5 ± 1.1	B	132 ± 21
Ehrlich	Carcinoma	++	15 ± 3.0	600 ± 15.3	15.5 ± 2.8	B	85 ± 15
SRC57BL	Sarcoma	++–+++	15 ± 2.5	560 ± 12.2	8.5 ± 1.5	B	65 ± 13
SRDDD	Sarcoma	++	13 ± 1.5	520 ± 10.4	5.5 ± 1.5	B	38 ± 6
MH134	Carcinoma	++	12 ± 1.5	350 ± 32.6	3.5 ± 1.2	B	37 ± 6
S37	Sarcoma	+	12 ± 2.0	310 ± 8.6	4.5 ± 1.3	B	128 ± 17
S180	Sarcoma	−	15 ± 2.0	168 ± 15.5	6.5 ± 1.5	B	48 ± 7
MM2	Carcinoma	−	17 ± 1.5	85 ± 20.5	17.5 ± 2.4	B	122 ± 15
Hematological tumor							
X5563	Plasmacytoma	+–++	11 ± 2.0	38 ± 18.5	4.0 ± 1.4	B	35 ± 8
L1210	Leukemia	++++	7 ± 1.0	19 ± 6.5	2.0 ± 3.3	B	25 ± 3
OG	Lymphoma	++++	13 ± 1.0	18 ± 2.5	3.5 ± 0.2	C	22 ± 6
P815	Mastocytoma	+	8 ± 1.5	15 ± 9.4	4.5 ± 0.2	C	120 ± 6
RG	Lymphoma	++++	14 ± 1.5	6 ± 3.5	3.5 ± 0.3	C	20 ± 3
Normal mouse							
Serum				2 ± 0.3			
Peritoneal wall							5 ± 2

[a] Indicates the growth on the surface of or into the peritoneal lining tissues. −, not evident; +, slight; ++, moderate; +++, significant; ++++, extensive invasiveness.
[b] Ascites fluid either contained (B) or did not contain (C) more than 2×10^8 RBCs per mouse, at a certain stage.
[c] Mean ± SD.

permeability-enhancing activity. To examine this function, we performed the Miles assay using a guinea pig. As shown in Fig. 3*B*, the partially purified VEGF from MM2 ascites exerted a permeability-enhancing effect similar to that of r.m-VEGF, whereas the addition of the VEGF-neutralizing antibody completely blocked this effect.

The biologically active VEGF was also found in the cell-free ascites fluids of MM2, MH134, X5563, and P815 tumors (data not shown), indicating that VEGF accumulated in the tumor ascites fluids was almost biologically active.

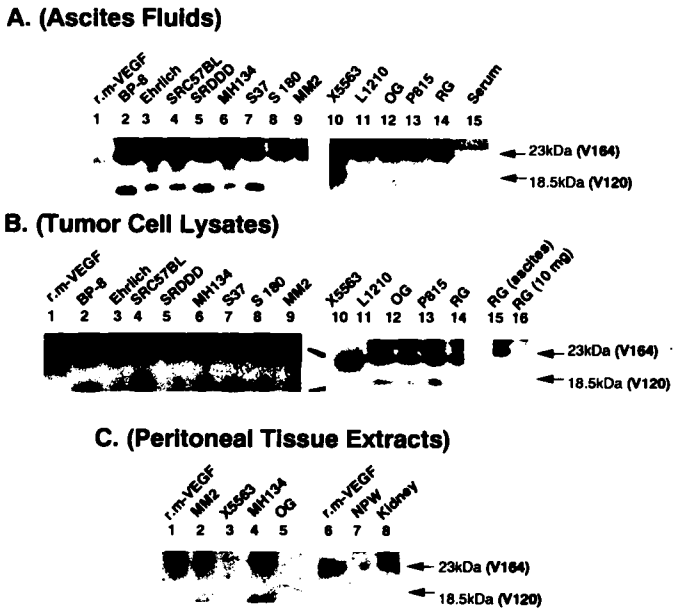

Fig. 2. IP-Western blot analysis of VEGF in the malignant ascites fluids, tumor cell lysates, and tissue extracts of the peritoneal wall from tumor-bearing mice. IP-Western blot analyses of VEGF were performed as described above. *A*, samples of malignant ascites fluids: 0.25 ml (*Lanes 2–9*) or 0.5 ml (*Lanes 10–14*) of the ascites fluids indicated, with 15 ng of r.m-VEGF (*Lane 1*) and 0.5 ml normal mouse serum (*Lane 15*) as controls. *B*, samples of tumor cell lysates: 5 mg (*Lanes 2–14*) or 10 mg (*Lane 16*) of protein of tumor cell lysates indicated, with 15 ng of r.m-VEGF (*Lane 1*) and 0.5 ml of RG ascites fluid (*Lane 15*) as controls. *C*, samples of tissue extracts of the peritoneal wall derived from tumor-bearing mice: 5 mg protein of tissue extracts from tumor-bearing mice (*Lanes 2–5*), with the same amounts of tissue extracts of normal peritoneal wall (*NPW*) (*Lane 7*) or kidney (*Lane 8*), or 15 ng (*Lane 1*) or 20 ng (*Lane 6*) of r.m-VEGF as controls. *Top* and *bottom arrows* indicate 23 kDa ($VEGF_{164}$) and 18.5 kDa ($VEGF_{120}$), respectively.

Characterization of Mouse VEGF in Tumor Ascites

Origin of VEGF. VEGF gene is expressed in a wide variety of normal adult tissues, such as vascular smooth muscel cells (2) and the elicited peritoneal macrophages (15), as well as in various tumor cells. To clarify the origin of VEGF in the tumor ascites fluids, we carried out IP-Western blot of the cell lysates derived from tumor cells. VEGF was easily identified in most tumor cells except for those from OG and RG lymphoma cells, especially in RG cells, where it was detectable only when the protein amounts of cell lysates were increased from 5 to 10 mg (Fig. 2*B*).

The expression of VEGF mRNA was found from the RNA of most tumor cells (12 of 13) by Northern blot (Fig. 4*A*) and of all 13 tumor cells by RT-PCR (Fig. 4*B*). RT-PCR results were afterward confirmed by the Southern blot analysis (data not shown). A rough correlation was observed among the levels of VEGF protein in the ascites fluids, the levels of VEGF protein in the tumor cell lysates, and the levels of VEGF mRNA derived from the tumor cells. These findings indicate that all ascites tumor cells tested secrete VEGF to different extents and contribute, at least in part, to the VEGF production in the ascites fluids.

A Constitutive Expression of VEGF. The microenvironment of ascites tumor is thought to be generally hypoxic (37). To elucidate whether the hypoxia thought to exist was involved in the regulation of VEGF gene in ascites tumors, we compared VEGF levels in the ascites or cell lysates of tumor cells grown in peritoneal cavity at different stages with those in normoxic cultures, using MM2, Ehrlich, and S180 tumors as the representatives. Interestingly, the former (*i.e.*, the level of VEGF in media or cell lysates of tumor cells grown under normoxic culture) was not lower than that in the latter (*i.e.*, the peritoneal cavity; Fig. 5). This result indicates that these ascites tumors expressed VEGF constitutively at high levels.

Subtypes of VEGF. The genomic organization of VEGF gene suggests that alternative splicing generates $VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$ isoforms, among which, $VEGF_{120}$ fails to bind to heparin because of lack of basic residues (6). The above results of IP-Western analysis (Fig. 2) and RT-PCR (Fig. 4*B*) indicate that in all 13 ascites tumors, $VEGF_{164}$ is the most abundant isoform, approximately 3–10 fold more than $VEGF_{120}$ isoform, whereas $VEGF_{188}$ is the least abundant. The chromatographic behaviors of the polypeptides of M_r 23,000 and M_r 18,500 in ascites further supported this idea. The M_r

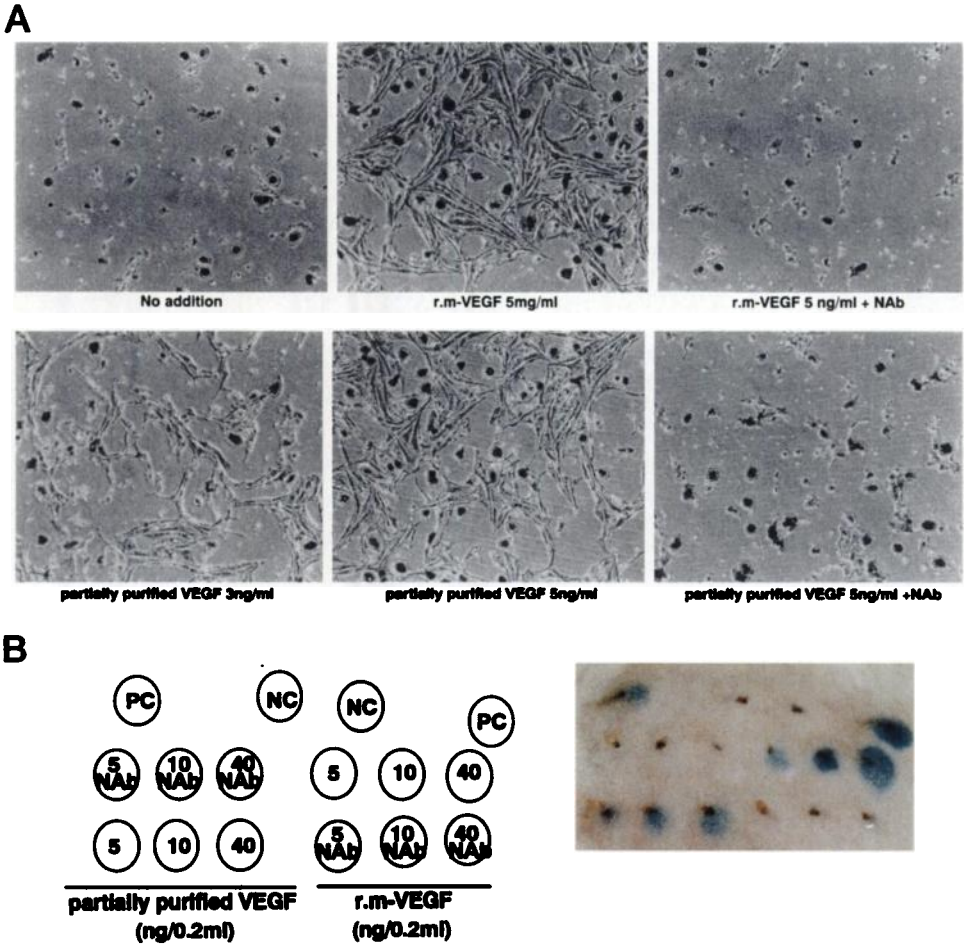

Fig. 3. Biological activities of partially purified VEGF from MM2 ascites fluid. *A*, growth of RLSECs in response to the partially purified VEGF from MM2 ascites fluid. r.m-VEGF (*top panel*) or the partially purified VEGF from MM2 ascites fluid (*bottom panel*) were added to triplicate cultures of RLSECs at the doses shown, in the absence or presence of neutralizing antibody (*NAb*) at 100-fold the amount of VEGF, for 4 days. *No addition*, cells were incubated with HCM medium only. *B*, vascular permeability activity of the partially purified VEGF from MM2 ascites fluid. Various amounts of r.m-VEGF or the partially purified VEGF indicated, in the absence or presence of neutralizing antibody (*NAb*) at the 100-fold amounts of VEGF, were injected (0.2 ml/spot) s.c. in the back of a guinea pig (see "Materials and Methods"). *PC*, positive control (40 ng r.m-VEGF/spot); *NC*, negative control (0.2 ml of PBS).

23,000 polypeptides significantly bound to heparin-affinity column, in agreement with that of $VEGF_{164}$ (2, 4, 8). In contrast, relatively much M_r 18,500 polypeptide was observed in the passthrough fraction, basically consistent with the pattern of $VEGF_{120}$ homodimer (Refs. 6, 7, and 38; Fig. 6). In addition, the presence of M_r 18,500 polypeptide in the 1.2 M NaCl fraction may suggest the heterodimer between $VEGF_{164}$ and $VEGF_{120}$.

These findings indicate that in VEGF expression, the ascites tumors tested share patterns of splicing variants similar to most normal tissues or tumor cells (5).

Relationship between VEGF Concentrations in Ascites Fluids and Vascular Densities in Peritoneal Walls of Tumor-bearing Mice

To understand the relationship between VEGF concentrations in the ascites fluids and the vascular densities in the peritoneal walls of tumor-bearing mice, we first examined whether the VEGF that accumulated in ascites fluid had access to the microvessels lining the peritoneal cavity by performing IP-Western blot of the peritoneal wall extracts and immunochemical staining of the peritoneal walls. These analyses (of four randomly selected tumors) revealed that VEGF remained significantly trapped in the peritoneal tissues and concentrated in the microvessels lining the peritoneal cavity (Figs. 2*C* and 7), basically correlated with the VEGF levels in their respective ascites fluids. Such a perivascular staining of VEGF in ascites tumor-bearing mice was previously reported with an anti-VEGF antiserum by Dvorak *et al.* (39).

We further investigated the relationship between VEGF concentrations in the ascites fluids and the vascular densities in the peritoneal walls. Table 1 shows that the VEGF concentrations basically correlated with, but were not strongly proportional to, the vascular densities of peritoneal walls of tumor-bearing mice, although their vascular densities were significantly greater than those of normal mice ($P < 0.001$). As exceptions, several tumors, such as MH134 and S180, which expressed high levels of VEGF, did not provoke correspondingly significant new vessels, whereas tumors such as P815 mastocytoma, which expressed relatively low level of VEGF, developed a mass of new microvessels (Table 1). These results may indicate that VEGF contributes to the angiogenesis in the peritoneal walls of tumor-bearing mice, but some other factors (*e.g.*, non-VEGF angiogenesis factors or inhibitors) are also involved in the determination of total levels of new vascular formation.

DISCUSSION

We successfully developed a sensitive mouse VEGF-measuring assay by combining pN2, an affinity-purified antibody (to the NH_2 terminus of mouse VEGF) that specifically identifies mouse VEGF, with ^{125}I-7N Flt-1, a ligand binding domain of VEGF receptor-1. A similar radioreceptor binding assay has been used to detect biologically active human VEGF in biological fluid (40). VEGF was easily detected in the ascites fluids of all 13 ascites tumors tested by using the radioreceptor binding assay (Table 1). The levels may well have biological significance because a VEGF concentration of 0.05 ng/ml was effective in cultured endothelial cells (40, 41). The functional analyses not only confirmed that the binding assay correctly measured

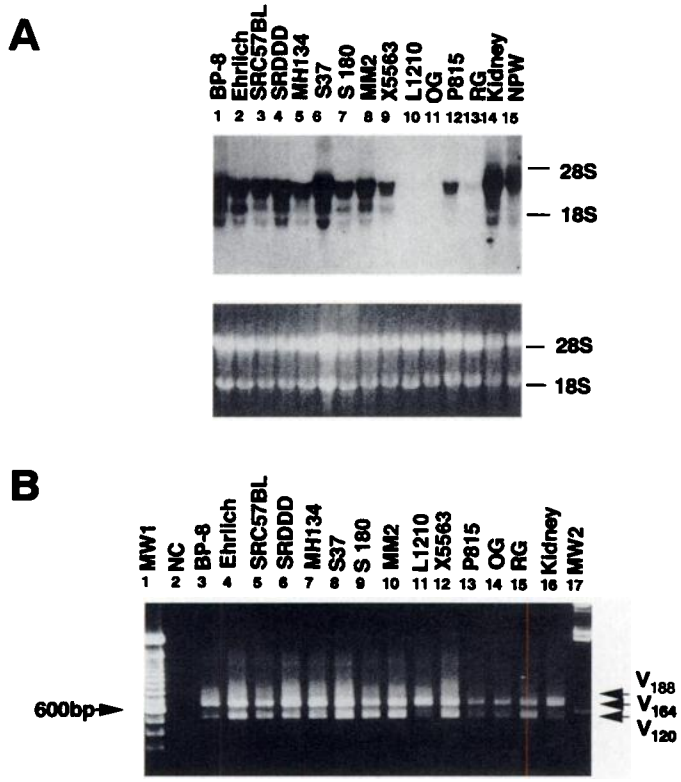

Fig. 4. VEGF mRNA expression in tumor cells, normal peritoneal wall, and normal kidney. *A*, Northern blot analysis of 15 μg of total RNA hybridized with mouse VEGF probe (see "Materials and Methods"). The positions and amounts of rRNA 18S and 28S are indicated. *B*, RT-PCR analysis for VEGF gene expression. The bands corresponding to the three isoforms ($VEGF_{188}$, $VEGF_{164}$, and $VEGF_{120}$) are indicated. *NPW*, normal peritoneal wall; *NC*, negative control containing all of the reaction products except for the reverse transcriptase. *MW1*, 100-bp ladder marker; *MW2*, λ-phage DNA marker (digested with *Hin*dIII).

VEGF in the ascites fluids but also revealed that most VEGF accumulated in the fluids was biologically active.

A surprising finding in this study is the great difference of VEGF levels in the ascites fluids among the 13 tumors, more than a 100-fold difference between the highest and the lowest ones. In the ascites tumors originated from sarcoma and carcinoma origins, which are the main source of malignant ascites in human, VEGF levels are extremely high, in great excess of the levels needed to enhance microvascular permeability or to promote the growth of endothelial cells both *in vitro* and *in vivo*. Because VEGF protein is concentrated in the microvessels lining peritoneal cavities of tumor-bearing mice (Fig. 7), by exerting its effects on the surrounding microvessels, VEGF may play an important role in this group of tumors, such as promoting and maintaining ascites formation and stimulating tumor expansion.

Another noteworthy point is that peritoneal bleeding was commonly observed in the ascites tumors with high levels of VEGF. Several independent pieces of evidence indicate that VEGF has a certain relationship to the coagulation system *in vivo*. VEGF stimulates endothelial cells to express tissue factor, urokinase-type plasminogen activator, tissue-type plasminogen activator, and plasminogen activator inhibitor-1 (1, 5). A recent study demonstrated that overexpression of the smaller VEGF isoforms, $VEGF_{121}$ and $VEGF_{165}$ but not $VEGF_{189}$, causes tumor-associated hemorrhage in an experimental system of brain tumor that requires a high level and constant secretion of VEGF (42).

Thus, it is quite possible that the constitutively excessive exposure of the smaller VEGF isoforms in ascites fluid to the surrounding endothelial cells of microvessels led to the peritoneal bleeding, directly, as a result of imbalance between coagulation and fibrinolysis, or indirectly, by inducing defective tumor-associated angiogenesis at great risk of injury and hemorrhage (43, 44). However, the non-VEGF-related possibilities, such as the direct invasive damage of vessels by tumor cells or other vessel-injuring factors secreted by tumor cells, have not been excluded.

In contrast to its high level in the tumors of sarcoma and carcinoma origin, VEGF is relatively low in those of lymphoma and hematological tumor origin. Some tumors of this group, such as L1210 leukemia and OG lymphoma, tended to grow in mass on the surface of the tissues lining peritoneal cavity or to invade into these tissues (Fig. 7 and Table 1). Therefore, the invasiveness of ascites tumor cells to peritoneal walls was not directly correlated with the levels of VEGF in ascites fluids. Rather, this invasiveness of tumor cells might directly relate to the ascites accumulation by leading to local obstruction of peritoneal draining lymphatics (45–47).

It has been proposed that among several mechanisms involved in the regulation of VEGF gene expression, hypoxia plays a major role

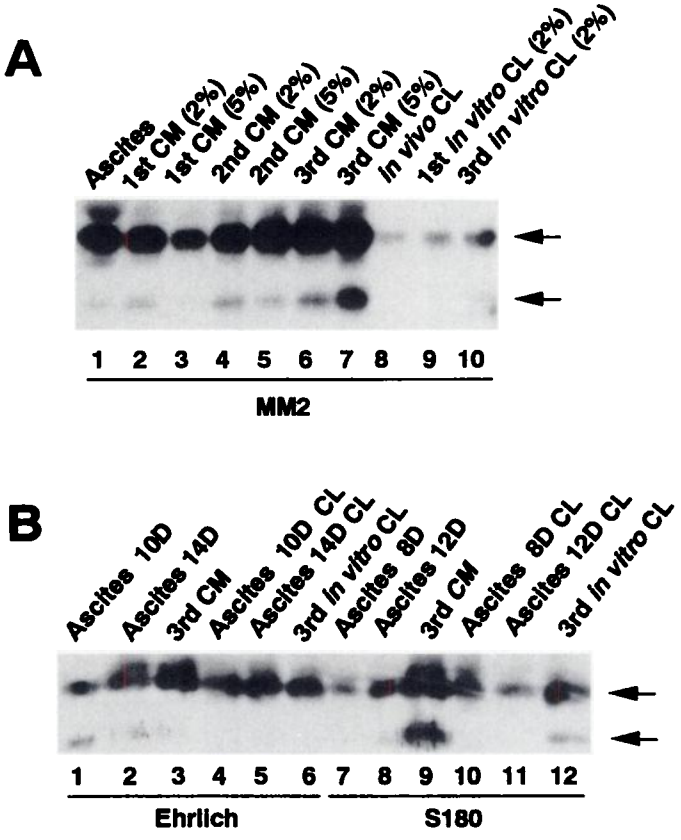

Fig. 5. No significant up-regulation of VEGF expression was seen in tumor cells grown in normoxic cultures. Fractionated cells of MM2, Ehrlich, and S180 were incubated at 4×10^6 cells/ml under normoxia and passed every 2 days. Cell-free media (*CM*) were prepared at intervals as indicated, as described in "Materials and Methods." The samples of ascites fluids (if not otherwise specified, used at a volume of 4.0×10^7 MM2 or 1.5×10^7 Ehrlich and S180 cells/each) or cell lysates (*CL*) (5 mg protein/each) were subjected to IP-Western blot analysis. *A*, analyses for MM2 tumor. The samples were as follows: the ascites fluid of day 12 tumor (*Lane 1*), the CM (for 4×10^7 cells/each) at intervals (*Lanes 2–7*), and the CL of cells grown in peritoneal cavity (*Lane 8*) or those grown in normoxic cultures (*Lanes 9* and *10*). The percentages of FCS used are indicated. *B*, analyses for Ehrlich and S180 tumors. The samples for Ehrlich tumor were as follows: the ascites fluids of day 10 tumor (*Lane 1*) or day 14 (for 2.0×10^7 cells, *Lane 2*), the third passage CM (for 3.0×10^7 cells, *Lane 3*), and the CL of the cells grown in peritoneal cavity (*Lanes 4* and *5*) or in normoxic cultures (*Lane 6*). The samples for S180 tumor were as follows: the ascites fluid of day 8 tumor (for 1.0×10^7 cells, *Lane 7*) or day 12 (*Lane 8*), third passage CM (for 3.0×10^7 cells, *Lane 9*), and the CL of these cells grown in peritoneal cavity (*Lanes 10* and *11*) or in normoxic cultures (*Lane 12*). 2% FCS was used for Ehrlich cells culture. *Arrows* indicate the positions of VEGF molecules.

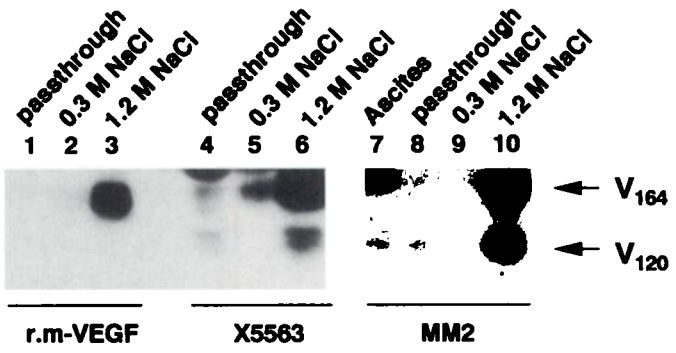

Fig. 6. Affinity of VEGF in the ascites fluids for heparin column. X5563 (*Lanes 4–6*) or MM2 (*Lanes 7–10*) ascites fluid (2.5 ml) diluted with the same volume of 20 mM Tris-HCl buffer was applied to a 5-ml heparin column. The void volumes were collected. Thereafter, the columns were washed with 20 mM Tris-HCl buffer containing 0.3 M NaCl and eluted with the same buffer containing 1.2 M NaCl. Fifty ng of r.m-VEGF (*Lanes 1–3*) diluted in 5 ml of PBS were used as a control. One-half volume (for X5563) or one-third volume (for MM2) of the passthrough and of the wash solution and the total (for X5563) or one-half (for MM2) volume of the eluted solution were subjected to IP-Western blot analysis. *Arrows* indicate the positions of VEGF molecules.

(13), which has been well established in many solid tumors or transformed culture cells (13). However, little is known about the regulation of VEGF gene expression in ascites tumor. Because oxygen, as well as nutrients, can only diffuse approximately 150 μm from vessels (48), the ascites fluid in which tumors grow can reasonably be thought of as hypoxic. Previously, Harris *et al.* (49) suggested that the microenvironment of ascites tumor cells *in vivo* is generally hypoxic, and afterward, this was confirmed in Ehrlich ascites tumor, metabolically and biologically, by others (50). Interestingly, in the present study, we found that the levels of VEGF expression by MM2, Ehrlich and S180 tumors grown in *in vitro* normoxic culture were no lower than those grown in the peritoneal cavity. An earlier report from Folkman and coworkers (51) described that S180 tumor cells grown in normoxic culture expressed high levels of VEGF mRNA. Although the supply of nutrients, including O_2, is thought to be increasingly impaired when the tumor mass in the peritoneal cavity grows (50), Dvorak and coworkers (18) did not find any significant change in VEGF mRNA or protein levels in two ascites tumors, which were recovered from the peritoneum at successive intervals after i.p. transplantation.

Collectively, these findings imply that VEGF expression in these ascites tumors became constitutive at a high level, without being obviously up-regulated further by hypoxia. It seems important to investigate whether a change of growth pattern from solid form to ascites-producing form is associated with a loss of hypoxia-responsive VEGF gene expression and, if so, what kind of molecular mechanism underlies the constitutive elevation.

In this study, we could not find a strong correlation between the VEGF concentrations in ascites fluid and the vascular densities in the peritoneal wall of tumor-bearing mice in some ascites tumors. Several explanations could, individually or collectively, account for this: (*a*) the rates at which angiogenesis developed may be not only dependent on VEGF. The report by Dellian *et al.* provided evidence that sometimes the formation of new vessels was dependent on both the cytokines and the microenvironment of the tissue in which the cytokines were exposed (52). (*b*) Angiogenesis provoked by ascites tumors was time-dependent. This view was confirmed by a published report (53) and our observations (36). New vessels, in response to VEGF expressed by ascites tumors, may have not developed enough during

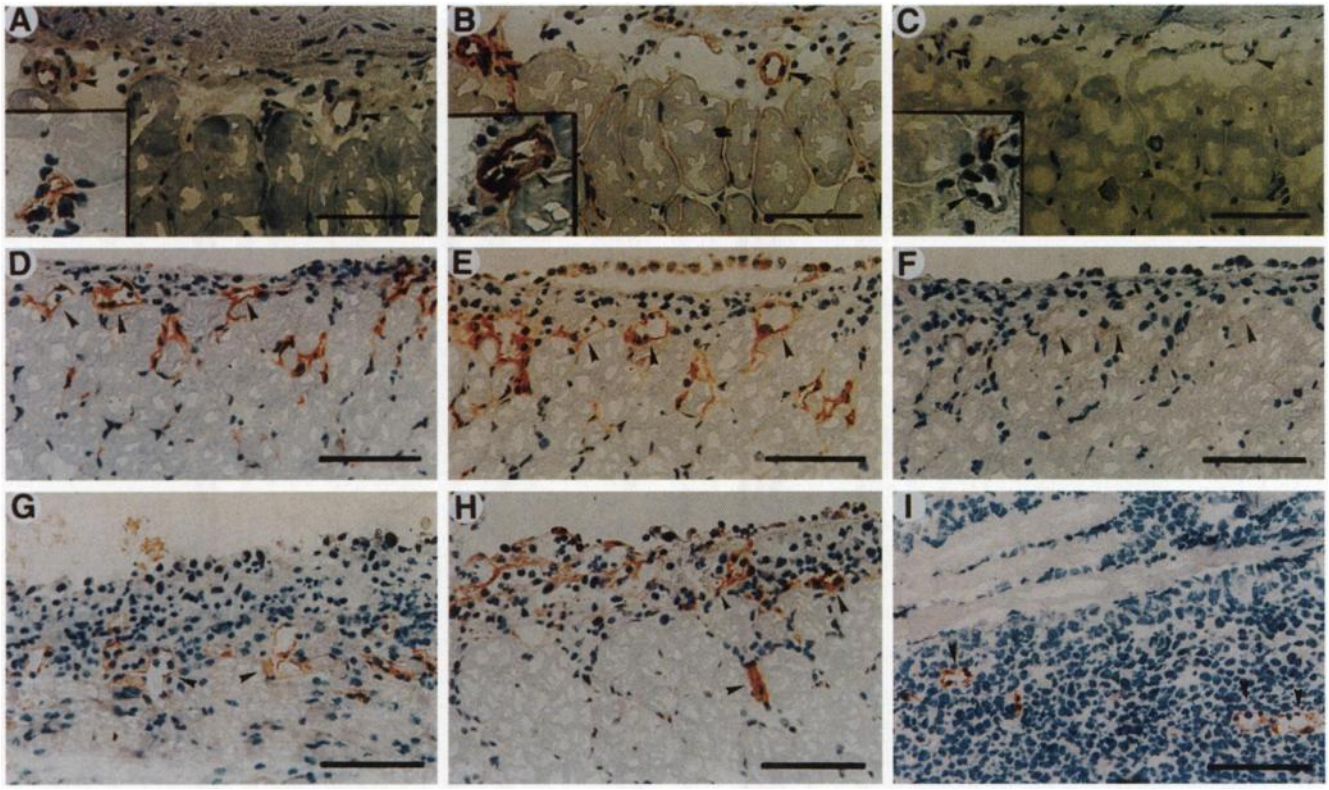

Fig. 7. Immunohistochemical localization of VEGF protein in the peritoneal walls of ascites tumor-bearing or normal mice. *A* and *D* show normal and BP-8 tumor-induced microvessels in the peritoneal walls, respectively, with antibody to vWF. Substantial and strong VEGF staining with anti-VEGF antibody pN2 was seen at the periphery of both normal (*B*) and tumor-induced (*E*) microvessels. In contrast, no staining was seen with normal rabbit IgG in the place of antibody pN2 (*C* and *F*). *G–I*, VEGF staining in the peritoneal microvessels induced by MM2, X5563, and OG, respectively. Incubation times used with peroxidase substrates for BP-8 (*E*), X5563 (*H*), and OG (*I*) sections were about 1.5, 5, and 7 min, respectively, to obtain a similar intensity of the staining. Note an area strongly invaded or infiltrated by OG tumor cells, at which microvessels localize, in/on the peritoneal wall. *Insets* in *A–C* are high-power (×132) views of one set of separate stainings, indicating that a typical VEGF staining for microvessels is abluminal, whereas vWF staining is luminal. Except for insets, ×66; *bar*, 100 μm.

the life span of some of the tumor-bearing mice tested. (*c*) angiogenesis inhibitor(s) or angiogenic cytokines other than VEGF were involved in some tumors tested. Several laboratories have demonstrated that some tumors produce angiogenesis inhibitors, such as angiostatin and thrombospondin, as they switch to the angiogenic phenotype (54), and various angiogenic factors secreted by tumors have been identified (5). Further work is now ongoing to determine which, if any, of these possibilities are responsible for the weak correlation.

Endothelial cells of the microvessels lining the normal peritoneal cavity are usually quiescent, and angiogenesis is rarely observed. Interestingly, we observed a substantial expression of VEGF in this area at both the RNA and protein levels (Figs. 2, 4, and 7), suggesting that VEGF plays a role in the maintenance of the basal permeability of the peritoneal microcirculation, as it is thought to do in other normal tissues, such as kidney, lung, and others. Physiologically, however, the basal permeability is not sufficient to lead to the ascites accumulation due to the presence of a peritoneal drainage system (mainly via diaphragmatic lymphatics). After the implantation of tumors, the microvessels lining the peritoneal cavity become so hyperpermeable, as a result of the stimulation by the permeability-enhancing factor(s) tumor cells secreted, that the fluid inflow from plasma to the peritoneal cavity exceeds the drainage capacity, and eventually, ascites fluid accumulates (18).

Because all ascites tumors tested significantly secreted VEGF (a powerful permeability-enhancing factor), we strongly suggest that VEGF plays a fundamental role in the fluid accumulation and tumor growth in ascites tumors. By secreting VEGF, ascites tumors enhance the permeability of preexisting microvessels lining the peritoneal cavity to stimulate ascites formation, by which tumors improve nutrient supply and waste removal for further expansion. Subsequently, the constitutive exposure of VEGF accumulated in ascites fluid on the surrounding microvessels provokes angiogenesis, which contributes to the long-term progressive growth of tumors. According to this view, the elimination of VEGF may inhibit the ascites tumor. Because greatly different levels of VEGF expression were observed between the ascites tumors originated from sarcoma and carcinoma and those from lymphoma and hematological tumor, and permeability-enhancing factors other than VEGF had been demonstrated to be involved in some tumors tested in this study (55, 56), the exact role of VEGF in the ascites tumors needs to be further defined.

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