Increased Production of Vascular Endothelial Growth Factor in Peritoneal Macrophages of Cirrhotic Patients With Spontaneous Bacterial Peritonitis

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Spontaneous bacterial peritonitis (SBP) is a common complication of cirrhotic patients with ascites that usually results in renal failure and death despite the efficacy of the current antibiotic therapy. The pathogenesis of these phenomena is poorly known but it has been related to the production of vasoactive cell mediators locally acting on the splanchnic vasculature. Because previous studies showed that peritoneal macrophages of cirrhotic patients may produce high quantities of vascular endothelial growth factor (VEGF), a powerful vessel permeabilizing agent, when stimulated by cytokines and bacterial lipopolysaccharide, the present study aimed to seek whether peritoneal macrophages of SBP patients are induced to produce increased amounts of VEGF. Our results indicate that the production rate and the messenger RNA (mRNA) and protein expression of this substance are increased in macrophages of patients with SBP in comparison with those of noninfected cirrhotic patients. This characteristic feature is absent in circulating monocytes of these patients. Moreover, enhanced endothelial cell proliferation induced by conditioned medium of macrophages isolated from the ascites of patients with SBP is abolished by anti-VEGF antibody, and peritoneal tissue of cirrhotic patients expresses both VEGF receptors, Flt-1 and KDR. These results, therefore, are consistent with the concept that locally released macrophage-derived VEGF may result in increased vascular permeability and plasma leakage in the peritoneal vessels of cirrhotic patients with SBP. (HEPATOLOGY 2001;34:487-493.)

Abbreviations: SBP, spontaneous bacterial peritonitis; VEGF, vascular endothelial growth factor; LPS, lipopolysaccharide; mRNA, messenger RNA; PMN, polymorphonuclear cells; HUVEC, human umbilical vein endothelial cells; RT, reverse transcription; cDNA, complementary DNA; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; MT3, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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Peripheral monocytes were seeded on 24-well plates (6 rpm (15 minutes, room temperature), and leukocytes were fractionated with acid citrate dextrose as anticoagulant. Blood was centrifuged at 800 g to isolate. Cells were obtained from fresh blood by venipuncture using heparin. Patients with ascites without (n = 5) and with cirrhosis, ascites, and SBP, were prepared and isolated by Ficoll-Hypaque gradient centrifugation as previously described. Cells were seeded on 24-well plates (Costar, Cambridge, MA) at a concentration of 6 × 10^5 cells/well and incubated for 12 hours with phenol red-free RPMI 1640 medium supplemented with 2% heat-inactivated fetal calf serum, penicillin/streptomycin (50 U/mL and 50 μg/mL), HEPES (250 μmol/L), and L-glutamine (2 mmol/L) at 37°C in a humidified atmosphere (95% air and 5% CO2). More than 96% of the adherent cells were positive for human Factor VIII antigen or hepatitis C antibody associated in 8 patients with or without SBP, were determined by liver biopsy in 6 cases and by clinical, laboratory, and ultrasonographic findings in the remaining cases. All patients had advanced cirrhosis: 10 belonged to Child-Pugh class B and 7 to Child-Pugh class C. SBP was diagnosed on the basis of a polymorphonuclear (PMN) cell count in ascitic fluid equal to or greater than 250 cells/mm^3 in the absence of clinical, radiologic, or laboratory data suggesting secondary peritonitis or other abdominal disorders resembling SBP (e.g., hemorrhage into ascites, pancreatitis, peritonitis, tuberculosis, or carcinoma).

**Cell Cultures.** Human peritoneal macrophages, either from patients with or without SBP, were prepared and isolated by Ficoll-Hypaque gradient centrifugation. Total RNA was extracted from peritoneal macrophages using a commercially available kit (Trizol Reagent; Life Technologies, GIBCO BRL; Gaithersburg, MD). The final RNA pellets were resuspended in diethylpyrocarbonate-treated water and stored at −80°C until used. Reverse transcription (RT) was performed with random primers using a complementary DNA (cDNA) synthesis kit (Promega, Madison, WI). First strand cDNA synthesis was performed at 42°C for 45 minutes in the DNA thermal cycler (PTC-100, MJ Research Inc., Watertown, MA). Afterwards, the tubes were incubated at 95°C for 5 minutes to stop the reaction. Then each tube was kept at 4°C until polymerase chain reaction (PCR) was performed. PCR was done using a DNA amplification reagent kit (Gibco BRL, Life Technologies) with human VEGF primers prepared by a DNA synthesizer (394 DNA/RNA Synthesizer; Applied Biosystems, Foster City, CA). VEGF primers flanking the insertion/deletion site of human VEGF were designed as described by Mohle et al. Primers were also synthesized to amplify the cdNA encoding β-actin, a constitutively expressed gene, as control. 

**Immunocytochemical Analysis of VEGF Protein Expression.** VEGF protein expression was assessed in peritoneal macrophages and cytokeratin preparations of ascitic fluid of cirrhotic patients. Macrophages were isolated and seeded on slides placed in P-100 plates as described above. Adherent cells were maintained for 48 hours on culture medium with or without MIX and stored at −20°C until further processing. Cytokeratin preparations were obtained after ascites cell concentration by centrifugation (10 minutes, 1800 rpm, 4°C). Thereafter concentrated ascites samples were placed in a plastic chamber and centrifuged for 5 minutes at 500 rpm (Cytospin 3; Shandon Scientific, Astmoor, England) to seed the cells on the slides, which were immediately treated as described. Preparations were fixed with 100% methanol for 7 minutes at 4°C immediately before the immunostaining. After fixation, they were hydrated with phosphate-buffered saline (PBS) solution for 10 minutes at 4°C and then incubated with 3% H2O2 for 30 minutes at room temperature. After 3 washes with PBS solution, nonspecific staining was blocked with normal rabbit serum (1:50 in PBS; Vector Laboratories, Burlingame, CA) for 20 minutes at room temperature. Cell preparations were incubated with goat anti-VEGF polyclonal antibody overnight with a 1:300 dilution in 5% rabbit serum at 4°C. After washing with PBS (3 times for 5 minutes each), cells were incubated with biotinylated rabbit anti-goat Ig G (Vector Laboratories) for 30 minutes. After washing with PBS for 5 minutes at room temperature, preparations were incubated for 30 minutes with horseradish peroxidase–conjugated avidin (Vector Laboratories) to visualize the reaction product. Immunostaining was visualized using the 3,3′-DAB substrate kit for peroxidase (SK-4100, Vector Laboratories). Cells were counterstained with Mayer’s hematoxylin. Negative controls were prepared by omitting the primary antibody and their replacement with serum or by preabsorption of the primary antibody with a 5-fold excess of human recombinant VEGF (R&D Systems, Minneapolis, MN).

**Endothelial Cell Proliferation Assay.** To examine the effect of conditioned medium of peritoneal macrophages cultured for 48 hours on cell proliferation, a microculture (3-[4,5-diethylthiazol-2-yl]-2,5-diphenyterazolium bromide) (MTT) assay was performed. HUVEC were grown to confluence as described. Cells from confluent culture phase were counted and dispensed within preliminarily 96-well culture plates in 200 μL of medium (approximately 4,800 cells/well). After 24 hours at 37°C, the culture medium was removed, and fresh medium (200 μL) containing 100 μL of culture supernatant, or vehicle (medium), was added to 12 replicates for each condition studied. Experiments were performed with culture supernatants of peritoneal macrophages isolated from noninfected cirrhotic patients and patients with SBP. Culture plates were incubated for up to 24 hours at 37°C in a 5% CO2 atmosphere. At the end of this period, 25 μL of freshly prepared MTT in Hanks’ balanced salt solution (5 mg/mL) was added to the wells and MTT incorporated by HUVEC was measured as previously described. In addition, anti-human recombinant VEGF antibody (20 μg/mL) was added to the conditioned media and incubated for 2 hours at 37°C. After this preincubation period, the media were used to determine whether the plasma leakage in the peritoneal vasculature of patients with cirrhosis, ascites, and SBP.
effect on HUVEC proliferation was in fact caused by VEGF. In preliminary experiments we observed that, in the range of 2 to $10 \times 10^3$ HUVEC/well, a direct linear correlation ($r = 0.963$) exists between the obtained absorbance and the amount of cells seeded in the well. Viability was assessed routinely using trypan blue exclusion assay.

VEGF Receptors mRNA Expression in Peritoneal Tissue of Cirrhotic Patients. VEGF receptor transcript expression was assessed in 3 cirrhotic patients undergoing orthotopic liver transplantation. After laparotomy, peritoneal tissue (approximately 200 mg) was collected by serosal scraping from the antimesenteric border of the terminal ileum. Total RNA was extracted from the tissue as described above, and the final RNA was stored at $-80^\circ$C until used. RT was performed with DNA-free RNA samples as indicated above. PCR was performed by using a DNA amplification reagent kit. Appropriate sense and antisense primer oligonucleotide was selected from the human cDNA sequences of the Flt-1 receptor$^{13}$ and the KDR receptor$^{16}$ as described by Couffignal et al.$^{7}$ and Mohle et al.$^{16}$ respectively. The cDNA amplification products were predicted to be 735 bp and 790 bp, respectively.

For the Flt-1 receptor 40 cycles of the following sequential steps were performed: 30 seconds at 95°C, 5 seconds at 56°C, and 1 minute at 72°C and final incubation at 72°C for 10 minutes. For the KDR receptor and after a first incubation at 94°C for 5 minutes, 40 cycles of the following sequential steps were performed: 1 minute at 94°C, 1 minute at 62°C, and 2 minutes at 72°C and final extension was identical to that of Flt-1. Authenticity of VEGF receptor RT-PCR products was confirmed by direct sequencing (373A DNA Sequencer, Applied Biosystems). The sequences were found to be identical to those reported for human Flt-1 and KDR receptors.$^{15,16}$ Control experiments included RNA amplification of each sample without RT and negative control for the PCR step (no cDNA added) to control for contamination of PCR components.

Materials. LPS (E. coli serotype 0127:B8), bovine serum albumin, bovine serum, and MTT were purchased from Sigma Chemical Co. (St. Louis, MO). Hanks' balanced salt solution, and Dulbecco's phosphate-buffered saline were from Bio Whittaker (Walkersville, MD); RPMI 1640, L-glutamine, and streptomycin/penicillin were from Gibco BRL (Life Technologies, Paisley, Scotland); fetal calf serum was from Reactiva Biological Industries (Kibbutz Beit Haemek, Israel); tumor necrosis factor $\alpha$ was from Immunokontact (Frankfurt, Germany); interleukin 1$\beta$ was from Genzyme (Cambridge, MA); Nu/serum was from Collaborative Res. Inc. (Lexington, MA); endothelial mitogen was from Biomedical Technologies Inc. (Stoughton, MA); human recombinant VEGF$^{165}$ and anti-human VEGF$^{165}$ antibody for the cell proliferation assays were from R&D; and goat anti-human VEGF$^{165}$ antibody for the immunocytochemical studies was from Santa Cruz Biotechnology (SC-132-G; Santa Cruz, CA). Other reagents were obtained from Merck Darmstadt (Darmstadt, Germany). Drugs were prepared fresh daily and concentrations are expressed as the final concentration in the well. VEGF concentration was measured using an enzyme-linked immunosorbent assay (Quantikine Human VEGF Immunoassay, R&D Systems) that recognized the soluble isoforms VEGF$_{121}$ and VEGF$_{165}$.

Statistical Analysis. Statistical analysis of the results was performed by using unpaired Student’s t test. Data are expressed as mean $\pm$ SEM and were considered significant at a P level of .05 or less.

The study was performed according to the criteria of the Investigation and Ethics Committee of the Hospital Clinics Universitari.

RESULTS

Eleven patients included in the study were diagnosed with SBP. The clinical and laboratory data of patients without peritonitis or with SBP are shown in Table 1. Marked hepatic failure was present in the 2 groups of patients, as indicated by high serum bilirubin and low serum albumin and prothrombin activity.

### Table 1. Baseline Characteristics of the Patients Included in the Study

<table>
<thead>
<tr>
<th></th>
<th>No SBP (n = 6)</th>
<th>SBP (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>60 $\pm$ 4</td>
<td>54 $\pm$ 2</td>
</tr>
<tr>
<td>Male sex</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Alcohol-induced cirrhosis (n)</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Time since diagnosis of cirrhosis (mo)</td>
<td>43 $\pm$ 18</td>
<td>75 $\pm$ 20</td>
</tr>
<tr>
<td>Child-Pugh score</td>
<td>8.5 $\pm$ 0.6</td>
<td>10 $\pm$ 0.4</td>
</tr>
<tr>
<td>Serum bilirubin (mg/dL)</td>
<td>2.2 $\pm$ 1.3</td>
<td>3.1 $\pm$ 0.5</td>
</tr>
<tr>
<td>Serum albumin (g/dL)</td>
<td>29.3 $\pm$ 1.5</td>
<td>30.7 $\pm$ 1.7</td>
</tr>
<tr>
<td>Prothrombin activity (%)</td>
<td>60 $\pm$ 26.5</td>
<td>56.6 $\pm$ 6.3</td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>1.5 $\pm$ 0.4</td>
<td>1.4 $\pm$ 0.26</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>33.0 $\pm$ 6.2</td>
<td>34.1 $\pm$ 7.6</td>
</tr>
<tr>
<td>Serum Na (mEq/L)</td>
<td>133 $\pm$ 2</td>
<td>132 $\pm$ 2</td>
</tr>
<tr>
<td>Ascitic fluid (cells/mm$^3$)</td>
<td>148 $\pm$ 56</td>
<td>1188 $\pm$ 426</td>
</tr>
<tr>
<td>Total leukocytes</td>
<td>148 $\pm$ 56</td>
<td>1188 $\pm$ 426</td>
</tr>
<tr>
<td>PMN cells</td>
<td>35.3 $\pm$ 6.1</td>
<td>76.4 $\pm$ 351.4</td>
</tr>
<tr>
<td>Serum K (mEq/L)</td>
<td>5.1 $\pm$ 0.5</td>
<td>3.9 $\pm$ 0.18</td>
</tr>
</tbody>
</table>

NOTE. The values of continuous variables are expressed as mean $\pm$ SEM.

VEGF Production and VEGF mRNA Expression in Peritoneal Macrophages. To assess whether cytokines and LPS may affect VEGF production in human macrophages of cirrhotic patients regardless of whether they were isolated from subjects with or without SBP, MIX was added for 48 hours to the cell culture medium of peritoneal macrophages of cirrhotic patients obtained after paracentesis. Confirming previous studies,$^7$ peritoneal macrophages from noninfected cirrhotic patients with ascites markedly increased VEGF production under MIX stimulatory conditions (from 15.6 $\pm$ 2.3 pg/mL to 1,033 $\pm$ 146 pg/mL, P < .001). In addition, the concentration of VEGF in the cell culture medium of macrophages obtained from patients with SBP was significantly higher (705 $\pm$ 70 pg/mL, P < .001) than that found in patients without peritonitis after 48 hours in culture. Interestingly, MIX stimulation also resulted in a further increase in VEGF production in macrophages of patients with SBP (2,824 $\pm$ 272 pg/mL, P < .001), although this effect was of less intensity than that observed in cells obtained from patients without peritonitis.

Time course production of VEGF in peritoneal macrophages isolated from patients without peritonitis or with SBP is shown in Fig. 1. Macrophages from both groups of patients released significant amounts of VEGF. However, the production rate of this substance was markedly increased in the cells obtained from subjects with SBP, as compared with those without peritoneal infection. In fact, as early as 12 hours in culture the concentration of VEGF in the cell culture medium of SBP macrophages was significantly increased in comparison with that in the medium of noninfected macrophages. This difference tended to be more pronounced after 24 hours and 48 hours in culture.

To assess whether increased VEGF protein production in SBP macrophages is associated with enhanced abundance of VEGF mRNA, a semi-quantitative RT-PCR analysis was performed. As shown in Fig. 2, peritoneal macrophages expressed bands of 403, 535, and 607 bp corresponding to 121, 165, and 189 amino acid isoforms of VEGF, respectively. The expression pattern of these isoforms was found to be similar in macrophages of patients with or without SBP, indicating that the increased VEGF protein production in SBP macrophages is not caused by the existence of unique alternative splicing
products of VEGF in these cells. However, analysis of PCR products indicates greater abundance of all 3 transcripts in macrophages of SBP patients. Under the exponential phase of PCR amplification (25 cycles), the 3 bands of VEGF isoforms could be detected in SBP macrophages harvested immediately after paracentesis, the weakest signal corresponding to that of VEGF_{189} and the strongest to VEGF_{165}. Macrophages of non-infected cirrhotic patients showed the same bands but of less intensity, with the band corresponding to VEGF_{189} being barely detectable. This difference in the intensity of the expression of the 3 transcripts between macrophages of patients with or without SBP was also observed when the cells were maintained in culture for 24 hours and 48 hours. Bands of equal strength were obtained using primers for β-actin after 22 PCR cycles.

**Conditioned Medium-Induced Cell Proliferation in HUVEC.** The effect of conditioned medium on endothelial cell proliferation, as assessed by MTT incorporation by these cells, is shown in Fig. 3. After culturing peritoneal macrophages of patients with or without SBP for 48 hours, the conditioned medium was mixed with standard medium at a proportion of 50% and endothelial cell growth in these conditions compared with that observed with standard medium. Cell proliferation induced by conditioned medium obtained from macrophages of patients with SBP was significantly higher than that induced by standard medium or conditioned medium of macrophages without bacterial peritonitis. This difference was abrogated when the conditioned medium of macrophages of patients with SBP also contained the VEGF neutralizing antibody.

**Circulating Monocytes Do Not Produce VEGF.** To examine whether the increased VEGF production in peritoneal macrophages of cirrhotic patients with SBP is a specific characteristic of these cells or also occurs in other cells of the monocyte/macrophage cell lineage, the in vitro production of VEGF was also assessed in circulating monocytes isolated from healthy subjects and from cirrhotic patients with ascites, with and without SBP. Neither cells from controls (from 10.2 ± 1.7 to 9.3 ± 1.0 pg/mL) nor those from cirrhotic patients with ascites (from 7.2 ± 2.1 pg/mL to 7.7 ± 1.1 pg/mL) and without SBP (from 5.9 ± 0.7 pg/mL to 5.8 ± 0.6 pg/mL) produced significant amounts of VEGF after 48 hours in culture.

**Immunocytochemical VEGF Protein Expression.** A representative cell culture of macrophages isolated from ascites of patients with cirrhosis is depicted in Fig. 4A. In agreement with our previous findings showing constitutive VEGF production in peritoneal macrophages of cirrhotic patients with ascites,
immunoperoxidase staining for VEGF was positive in most of these cells. As expected, the presence of MIX in the cell culture medium of peritoneal macrophages resulted in cell clustering, a characteristic phenomenon of activated cells (Fig. 4B). In addition, MIX induction was also associated with a stronger positive signal for immunoperoxidase VEGF-specific staining.

In an attempt to ascertain whether VEGF protein expression is restricted to peritoneal macrophages or is a generalized characteristic of other cell types found in the ascitic fluid, cytocentrifuge preparations obtained from concentrated ascites were immunostained with the specific VEGF antiserum. As shown in Fig. 4C, mainly macrophages showed a positive reaction for the VEGF antibody, which was not observed in erythrocytes, lymphocytes, or PMN leukocytes. No staining was detected in negative controls (data not shown).

VEGF Receptors in Peritoneal Tissue of Cirrhotic Patients. To explore whether the peritoneum could be a target for the VEGF released by peritoneal macrophages, mRNA expression of the VEGF receptors, Flt-1 and KDR, was assessed in peritoneal tissue of 3 cirrhotic patients. As shown in Fig. 5, samples from all patients showed a clear band migrating at about 735 bp, which is compatible with the predicted size of the Flt-1 receptor. Similarly, the amplification products corresponding to KDR primers showed a single band of 790 bp in all patients. In both cases, the authenticity of these PCR products was confirmed by direct sequencing.

DISCUSSION
In the current investigation we examined the production of VEGF in ascites fluid macrophages of 11 cirrhotic patients with SBP and 6 patients with cirrhosis and ascites without SBP. Because it is well known that hepatocellular carcinoma is associated with hepatic VEGF gene induction, no patient with this or any other type of cancer was included in the present set of experiments. All but 3 SBP patients showed an enhanced production rate of VEGF as compared with macrophages obtained from cirrhotic patients without SBP. These findings indicate that, despite the clinical heterogeneity that may exist from patient to patient (time of evolution of the disease, days of antibiotic treatment, time of diagnosis of the infection, etc.), induction of VEGF protein overproduction is a rather extended phenomenon in cirrhotic patients with SBP.

The results of our RT-PCR indicated that mRNA encoding the 3 most common isoforms, VEGF121, VEGF165, and to a lesser extent VEGF189, are all expressed by peritoneal fluid macrophages of cirrhotic patients. An identical pattern of alternatively spliced VEGF transcripts was observed in cells isolated from ascites of SBP patients. However, the signal intensity was stronger in the PCR products obtained from macrophages of these patients. This is likely because of both increased transcriptional rate of the gene and greater mRNA stability, because previous experiments showed that LPS and cytokines promote higher VEGF mRNA abundance in cultured peritoneal macrophages of cirrhotic patients through these mechanisms.

To determine the biological activity of macrophage-derived VEGF on human endothelial cells, we investigated the effect of macrophage-conditioned media that contained immunoreactive VEGF on the proliferation of HUVEC in vitro. Macrophage-conditioned medium triggered enhanced cell proliferation when derived from SBP patients and this difference was abolished on inhibiting the bioactivity of VEGF by addition of anti-VEGF to the cell culture medium. Together, these findings provide strong evidence that VEGF produced by macrophages from cirrhotic patients with SBP can elicit biological responses from its target cells, the endothelial cells.

Further insight on the cellular origin and cell specificity of the increased VEGF production by ascites fluid macrophages of cirrhotic patients was gained by following 2 distinct exper-
The biological responses elicited by VEGF-related peptides are mediated by the interaction of these proteins with 2 high-affinity protein tyrosine kinase receptors, Flt-1 (or VEGF-R1) and KDR (VEGF-R2), the human homologue of murine Flk-1.28 They are predominantly expressed in endothelial cells, although a few additional cell types express one or both of these receptors.29-33 In our study, PCR products corresponding to Flt-1 and KDR messengers were obtained in all 3 tissue samples obtained from cirrhotic patients, thus showing the expression of both transcripts in the peritoneal tissue of human cirrhosis.

In summary, the results of the present study show that ascites fluid macrophages of patients with cirrhosis and SBP produce higher quantities of the angiogenic/vasodilator/vasopermeabilizing factor VEGF than ascites fluid macrophages of cirrhotic patients without SBP. Moreover, enhanced endothelial cell proliferation induced by conditioned medium of macrophages isolated from the ascites of patients with SBP is abolished by anti-VEGF antibody. In addition, this characteristic feature is absent in circulating monocytes of these patients and peritoneal tissue of cirrhotic patients expresses both VEGF receptors, Flt-1 and KDR. These results, therefore, are consistent with the concept that locally released macrophage-derived VEGF may result in increased vascular permeability and plasma leakage in the peritoneal vessels of cirrhotic patients with SBP.

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