**Advances in Brief**

**Vascular Endothelial Growth Factor Levels and Induction of Permeability in Malignant Pleural Effusions**

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**Abstract**

Vascular endothelial growth factor (VEGF) is an important mediator of angiogenesis and vascular permeability. We hypothesized that malignant pleural effusions may contain high levels of VEGF protein as well as other cytokines implicated in these processes. Pleural effusions cytologically proven to be malignant were collected from 39 patients with various types of cancer, and VEGF, interleukin-8, and angiogenin levels in the effusions were determined by immunnoassy. Negative controls were nonmalignant ascites and serum samples from healthy individuals. VEGF levels were significantly higher than those of control samples in pleural effusions secondary to breast, mesothelioma, and non-small cell lung cancer and when all malignant pleural effusion samples were pooled. Neither interleukin-8 nor angiogenin levels were elevated in malignant pleural effusions relative to the control samples. Vascular permeability, which was measured by using the Miles assay in nude mice, was increased proportionately with VEGF levels in the malignant pleural effusions; this increase in permeability induced by injection of recombinant VEGF or the malignant effusions was reduced by pretreating the mice with a VEGF receptor antibody.

**Introduction**

The formation of malignant pleural effusions as part of the continuum of the malignant process indicates the presence of end-stage disease and survival times as short as 4–6 months (1, 2). Approximately half of all patients with metastatic cancer develop pleural effusions (3). The presence of malignant pleural effusions is associated with significant morbidity and poor quality of life (4).

The proteinaceous milieu of malignant pleural effusions contains many growth factors and cytokines that may influence the dynamics of its formation. Many angiogenic cytokines, including VEGF (5), IL-8 (6), and angiogenin (7) have been found to be elevated in malignant effusions and ascites from many different tumor types. Malignant pleural effusion formation is thought to be associated with high levels of these angiogenic proteins, and these high levels are thought to induce vascular permeability. The purpose of the present study was to measure the levels of VEGF, IL-8, and angiogenin in malignant pleural effusions and to investigate the role of these cytokines in the induction of vascular permeability in vivo animal studies.

VEGF, also known as vascular permeability factor, is a unique angiogenic dimeric glycoprotein with a molecular mass of 34–42 kDa. VEGF is expressed by nearly all cell types, but many malignant tumor cells overexpress it (8, 9). VEGF stimulates capillary-tube formation and has specific mitogenic and chemotactic effects on vascular endothelial cells (10). It is also a potent inductor of vascular permeability, 50,000 times more so than histamine (11, 12). VEGF has been implicated in many pathological conditions for its angiogenic and permeability-inducing effects, the latter of which has been implicated in such disease states as ovarian hyperstimulation syndrome (13) and malignant ascites (14).

IL-8, a member of the “C-X-C” chemokine family, is also associated with inflammation and angiogenesis, and its increased expression has been implicated in a variety of malignancies (2, 15, 16). Pleural effusions have been shown to contain elevated levels of IL-8 in various disease states, and this elevation has been implicated in the pathogenesis of inflammation-driven effusions (6).

Angiogenin is another potent angiogenic cytokine that has been implicated in various malignant processes. Patients with ovarian cancer, a disease characterized by the formation of malignant ascites, have been found to have elevated serum angiogenin levels (17).

Given that the above cytokines may participate in the development of malignant effusions, we investigated the possibility that malignant pleural effusions may contain increased levels of VEGF, IL-8, and angiogenin proteins. Our studies demonstrated that VEGF was significantly elevated in malignant pleural effusions. Therefore, follow-up studies were done.

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3 The abbreviations used are: VEGF, vascular endothelial growth factor; IL-8, interleukin-8; NSCLC, non-small cell lung cancer.
to determine whether blockade of VEGF activity in malignant pleural effusions could diminish permeability.

Materials and Methods

Reagents. Recombinant human IL-8 and recombinant human VEGF were purchased from R&D Systems (Minneapolis, MN). Evans Blue dye, polyethylene glycol, and poloxymethylene sorbitan monooleate were purchased from Sigma Chemical Company (St. Louis, MO). Antibody to the VEGF receptor Flk-1 (DC101; Ref. (18) was provided by Imclone Systems (New York, NY).

Patient Samples. Samples of pleural effusions and ascites cytologically proven to be malignant were collected from patients at The University of Texas M. D. Anderson Cancer Center at the time of therapeutic or diagnostic thoracentesis, paracentesis, or laparotomy under approved protocols. Cytological results were obtained from the patients' pathology records. Samples were centrifuged at the time of collection to remove debris, filtered through a 0.22-µm filter, and then stored at ≈70°C until analysis. Samples from 39 patients with a variety of primary cancers (6 lymphoma, 7 breast, 15 NSCLC, 4 mesothelioma, 4 sarcoma, and 3 renal cell) were examined. Negative controls consisted of four samples of serum from healthy individuals and four samples of ascites from patients with nonmalignant cirrhosis.

Determination of VEGF, IL-8, and Angiogenin Protein Levels. VEGF, IL-8, and angiogenin protein levels were measured with Quantikine human immunoassay kits (R&D Systems, Minneapolis, MN). The malignant pleural effusions, ascites, and serum samples were treated as serum/plasma samples. Some samples exhibited high levels requiring up to a 500-fold dilution of the sample with assay diluent to have concentrations that could be read on the standard curve. Total protein levels were quantified with Bio-Rad (Bradford) protein assay kits (Bio-Rad Laboratories, Hercules, CA).

In Vivo Permeability Assays (Miles Assay). To determine the effect of human malignant pleural effusion samples and recombinant cytokines on vascular permeability in vivo, we performed a modified Miles assay (19) as described below. In brief, male nude mice (BALB/c background) were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with present regulations and standards of the United States Department of Agriculture, the United States Department of Health and Human Services, and the NIH. In accordance with institutional guidelines, the experiments were conducted when the mice were 8–12 weeks old.

The mice received i.v. injections of 200 µl of Evans blue dye (0.5%) via the tail vain. Ten minutes later, mice received intradermal injections of 50 µl of (a) PBS, (b) recombinant human IL-8 (50 ng/ml), (c) recombinant human VEGF 165 (50 ng/ml), (d) malignant effusion containing low levels of VEGF (~2 pg/mg protein) from a patient with NSCLC, (e) malignant effusion containing high levels of VEGF (1364 ng/mg protein) from a patient with sarcoma, (f) malignant effusion containing high levels of VEGF (321 ng/mg protein) from a patient with breast cancer, and (g) malignant effusion containing high levels of VEGF (336 ng/mg protein) from a patient with NSCLC. The subdermis was harvested and photographed 30 min later to document any leakage of the dye into the dermal tissue.

To determine whether permeability was mediated by VEGF activity, we used anti-Flk-1 antibodies to block VEGF receptor activity in mice as follows. Mice were treated every third day for 1 week with i.p. injections of either PBS or the DC101 antibody at 1 mg/mouse by i.p. injection before being injected with the test compounds listed above.

Statistics. Data were analyzed by the Mann-Whitney U test using the Instat program for the Macintosh (GraphPad Software, San Diego, CA). The data did not fit a Gaussian distribution because of significant differences in the SDs of the various test groups. Ps of ≤0.05 were considered statistically significant.

Results

VEGF, IL-8, and Angiogenin Levels in Malignant Pleural Effusions. VEGF protein was detectable in 37 of the 39 malignant pleural effusion samples, and IL-8 protein and angiogenin were detectable in 36. VEGF protein levels were higher in all malignant effusions than in control specimens (P = 0.0112; Fig. 1A). Control values represent mean serum cytokine levels from healthy adults because these values were greater than those in nonmalignant ascites (mean value in nonmalignant ascites was 121 pg/mg; Ref. 14). Cytokine levels in malignant effusions varied markedly among the various types of malignancies (Table 1).

When the VEGF values from all malignant effusions were combined, the VEGF protein level was higher than that of controls (P = 0.01). However, when VEGF in malignant effusions was examined by disease type, VEGF was higher than the control only for the breast, mesothelioma, and NSCLC groups (Fig. 1A) because of large SDs for the other cancer types.

IL-8 levels in malignant pleural effusions were not significantly different from those in control samples (Fig. 1B). As was true for VEGF, the amounts of IL-8 varied substantially among the types of cancer, but no statistically significant difference was found between IL-8 in pooled malignant samples versus control samples.

Angiogenin levels in malignant pleural effusions were no different than those in control samples (Fig. 1C), and the variation among cancer types was less than that for VEGF or IL-8.

In Vivo Vascular Permeability Assay. As indicated by a Miles assay, VEGF prompted more vascular permeability (indicated by dermal dye leakage) than did PBS or IL-8 (Fig. 2). Among the malignant-effusion samples, the least amount of dye leakage resulted from injection of the sample with the lowest amount of VEGF [condition (Fig. 2D) compared with Fig. 2, E-G]. Treating the mice with the anti-Flk-1 DC101 antibody before injection of the test compounds decreased the vascular permeability relative to that in the control mice for the injection sites containing either recombinant VEGF (Fig. 2B) or malig-
nant pleural effusions (Fig. 2, D-G). Duplication of this experiment produced similar findings.

Discussion

The development of malignant effusions is likely due to a combination of factors. Tumors that line the pleural cavity can produce differential osmotic and oncotic pressures that may lead to fluid accumulation. Vascular integrity may be physically disrupted by tumor infiltration of lymphatic channels and capillaries. In addition, permeability factors such as VEGF may be secreted by tumor cells, thus leading to increased capillary permeability and fluid accumulation.

We have previously shown that VEGF levels are elevated in malignant ascites and that this elevation of VEGF leads to increased endothelial cell permeability in vitro. Because most malignancies express VEGF, we hypothesized that elevated levels of VEGF may be associated with malignant pleural effusions. Given that other cytokines can be elevated in malignant effusions, we also investigated IL-8 and angiogenin in malignant pleural effusions. We found that amounts of VEGF in malignant pleural effusions were significantly higher than in serum from healthy individuals, in nonmalignant effusions from patients with nonmalignant ascites, or in cerebrospinal fluid from healthy individuals (14). Thus, in nonmalignant states, VEGF expression is lower than that in fluids associated with malignant effusions.

In more detailed analyses, we found that VEGF levels were significantly higher in pleural effusions from patients with breast cancer, mesothelioma, and non-small cell lung carcinomas than in control samples. The large range of VEGF levels in effusions from patients with lymphoma, sarcoma, or renal cell cancer may have obscured differences between VEGF in these samples versus in control samples; alternatively, the number of specimens studied may have been too small to reach statistical significance.

We also assessed IL-8 and angiogenin levels in malignant effusion samples. No significant differences in either

<table>
<thead>
<tr>
<th>Sample type (n)</th>
<th>VEGF levels (pg/mg protein) [median]</th>
<th>IL-8 levels (pg/mg protein) [median]</th>
<th>Angiogenin levels (ng/mg protein) [median]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesothelioma (4)</td>
<td>23–223 [142]</td>
<td>6–134 [29]</td>
<td>0–12 [8]</td>
</tr>
<tr>
<td>NSCLC (15)</td>
<td>0–402 [75]</td>
<td>0–302 [8]</td>
<td>0–6 [6]</td>
</tr>
</tbody>
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Fig. 1 VEGF (A), IL-8 (B), and angiogenin (C) levels in malignant pleural effusions. VEGF levels were significantly higher than those of control samples (*P < 0.05) in pleural effusions secondary to breast, mesothelioma, and NSCLC and when all malignant pleural effusion samples were pooled.
compound were found between malignant and control samples, perhaps because of the large range of values. IL-8 is sometimes shed from tumor cells (20) and thus may serve as a tumor marker; however, results from our in vivo assay indicated that IL-8 was not associated with an increase in vascular permeability.

Once we found that VEGF levels in malignant pleural effusions were relatively high compared with VEGF levels in control fluids, we sought to investigate whether blockade of VEGF activity could block permeability in an in vivo permeability assay. Using the Miles mouse assay, we demonstrated that treating mice with DC101, an antibody to Flk-1 (the predominant VEGF receptor), inhibited subsequent VEGF-induced permeability. This pretreatment also inhibited the permeability induced by injection of malignant specimens with relatively high VEGF content (Fig. 2, E-G).

In summary, these results demonstrate that VEGF expression is associated with malignant pleural effusions. Blockade of VEGF activity may decrease the permeability associated with increased VEGF expression. These studies form a foundation for future studies in mice and potentially in humans, where VEGF antagonists may be useful in the treatment and prevention of malignant pleural effusions.

Acknowledgments

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References

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