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ARTICLE

Isolation and Purification of Vascular Endothelial Growth Factor (VEGF) from Ascitic Fluid of Ovarian Cancer Patients

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Vascular Endothelial Growth Factor (VEGF) or Vascular Permeability Factor (VPF) is an angiogenic cytokine expressed by many human and animal tumors. Because of the importance of VEGF in animal tumors, we purified VEGF/VPF from ascitic fluid of ovarian cancer patients with heparin sepharose column. The purified protein gave protein bands of 37 and 26 kD, respectively in 12% SDS PAGE. The specificity of the purified protein was determined with dot blot, trans-immunoblot and ELISA using polyclonal goat anti-VEGF antibody (Santa Cruz Biotechnology). The vasodilatatory effect of the purified protein was confirmed by a vascular permeability assay on mouse. A polyclonal mouse antibody was raised against the purified protein, which recognized the same protein by ELISA, transimmunoblot and dot-blot analysis. It has been also found that the raised polyclonal antibody in mouse- and the commercial VEGF polyclonal antibody (Santa Cruz Biotechnology) both inhibited *in vitro* cell proliferation of human MCF-7 cell line. This study shows for the first time an effort to purify VEGF from human source. (Pathology Oncology Research Vol 10, No 2, 104–108)

Keywords: VEGF, ascitic fluid, ovarian cancer

Introduction

VEGF is a key mediator of tumor angiogenesis.[4] It is commonly expressed in a wide variety of animal tumors,[5] and has been found to be related to the fundamental features of tumors such as growth rate,[7] microvessel density,[16] vascular architecture and development of tumor metastasis.[15] Kraft et al reported that VEGF level was several-fold higher in malignant effusion than in matched serum samples, indicating local release of this cytokine within the peritoneal cavity.[8] In human, elevated expression of VEGF has been reported in tumors of the gastrointestinal tract, kidney, ovary and breast. VEGF is expressed in many normal tissues, including those from lung, kidney and adrenal gland, heart, liver, stomach mucosa and blood mononuclear cells as well as activated macrophages. Thus, VEGF in effusion and serum of cancer patients may originate from normal tissues as well as from tumor cells. In breast carcinoma, strong VEGF expression has been demonstrated to correlate with high microvessel density, early metastatic spread and poor prognosis. Little is known, however, about the relevance of soluble VEGF in malignant effusion. It has been proposed that VEGF secretion by tumor cells is responsible for initiating and maintaining the ascitic pattern of tumor growth.[14] In fact, the expression of VEGF in human ascitic tumor has not been systematically investigated. To address this, in this study we aimed to purify and characterize the VEGF/VPF protein from abdominal malignant effusion of ovarian cancer patients. We also report the effect of antibody, raised against the purified protein on human metastatic breast cancer cell line.

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Materials and Methods

Collection of ascitic fluid

Ascitic fluid samples were collected from stages I–IV (n=24) ovarian carcinoma patients attending the gyneco-oncology department of the Institute. The mean age of patients was 45 years (range 30–59 years). Ascitic fluid was filtered, 0.35 mg/ml PMSF was added to it, and stored at –20°C.

Characterization of VEGF

Affinity column purification. Fifteen ml of pooled ascitic fluid, diluted 2-fold with 10 mM sodium phosphate buffer (pH 7), was applied to a 5 ml heparin sepharose column (Amersham Pharmacia Biotech), previously equilibrated with 10 mM sodium phosphate buffer (pH 7) according to Senger et al[14] & Luo.[6] The column was eluted with 10 mM sodium phosphate buffer (pH 7) containing 1.2 M NaCl. The eluted sample was dialyzed in PBS (pH 7.4).

SDS gel electrophoresis of the purified protein. The protein obtained after passing through the affinity column was run in 12% SDS-PAGE according to the method of Laemmli.[10]

Production of antibody in mice. The antisera against the purified protein was raised in Swiss albino mice by simultaneous injection of the enzyme emulsified with Freund's complete and incomplete adjuvant (Sigma) alternatively at 7 days intervals. After the 4th injection the blood was collected from corneal artery. Then, after clotting the serum was collected and stored at –20°C.

Western blot analysis was carried out essentially as described.[17] Samples were subjected to 12% SDS-PAGE and then transferred to nitrocellulose. After blocking with BSA-Tween20-TBS solution, the nitrocellulose was incubated with goat anti-VEGF antibody (VEGF-C; C-20; Santa Cruz Biotechnology), and kept overnight at 4°C. Then it was incubated with the respective peroxidase conjugated antibody for 1 hour at room temperature. Staining was carried out by immersing the nitrocellulose papers into the solutions containing 10 mg of DAB (3' 3' diaminobenzidine) in 10 ml of TBS containing 0.05% (V/V) hydrogen peroxide.

Dot blot analysis has been performed according to Escribano et al.[3] On a PVDF membrane the purified proteins were spotted in a volume of 10 µl, and the membrane was dried overnight. The PVDF membrane was blocked with 1% gelatin in TBS for 1 hour with shaking. After blocking the membrane was incubated with anti-VEGF antibody (Santa Cruz Biotechnology) overnight. Then it was washed with TBS and incubated for 1 hr with anti-goat IgG (Sigma) labelled with peroxidase. Peroxidase activity was visualized using DAB solution (1 mg/ml TBS) and 20 µl of H_2O_2.

Immunoprecipitation and gel electrophoresis. Western blot analysis was performed with the purified protein using the antisera raised against the purified protein (PAb) according to Towbin et al.[17] 200 µl of rhVEGF (R & D System, Cat No-293-VE) (10 µg/ml) was mixed in a 1.5 ml eppendorf tube with 300 µl of antisera, sealed with Parafilm and shaken for 4 hours at 4°C. Fifty µl of protein-A-Sepharose bead solution was added to it and kept in shaking condition overnight at 4°C. The supernatant was discarded and the precipitate was washed in PBS thrice. Then 25 µl of sample buffer was added and heated in boiling water bath for 5 minutes. The samples were separated by 12% SDS-PAGE and stained with Coomassie blue stain.

Vessel hyperpermeability. Unanaesthesized Swiss albino mice weighing 20-25 g were given a bolus injection in the caudal vein of trypan blue dye at a dose of 20 mg/kg body weight in PBS in a final volume of 200 µl. The dye was allowed to circulate for 10 min before sacrificing the mice. The pancreas and peritoneal membrane were dissected, weighed and a portion of each was immersed in formamide (4 ml/g wet weight, at 24°C for 24 hrs). The concentration of trypan blue dye extracted in formamide from these tissues was determined spectrophotometrically at 620 nm and expressed as µg of trypan blue/g of dry tissue after comparison to a standard curve.[1]

ELISA was performed according to Voller et al.[18] The purified VEGF from different patients were added into wells of a 96-well microtiter plate and incubated initially for 2 hrs at 37°C, followed by 12 hrs at 4°C. After a thorough wash, mouse antibody raised against the affinity purified protein was added to each well and the plate was incubated for 2 hrs at 4°C. After washing, peroxidase labeled anti-mouse secondary antibody was added to each well, and the plate was incubated for 1 hr at room temperature. Finally, after washing, the substrate solution containing ortho-phenyl-diamine and hydrogen peroxide was added to each well, colours were developed and optical density was measured in ELISA reader at 490 nm.

Cell proliferation assay. $2x10^4$ MCF-7 cells were cultured in a 96-well cell culture plate (Nunc) using MEM media at humidified atmosphere (37°C, 5% CO_2) for 72 hrs in the presence of different concentrations of anti-VEGF antibody (Santa Cruz Biotechnology) or antisera developed against the purified VEGF protein (1:100 dilution), or the purified protein. Cell proliferation assay was performed using cell proliferation kit I (MTT, 1465007 Boehringer Mannheim), according to manufacturer's guidelines.

Results

Purification of VEGF protein from ascitic fluid of ovarian cancer patients

We purified heparin-binding protein (mainly VEGF) from ascitic fluid. Ascitic fluid of 4 ovarian cancer patients of stage II and III were pooled and subjected to purifica-

tion of VEGF with the heparin sepharose column. The sample was purified 5.8-fold. The purified protein gave mainly two bands in 12% SDS-PAGE having the Rf value of 0.448 and 0.535 (*Figure 1*). Western blot analysis has been carried out with the protein in the presence of anti-

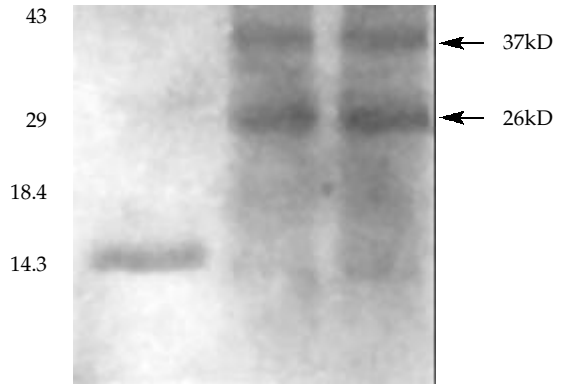

Figure 1. *Gel electrophoresis of affinity purified protein from ovarian cancer ascitic fluid, having molecular weights of 37 kD and 26 kD.*

VEGF antibody (Santa Cruz Biotechnology), and after immunochemical reaction, two bands were observed at 37 kD and 26 kD (*Figure 2*). The identity of the protein was also confirmed by dot blot analysis with anti-VEGF antibody (data not shown). Thus, with the help of heparin sepharose column, we were able to purify VEGF-like protein from human ascitic fluid.

Confirmation of VEGF with immunoprecipitation and gel electrophoresis

Another Western blot was carried out to show the immunological reaction of VEGF antibody (PAb) with the purified protein. *Figure 3* shows the results of Western blot analysis with our antibody raised in mice (PAb), with the partially purified VEGF. It shows similar type of bands as *Figure 2*.

Next we compared the immunoreactivity of the standard anti-VEGF (R & D System) with our newly raised mouse antibody (PAb). *Figure 4* shows immunoprecipitation followed by gel electrophoresis analysis of PAb by commercially available VEGF (R & D System). Two distinct bands were observed in lane 4 & 5, where the immunoprecipitated sample was loaded. The upper band corresponds to the band in lane 3 where only recombinant human VEGF was loaded. The lower bands of lane 4 & 5 correspond to the bands found in lane 1 where PAb was loaded. These data indicated that the polyclonal mouse antibody against the VEGF-like protein reacts with the commercially available rhVEGF. A dot blot analysis was also performed to confirm the immunoreaction of PAb with commercially available VEGF and indicated similar reaction than that of the commercial anti-VEGF antibody (data not shown).

Vessel permeability study

It is an important biological function of VEGF to increase vascular permeability. We therefore investigated the effect of purified protein on vessel permeability in mouse model. The amount of dye extravasated from peritoneal membrane (in unit area) after i.v. VEGF administration was calculated from the standard curve and compared to that of control mice which were given PBS (*Figure 5*). VEGF purified from stage II-III ovarian carcinoma patients were tested for vascular permeability in two different dilutions. The partially purified VEGF exerted a permeability enhancing effect when compared with the control mice (*Figure 5*).

ELISA was performed to test the antisera raised against the purified human protein. The antisera developed against purified VEGF (in 1:10 dilution) showed high optical density at 490 nm, unlike unimmunized mouse serum or control sample (PBS) (data not shown).

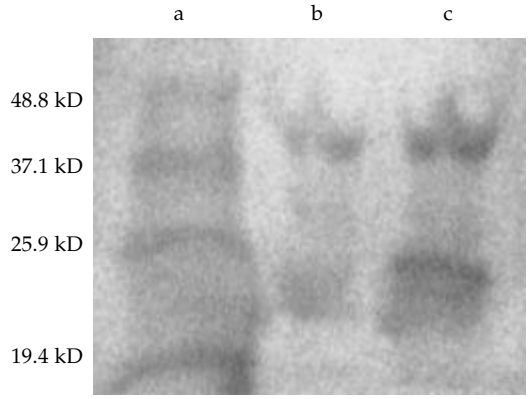

Figure 2. *Immunoblot analysis of purified protein with polyclonal anti-VEGF antibody (Santa Cruz Biotechnology), showing bands at 37 kD and 26 kD (Lane b & c).*

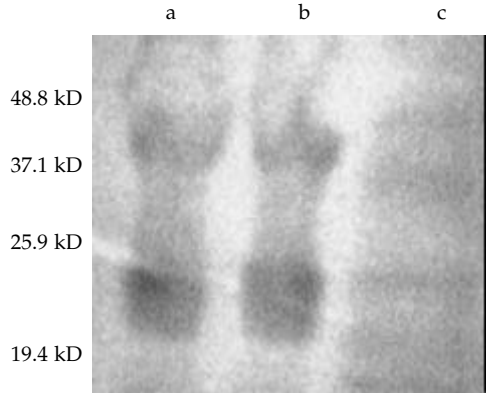

Figure 3. *Immunoblot analysis of purified protein with antibody raised in mice (PAb)(Lane a & b).*

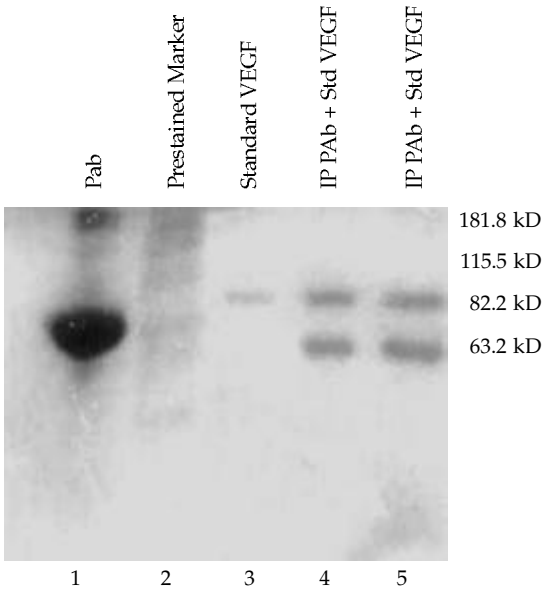

Figure 4. *Immunoprecipitation of PAb with commercially available VEGF (R&D System) confirms the interaction of PAb with rHuVEGF.*

Cell proliferation Assay

Biological activity of the raised anti-VEGF antibody was studied on MCF-7 cells *in vitro*. Results show that after 24 hours exposure, 40% inhibition of the cell density could be achieved with our antibody, PAb, when compared with the control (*Figure 6*). As a comparison, the commercially available anti-VEGF antibody (Santa Cruz Biotechnology) showed 44% inhibition when compared with the control group. It is accepted that VEGF is a mitogen for certain tumor cells. Accordingly, partially purified VEGF in two dilutions (VA1 & VA2) was applied to MCF-7 cells *in vitro* for 24 hrs. Cell proliferation was increased by 86% and 27% with higher and lower dose of VEGF, respectively.

Discussion

Vascular endothelial growth factor/vascular permeability factor is a multifunctional cytokine that has potent angiogenic activity and enhances microvascular permeability by direct action on vascular endothelium.[11,13] Expression of VEGF by tumor cells was shown to increase tumor growth, angiogenesis and experimental metastasis.[2] Elevated plasma and serum concentration of this cytokine in tumor bearing mice, in patients with advanced cancer, and in patients with systemic inflammatory disease have been reported previously.[9,20] We found detectable levels of VEGF in the ascitic fluid of ovarian cancer patients (unpublished data). There was a direct correlation observed between the disease and ascitic fluid VEGF level.[21] Although there is abundant evidence to show that VEGF plays a central role in the development and growth of malignant tumors, no information is available regarding purification of VEGF from human ascitic fluid, although it was previously purified from mouse ascitic fluid.[6] In this study we have purified ovarian cancer cell-secreted VPF/VEGF with heparin sepharose affinity column. It had Mr values of 26 and 37 kD. It also exhibited vessel permeability activity, and a polyclonal antibody raised against this protein gave cross reaction with commercially available VEGF. The antisera raised against the purified protein was immunoprecipitated with the isolated and the standard VEGF protein in dot blot reaction. There is abundant evidence showing that VEGF plays a central role in the development and growth of malignant tumors but no correlation was detected between serum VEGF and stages of disease in the study of Ober-

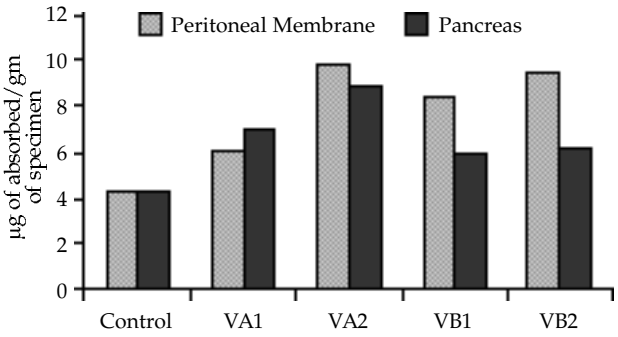

Figure 5. *Quantity of dye absorbed in mice tissue after i.v. administration of VEGF purified from two ascitic fluid samples in two different concentrations (VA1=1500 pg/ml, VA2=3000 pg/ml, VB1=1000 pg/ml, VB2=2000 pg/ml). Figure shows that the purified protein increases vascular permeability and thereby µg of dye absorbed/g tissue in a concentration dependent manner when compared with the control mice tissues (given PBS i.v.).*

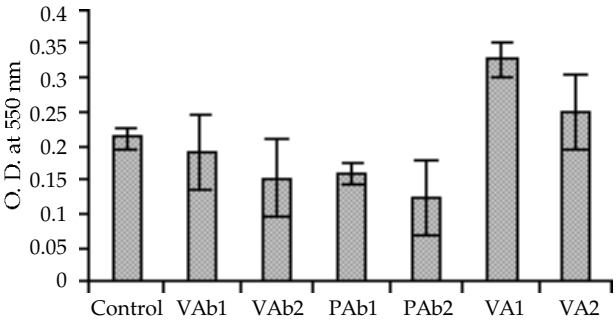

Figure 6. *Cell proliferation assay of MCF-7 cell in the presence of purified ascitic fluid-VEGF and raised polyclonal mouse antibody (PAb and purchased anti-VEGF antibody V Ab Santa Cruz Biotechnology).*

mair et al.[13] Little is known about the role of VEGF in tumor-associated ascites formation in humans. Using a mouse tumor model, our previous observation and work of *Nagy et al* demonstrated that tumor cells implanted into the peritoneal cavity secrete VEGF and thereby transform the microvessels supplying the peritoneal lining hyperpermeable. In this animal model, VEGF concentration, ascitic fluid volume, tumor cell number and hyperpermeability of microvessel in the peritoneal lining were found to increase in parallel.[12] Using antisera against VEGF,[8] or soluble VEGF receptor[19] were shown to inhibit angiogenesis and tumor growth. In this study, neutralization of VEGF by various polyclonal antibodies inhibited the proliferation of human breast cancer cells, MCF-7, suggesting direct immunological targeting of cancer cells.

In summary, soluble VEGF can be detected in certain malignant effusions, maximum VEGF concentrations were detected in advanced stages of ovarian cancer patients. Thus the perspective of measuring VEGF levels in ascitic fluid should be helpful in determining the stage of the disease and may have prognostic value. In addition, the biological role of anti-VEGF antibody in human ovarian cancer biology deserves further investigation.

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