Development of Time-Resolved Immunofluorometric Assay of Vascular Permeability Factor
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We describe a two-site time-resolved immunofluorometric assay for guinea pig vascular permeability factor (VPF) for quantifying VPF in different biological fluids. Antibody against the carboxy terminus (C-IgG) is immobilized on microtiter wells, and antibody against the amino terminus (N-IgG) is labeled with Eu³⁺-chelate. Line 10 tumor culture medium, known to be rich in VPF, is assayed in a two-step incubation. Bound Eu³⁺ is then quantified by dissociation into a fluorescent enhancement solution, with measurement of the time-resolved fluorescence. The analytical sensitivity is 0.35 VPF unit, and the intra-assay CV is about 20%. The assay is specific for VPF, because pre-treatment with the appropriate C- or N-peptide, or pre-extraction of VPF, greatly decreases fluorescence. The VPF immunoassay is highly correlated (r² = 0.94) with the Miles permeability assay, the classical bioassay of VPF. In addition, the immunofluorometric assay is about 30-fold more sensitive than the Miles assay.

Additional Keyphrases: vascular endothelial growth factor · angiogenesis · line 10 tumor culture medium · bioassay compared

Vascular permeability factor (VPF), which is secreted by various tumor cells, is a highly conserved protein (Mr, 34 000–42 000) with potent vascular permeability-enhancing activity that causes accumulation of ascites fluid associated with tumor growth (1, 2). In addition, recent studies have shown that VPF is similar or identical to vascular endothelial growth factor (VEGF), a mitogen specific for endothelial cells (3–7). Thus, tumor-secreted VPF (VEGF) may promote tumor angiogenesis directly by its mitogenic activity for endothelium. VPF (VEGF) may also elicit angiogenesis indirectly by its vascular permeability effect, which causes extravasation of plasma proteins, including fibrinogen, and deposition of an extravascular fibrin gel, which provokes ingrowth of vascular endothelial cells (8). Currently, the precise role of VPF (VEGF) in the pathogenesis of solid tumor growth is an area of intense investigation. Important to this investigation is the development of a simple, sensitive, and specific assay for VPF, preferably one that can be used for assaying VPF in various biological fluids and tissue homogenates.

VPF was first measured by using the Miles assay (9), which measures the extravasation of intravenously injected Evans Blue dye into the dermis of guinea pigs in response to intradermal injections of VPF (1). The amount of accumulated dye can be quantified by extraction and measuring the absorbance at 620 nm (10). The Miles assay has been used widely to detect VPF in cell-free culture medium of tumor cells as well as in tumor ascites fluid (1, 2). However, this assay is not specific because it will detect permeability changes in response to other inflammatory mediators besides VPF. Also, fluids from different animal species cannot be used because foreign proteins commonly elicit nonspecific permeability changes, leading to a false-positive Miles test.

We report here a sensitive and specific immunofluorometric assay for detecting VPF. Antibodies against the C-terminus of VPF (C-IgG) were immobilized on microtiter wells and served as the "capture" antibody. Antibodies raised against the amino terminus of VPF (N-IgG) were labeled with Eu³⁺-chelate and served as the "detector" antibody. In the presence of VPF, a "sandwich" configuration is formed. After the final wash step, bound Eu³⁺ is dissociated in the presence of β-diketone, forming a highly fluorescent chelate that can be read in a time-resolved fluorometer. This approach is termed "dissociation enhanced lanthanide fluorimunooassay," or DELFIA (11–14). The reagents and equipment required are commercially available.

Materials and Methods

Reagents. DELFIA Eu³⁺-labeling kits were purchased from Pharmacia-LKB Nuclear, Inc. (Gaithersburg, MD). Each kit contained 0.2 mg of labeling reagent [N³⁺-(p-isothiocyanatobenzyl)-diethylenetriamine-N³,N²,N⁶,N³⁺-tetraacetate-Eu³⁺], a 100 nmol/L Eu³⁺ standard, highly purified bovine serum albumin (BSA; 75 g/L in Tris·HCl, pH 7.8 plus, NaN₃, 0.5 g/L) stabilizer, enhancement solution (per liter, 15 μmol of 2-naphthoyltrialauroacetone, 50 μmol of tri-n-octylphosphine oxide, 100 mmol of acetic acid, 6.8 mmol of potassium hydrogen phthalate, and 1.0 g of Triton X-100 detergent), assay buffer (Tris·HCl solution, pH 7.8, containing BSA, bovine gamma globulin, Tween 40, diethylenetriaminepentaacetic acid, and NaN₃, 0.5 g/L), and wash concentrate solution (25-fold concentration of Tris·HCl/NaCl, pH 7.8, plus Tween 20) (11–14). PD-10 columns, Sepharose CL-6B, and Sephadex G-50 were from Pharmacia LKB Biotechnology (Piscataway, NJ). Macrosolute concentrators were from Amicon (Danvers, MA). Maxisorp microtiter plates and strips (96-well) were obtained from Nunc.

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1 Nonstandard abbreviations: VPF, vascular permeability factor; VEGF, vascular endothelial growth factor; C-IgG, antibody against carboxy terminus of VPF; N-IgG, antibody against amino terminus of VPF; ELISA, enzyme-linked immunosorbsorbent assay; and BSA, bovine serum albumin.

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Inc. (Naperville, IL). Serum-free medium (HL-1) was purchased from Ventrex Labs. Inc. (Portland, ME). Hemoglobin crystals were from Sigma Chemical Co. (St. Louis, MO). The GammaGone IgG removal device was from Genex Corp. (Gaithersburg, MD).

Buffers. The labeling buffer was 50 mmol/L NaHCO₃, pH 8.5, containing NaCl, 9 g/L. The elution buffer was 50 mmol/L Tris·HCl, pH 7.8, containing 9 g of NaCl and 0.5 g of NaN₃ per liter. The coating buffer contained phosphate-buffered saline, pH 7.0, and the blocking reagent was 30 g/L hemoglobin solution.

Polyclonal antibodies. Polyclonal antibodies were raised against two synthetic peptides that correspond to the N- and C-termini of guinea pig VFP (designated N-IgG, C-IgG, respectively). In the single letter code, the 25-amino acid sequence of the N-terminus is APMAEGPKPREVKFMDVYKRSYC (15), and the 20-amino acid sequence of the C-terminus is YKARQELNERT-CRCDKPRR (4). The C-terminal peptide was synthesized by Multiple Peptide Systems (San Diego, CA), and both peptides were used for generation of antibodies in rabbits as described (15), except that the C-terminal peptide was coupled to keyhole limpet hemocyanin with bis-diazobenzidine. The antibodies (N-IgG and C-IgG) were affinity-purified from rabbit antisera by using the respective peptides coupled to CNBr-Sepharose (Pharmacia LKB). Bound antibodies were eluted from Sepharose–peptide columns with 0.1 mol/L glycine, pH 2.5, and the activity against each peptide was determined by an ELISA (16). Briefly, we used a 1 g/L solution of peptide in 10 mmol/L NaCl and 10 mmol/L Tris, pH 8.5, to coat a 96-well microtiter plate. After blocking with normal human serum (100 mL/L) in phosphate-buffered saline, we added the respective anti-peptide IgG solution (200–2000–fold dilution). Antibody binding was detected with a peroxidase-labeled goat anti-rabbit antibody (Kirkegaard and Perry Labs, Inc., Gaithersburg, MD) with 2,2’-azino-di-[3-ethylbenzthiazoline sulfonate] as the enzyme substrate. Color development was determined with a THERMOMax microplate reader at 405 nm (Molecular Devices, Menlo Park, CA). All affinity-purified IgG preparations retained strong anti-peptide activities, even at 10,000-fold dilution. Moreover, both N-IgG and C-IgG (when bound to Protein A-Sepharose) adsorbed VFP efficiently from solution, as determined with the Miles vessel permeability assay (T. Sioussat et al., manuscript in preparation).

Eu³⁺-labeling of N-IgG. We performed Eu³⁺-labeling of the affinity-purified N-IgG according to the DELFIA kit protocol with some modifications and pooled and concentrated the affinity-purified antisera to ~0.5 g/L, using an Amicon Macrosolute concentrator. The PD-10 column was pre-equilibrated with 40 mL of labeling buffer, and 2 mL of the antisera (0.5 g/L) was loaded on the column. We rinsed the column with labeling buffer, collected 1.0-mL fractions, and measured the absorbance at 280 nm with a Model U-2000 spectrophotometer (Hitachi Instruments Inc., Danbury, CT 06810). Fractions corresponding to peak absorbance were pooled, and concentrated to ~1 mL, which typically contained 1.5 g/L IgG concentration (we used an absorptivity value of 1.34 for 1 g/L of IgG solution to calculate IgG concentration). We added 1.0 mL of the IgG solution to 0.2 mg of labeling reagent (containing the Eu³⁺-chelate) and mixed it gently on a rotator for 16 h at room temperature.

Purification of Eu³⁺-labeled IgG. Sepharose CL-6B was poured into a 1.5 × 30 cm column to a height of 18 cm. Next, we added pre-swollen Sephadex G-50 to a height of 28 cm and equilibrated the column with 180 mL of elution buffer. The Eu³⁺-labeled IgG reaction mixture was added and fractionated on this column. We rinsed the column with elution buffer, collected 60 1-mL fractions, and measured their absorbance at 280 nm. We diluted a small aliquot of each fraction 10 000-fold with enhancement solution and determined the fluorescence with a 1232 DELFIA time-resolved fluorometer (Pharmacia Diagnostics, Fairfield, NJ), in which a pulsed xenon flash at 340 nm and electronic gating were used to detect fluorescence at 613 nm between 400 and 800 μs after the excitation flash.

Characterization of Eu³⁺-labeled N-IgG. Fractions corresponding to peak IgG absorbance (280 nm) and fluorescence were pooled (usually, fractions 25 to 33) and the resulting absorbance (280 nm) and fluorescence (10 000-fold dilution) were determined. The yield of Eu³⁺/IgG was calculated as described in the DELFIA kit protocol (typically, 10 Eu³⁺/IgG). To increase the stability of the Eu³⁺-labeled N-IgG, purified BSA was added to a final concentration of 1.0 g/L.

Coating of microtiter strips. We added 50 μL of a 50-fold dilution of C-IgG (stock concentration of 0.64 g/L in phosphate-buffered saline) to each well of the microtiter strips, and incubated the plate overnight at 4 °C on a shaker. Thereafter, we washed the wells six times with DELFIA wash buffer, and blocked by incubation with 30 g/L hemoglobin solution at 20 °C for 2 h with gentle shaking. Plates were washed six times with DELFIA wash buffer before use.

Line 10 cell cultures. Line 10 tumor cells from guinea pig were grown as suspension cultures in serum-free defined medium HL-1 as described previously (17). Conditioned line 10 medium, which contains large amounts of VFP, was centrifuged and frozen at ~70 °C to serve as calibrators.

Immunoassay procedure. We used freshly coated microtiter strips on the same day to assay VFP. We added 50 μL of various dilutions of line 10 conditioned media (using HL-1 medium as the diluent) to each well and incubated at 20 °C for 2 h with gentle shaking. After six washes with wash buffer, we added 50 μL of Eu³⁺-labeled N-IgG (diluted appropriately in assay buffer), incubated for another 2 h at 20 °C, and again washed six times. Finally, we dispensed 200 μL of enhancement solution into each well, and after 5 min of gentle shaking, read the absorbance of the plate in the 1232 DELFIA fluorometer.

Miles vessel permeability assay. We assayed the bio-
activity of VPF in the line 10 culture medium preparations, using the Miles assay as described previously (1, 17).

Results

Preparation and purification of Eu³⁺-labeled N-IgG. Eu³⁺ labeling of affinity-purified antibodies directed against the N-terminal region of VPF was performed as described in Materials and Methods. The Sepharose 6B/Sephadex G-50 chromatographic profile in Figure 1 shows two distinct peaks. The first peak (I) corresponds to Eu³⁺-labeled N-IgG, and the second peak (II) represents unreacted Eu³⁺-chelate. For this reason, we showed in a separate experiment that ~90% of the fluorescence associated with peak I could be removed by an IgG-removing device (Gammagone), indicating that peak I comprised mainly Eu³⁺-labeled N-IgG. We pooled fractions 25-33, corresponding to Eu³⁺-labeled N-IgG, and determined the corrected protein concentration to be 115 mg/L (using the absorbivity value of 1.34 mentioned above, with corrections for absorbance of the thiourea bonds of ~0.008 A per µmol/L). We calculated the specific activity of the Eu³⁺-labeled N-IgG to be ~10 Eu³⁺/IgG, using a 1 nmol/L Eu³⁺ standard as described in the DELFIA kit protocol.

Optimization of VPF immunoassay. To determine the optimal dilution of Eu³⁺-N-IgG, we studied the effect of various amounts of N-IgG on the VPF binding curve. Microtiter plate wells were immobilized with a constant amount (225 ng/well) of C-IgG. Because pure VPF is not available, we used line 10 tumor cell conditioned medium, which is rich in VPF (17), to standardize the assay. We used the same lot of line 10 conditioned medium in all experiments. The concentration of VPF was expressed in arbitrary units, i.e., 100 units is defined as the amount of VPF in our batch of undiluted line 10 tumor cell conditioned medium. We arbitrarily defined “signal” as the fluorescence obtained with undiluted line 10 conditioned medium, and “noise” as the nonspecific fluorescence associated with HL-1 medium (0 unit). Thus, the signal-to-noise ratio is defined as fluorescence100 units/fluorescence0 unit. The effect of varying the N-IgG dilution (from five- to 50-fold) is shown in Figure 2A. We determined that 50-fold diluted N-IgG gave a maximal signal-to-noise ratio of 83 (Figure 2B).

In a separate experiment, we studied the effect of varying the C-IgG dilution, keeping Eu³⁺-N-IgG constant at 115 ng/well. As shown in Figure 3, we obtained a maximal signal-to-noise ratio of 89 with 30-fold diluted C-IgG (1000 ng/well). However, because of our limited supply of C-IgG, we decided to use a 50-fold dilution of C-IgG (640 ng/well) to coat the microtiter wells; at this concentration, the signal-to-noise ratio was close to maximal at 80. For all subsequent experiments, we coated microtiter plate wells with 50-fold dilution of C-IgG, and bound VPF was detected with 50-fold dilution of Eu³⁺-N-IgG.

Sensitivity and intra-assay CV of VPF immunoassay. To assess the analytical sensitivity of the VPF assay, we prepared line 10 conditioned medium corresponding to 0.25 unit, 0.50 unit, and 1.00 unit by diluting with HL-1 medium, then assayed these 10 times. HL-1 medium devoid of VPF served as the zero standard. The sensitivity or minimal detectable dose (defined as +2 SD above the zero standard), determined by extrapolation from the standard curve, was ~0.35 unit (Figure 4A). The intra-assay CV was <20% at 0.50 unit (Figure 4B).

Specificity of VPF immunoassay. Because the format of this assay depended on C-IgG as the capture antibody and Eu³⁺-N-IgG as the detector antibody, we used peptides corresponding to the N- and C-termini of VPF to demonstrate the assay specificity. As shown in Figure 5, inclusion of C-VPF, N-VPF, or both peptides in the assay inhibited the binding of VPF in line 10 medium by ~80%. In addition, when VPF was selectively removed from line 10 conditioned medium (by unlabeled N-IgG followed by incubation with Protein A–Sepharose and...
centrifugation), little fluorescent signal remained in the supernatant solution. In addition, when guinea pig serum containing platelet-derived growth factor and other growth factors was assayed, no VPF was detected (data not shown).

Correlation of VPF immunoassay with Miles permeability assay. We prepared and tested various concentrations of VPF from line 10 medium in both the Miles permeability assay and the VPF immunofluorometric assay. For the Miles assay, the amount of local dye development due to VPF permeability-enhancing activity was quantified by absorbance at 620 nm as described earlier (17). The VPF immunofluorometric assay was more sensitive than the Miles permeability assay; at a dose of 0.35 unit of VPF, the immunoassay gave values that were markedly different from zero (Figure 4A). In contrast, the sensitivity of the Miles permeability assay extended to only ~10 units (Figure 6). There was an excellent linear correlation ($r^2 = 0.94$) between the Miles permeability assay and the VPF immunofluorescence at VPF concentrations >10 units (Figure 6, inset).

Discussion

Immunofluorometric assays involving Eu$^{3+}$-chelate as the label are characterized by low-end sensitivity and wide dynamic range. The principles underlying these advantages have been reviewed recently (18). The large Stokes shift of Eu$^{3+}$-chelate and the time-resolved nature of the fluorometric measurements yield a high signal-to-noise ratio. Because of the longer decay time of the Eu$^{3+}$-chelate (>500 μs), readings can be determined between 400 and 800 μs to decrease nonspecific fluorescence, which typically has shorter decay times (~0.01 μs). This feature minimizes the endogenous nonspecific fluorescence of various biological specimens, making it especially attractive as a method to measure VPF in both tumor cell culture medium and in biological fluids.

We prepared affinity-purified N- and C-termini antibodies to guinea pig VPF and used the DELFIA approach
to develop a sensitive and specific immunofluorometric assay of VPF. We also demonstrated that our fluoroimmunoassay provides a quantitative measure of VPF in line 10 tumor cell culture medium. Although absolutely pure VPF is unavailable for standardization, we showed good correlation between the VPF immunoassay and the Miles bioassay with line 10 medium, thus confirming that the immunoassay is measuring bioactive VPF. We are confident that the immunoassay is measuring VPF in the physiologically relevant range because the dynamic range correlates well with the dynamic range of the Miles bioassay.

The VPF immunofluorometric assay has a minimal detection limit of 0.35 unit, and is ~30 times more sensitive than the Miles permeability assay. In addition, the immunoassay is much more precise and simpler to perform, is readily automatable, and can measure several specimens rapidly and inexpensively. In a separate study, we used the VPF immunooassay to measure VPF in ascites fluid, serum, and urine to study the potential role of VPF in tumor-elicited ascites fluid accumulation (19). We have also currently adapted the VPF immunofluorometric assay to measure VPF in human fluids to study its potential diagnostic utility in the pathogenesis of tumor metastases and the often accompanying fluid accumulation found in pleural and peritoneal cavities (19).

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References