Vascular Endothelial Growth Factor 165 (VEGF 165) Activities Are Inhibited by Carboxymethyl Benzylamide Dextran That Competes for Heparin Binding to VEGF 165 and VEGF 165-KDR Complexes*

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We have previously shown that carboxymethyl dextran benzylamide (CMD7), a heparin-like molecule, inhibits the growth of tumors xenografted in nude mice, angiogenesis, and metastasis by altering the binding of angiogenic growth factors, including platelet-derived growth factor, transforming growth factor β, and fibroblast growth factor 2, to their specific receptors. In this study, we explore the effect of CMD7 on the most specific angiogenic growth factor, vascular endothelial growth factor 165 (VEGF 165). We demonstrate here that CMD7 inhibits the mitogenic effect of VEGF 165 on human umbilical vein endothelial cells (HUV-ECs) by preventing the VEGF 165-induced VEGF receptor-2 (KDR) autophosphorylation and consequently a specific intracellular signaling. In competition experiments, the binding of 125I-VEGF 165 to HUV-ECs is inhibited by CMD7 with an IC 50 of 2 μM. Accordingly, CMD7 inhibits the cross-linking of 125I-VEGF 165 to the surface of HUV-ECs, causing the disappearance of both labeled complexes, 170–180 and 240–250 kDa. We show that CMD7 increases the electrophoretic mobility of VEGF 165, thus evidencing formation of a stable complex with this factor. Moreover, CMD7 reduces the 125I-VEGF 165 binding to coated heparin-albumin and prevents a heparin-induced increase in iodinated VEGF 165 binding to soluble 125I-KDR-Fc chimera. Concerning KDR, CMD7 has no effect on 125I-KDR-Fc electrophoretic migration and does not affect labeled KDR-Fc binding to coated heparin-albumin. In the presence of VEGF 165, 125I-KDR-Fc binding to heparin is enhanced, and under these conditions, CMD7 interferes with KDR binding. These data indicate that CMD7 effectively inhibits the VEGF 165 activities by interfering with heparin binding to VEGF 165 and VEGF 165-KDR complexes but not by direct interactions with KDR.

Neovascularization is essential for tumor growth and is regulated by tumor cell-produced factors that have mitogenic and chemotactic effects on vascular endothelial cells (1, 2). Several studies have recently indicated that vascular endothelial growth factor (VEGF-A) expression in tumor cells may play a major role in tumor angiogenesis (3–6). VEGF-A was observed in vivo to act as a potent angiogenic factor and blood vessel permeabilizing agent (7, 8).

VEGF-A is an homodimeric glycoprotein, the monomer of which exists in five forms, VEGF 121, VEGF 145, VEGF 165, VEGF 189, and VEGF 206, as a result of alternative splicing from a single gene (9). These various isoforms of VEGF-A differ in their affinity for heparin and extracellular matrix components. VEGF-A forms induce endothelial cell proliferation and migration and are essential for embryonic vessel development (10). The best characterized VEGF-A is the heparin-binding 165-amino acid-long form, VEGF 165 (11, 12). The binding of VEGF 165 to its receptors requires cell surface heparan sulfates and can be modulated by the addition of exogenous heparin (13).

Two tyrosine kinase receptors have been identified as VEGF-A receptors, the VEGFR-1 (fms-like tyrosine kinase, Flt-1) (14) and the VEGFR-2 (kinase domain region (KDR) in human, and the homologous fetal liver kinase-1 (Flk-1) in mouse) (15). These transmembrane proteins with apparent molecular masses of about 200 and 220 kDa, respectively, have been shown to bind VEGF-A with high affinity. Both KDR/Flk-1 and Flt-1 contain seven immunoglobulin-like domains in the extracellular regions and large insert sequences in their intracellular kinase-domains (16). Recently, an additional binding site, neuropilin-1 (NRPI), with a molecular mass of about 130 kDa, was identified and shown to be expressed on the surface of endothelial and tumor cells. NRPI can act as a co-receptor, forming a complex with VEGFR-2 and thus enhancing VEGF 165-induced activities mediated by VEGFR-2 (17).

Several molecules with anti-angiogenic activity have been described in the last decade (18, 19). The mechanisms involved in action of most of these molecules are unclear, but several of these angiogenesis inhibitors were found to be either heparin analogs or heparin-binding substances (20, 21). These observations and recent results showing that heparin-degrading enzymes can inhibit the tumor angiogenesis (22) suggest that heparan sulfates may play an important regulatory role in the angiogenic processes.

Carboxymethyl dextran benzylamide (CMD7) is a noncytotoxic substituted dextran. Its chemical derivatization involves statistical distribution of chemical groups linked to the 1–6 glucosyl units forming the macromolecular chains (23). CMD7 mimics some properties of heparin, such as the interactor factor; FGF, fibroblast growth factor; HUV-EC, human umbilical vein endothelial cell; PBS, phosphate-buffered saline; FCS, fetal calf serum.
actions with angiogenic growth factors, including FGF2 (24, 25), transforming growth factor β, and platelet-derived growth factor (26), but it has no anticoagulant or anticomplement effects (27, 28). We have shown that CMDB7 specifically inhibits the mitogenic effect and receptor binding of FGF2, platelet-derived growth factor, and transforming growth factor β, and thus prevents the endothelial cell proliferation and migration as observed in vitro (25). In addition, CMDB7 inhibits in vitro the growth of breast tumor cells (24–26). In vivo studies demonstrated that breast HH9 (25) and MCF-7ras (26) xenograft growth in nude mice is blocked by CMDB7 treatment. In parallel, we observed the decrease of angiogenesis within these tumors. Furthermore, CMDB7 is able to inhibit by 88% the incidence of lung micrometastasis from breast carcinoma MDA-MB435 cell implants in the mammary pad of nude mice (29).

In the present study, we evaluated for the first time, in our knowledge, the possible effects of CMDB7 on activities of the most specific angiogenic growth factor, VEGF165. We show that CMDB7 inhibits (a) mitogenic activity of VEGF165 on endothelial cells, (b) VEGF165 binding to its specific receptors, and (c) VEGF165 signaling by KDR in human umbilical vein endothelial cells. We further demonstrate the CMDB7 ability to bind to VEGF165 and to compete for heparin binding to VEGF165 and VEGF165-KDR complexes.

**EXPERIMENTAL PROCEDURES**

**Dextran Derivative Preparation**—A water-soluble dextran derivative (CMDB7) was prepared as previously described (25). Its chemical composition, determined by acidimetric titration and elementary analysis of nitrogen, is 0% dextran, 70% carboxymethyl and 30% benzylamide. Average molecular weight was estimated as 80,000 g/mol.

**Cell Line and Cell Culture**—Human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (Rockville, MD). HUVECs were routinely grown in M199 (Life Technologies, Inc.) and were cultured at 37 °C in a 5% CO2-humidified atmosphere. Culture medium is supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 units/ml penicillin, and 50 μg/ml streptomycin (all obtained from Life Technologies, Inc.).

**Cell Proliferation Assay**—HUVECs were seeded at a density of 2 × 104 cells per 10-cm-diameter tissue culture flask (Falcon, Becton Dickinson, Franklin Lakes, NJ) in M199–10% FCS. After 24 h, the cells were washed three times with PBS buffer and then incubated for another 24 h and the proliferation of cells was measured by cell counting using a Coulter counter (Coultronics, Marseille, France). All experiments were performed in triplicate and data illustrate the mean cell numbers ± S.E. provided from one representative of three independent experiments.

**Western Blot Analysis and Anti-phosphotyrosine Assay**—5 × 105 HUVECs were plated into six-well tissue culture plates (Falcon) in M199–10% FCS. After 24 h, the cells were washed with PBS and then incubated in serum-free medium containing 0.01% bovine serum albumin. After an overnight incubation at 37 °C, the cells were washed with PBS and then incubated in serum-free medium containing 0.01% bovine serum albumin and 0.1 mM sodium orthovanadate for 15 min at 37 °C. The media were then removed, and the cells were incubated for 5 min at 37 °C with the serum-deprived medium containing 1.2 mM VEGF165 in the presence or absence of 40 μM CMDB7. The incubations were terminated by aspiration of the medium, two washings with cold PBS containing 1 mM sodium orthovanadate, followed by the addition of 200 μl of cold lysis buffer (20 mM Tris-HCl (pH 7.5), 1% Igepal CA-630, 10% (v/v) glycerol, 100 mM NaCl, 5 mM EDTA, 50 mM sodium fluoride, 1/10 (v/v) protease inhibitor mixture (Sigma), 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate) at 4 °C for 15 min. Insoluble material was removed by centrifugation at 4 °C for 15 min at 14,000 × g and the protein concentrations of soluble fraction were determined with the BCA protein assay kit (Sigma). Cellular proteins were resolved by SDS-polyacrylamide gel electrophoresis (6%) and transferred to nitrocellulose membranes (R&D Systems). Immunoblots were probed with the following antibodies: an anti-phosphotyrosine mouse monoclonal antibody PY99 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (1:1000 dilution), and an anti-KDR rabbit polyclonal (Sigma) (1:500 dilution) for 1 h and then washed in Tris-buffered saline containing 0.05% Tween-20. Antigen-antibody complexes were revealed with horseradish peroxidase-coupled secondary antibodies and the enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech). Membrane VEGF165 Binding to HUVECs—For displacement binding assays, HUVECs were grown to 80% confluence in 24-well tissue culture plates (Falcon). After an overnight incubation in serum-free medium and two washings with ice-cold binding buffer (PBS/0.2% gelatin), the cells were incubated at 4 °C for 2 h in 0.3 ml of binding buffer containing 7 pm 125I-VEGF165 (Amersham Pharmacia Biotech) in the presence or absence of CMDB7 at various concentrations. Incubation was terminated by gently removing the medium and washing the cell monolayer three times with ice-cold binding buffer. The bound radioactivity was measured using a γ-counter (LKB 1261 Multigamma) after cell lysis in 0.3 ml of 0.5 N NaOH for 30 min. Nonspecific binding was determined in the presence of an excess (5 nm) of unlabeled VEGF165 (R&D Systems). For the Scatchard analysis, the binding was accomplished using increasing concentrations (0–5000 pm) of unlabeled VEGF165 (R&D Systems) and 7 pm 125I-VEGF165 (Amersham Pharmacia Biotech) at 4 °C for 2 h. Parallel experiments were performed in the presence of 10 and 40 μM CMDB7. Each curve was analyzed according to the Scatchard procedure or by fitting to a logistic curve. All experiments were carried out in triplicate unless otherwise stated.

**Affinity Cross-linking of VEGF165**—Subconfluent cell cultures in six-well plates (Falcon) were washed twice with binding buffer (PBS/0.2% gelatin) and then incubated for 2 h at 4 °C with 100 pm 125I-VEGF165 (Amersham Pharmacia Biotech) in the presence of a 1000-fold excess of unlabeled VEGF165 (R&D Systems) or 40 μM CMDB7. Cells were then washed with ice-cold binding buffer and cross-linked for 15 min at room temperature with 0.5 mM disuccinimidyl suberate (Pierce/Perbio Science, France, Bezons, France). The reaction was quenched by addition of an excess of 1 M Tris-HCl (pH 7.5) for 2 min. Cells were lysed with ice-cold lysis buffer (10 mM Tris-HCl, pH 7.0, 1 mM EDTA, 1% Nonidet P-40 (v/v), 1/10 (v/v) protease inhibitor mixture (Sigma)) for 10 min. Cell extracts were then clarified by centrifugation, and supernatants mixed with 2% sample buffer (100 mM Tris-HCl, 4% SDS, 10% glycerol, 0.05% bromphenol blue) were boiled for 3 min before loading on a SDS-8% polyacrylamide gel. After sample running, gels were dried and exposed to X-ray films (20 days) to a Kodak X-Omat film at −80 °C for autoradiography.

**Agarose Gel Electrophoresis**—The CMDB7 effects on the electrophoretic mobility of 125I-VEGF165 (Amersham Pharmacia Biotech) and 125I-KDR-Fc (labeled in laboratory) were analyzed by nondenaturant agarose gel electrophoresis (LKB, Rockingham, N. H.). HUVECs were grown to 80% confluence in 24-well tissue culture plates (Falcon, Becton Dickinson, Franklin Lakes, NJ) were analyzed in 1% agarose gel at pH 7.0 using running buffer containing 125 mM sodium acetate, 50 mM 3-(N-morpholino)-2-hydroxypropylsulfonic acid, 6% sucrose, and 0.5% (w/v) bromphenol blue. After sample running, gels were dried and exposed to X-ray film. The protein concentrations of soluble fraction were determined using a 125I-VEGF165 or to KDR, an anodic shift in the migration of the tracer should be observed.

**VEGF165 Binding to Soluble KDR Receptors**—The surface of flat-bottomed polystyrene wells (Disposable Immulon 1 Remowarell, Dynatech, Cambridge, MA) were coated overnight at 4 °C with 200 μg/ml anti-Fc IgG (Sigma) in PBS buffer. The nonspecific interactions were saturated with PBS containing 0.2% gelatin and 0.1% Tween-20 (PGT buffer) for an additional overnight at 4 °C. Blocking buffer was then removed, and the plates were washed three times with 300 μl of PGT buffer. Then, 2 μg/ml purified recombinant human KDR-Fc chimera (R&D Systems), 40 μg 125I-VEGF165 (Amersham Pharmacia Biotech), and unlabeled VEGF165 (R&D Systems) at increasing concentrations (0–2500 pm) were added successively to a final volume of 200 μl in PGT buffer. The incubations were performed in the presence or absence of CMDB7 or heparin (Sigma). After an overnight incubation at 4 °C, wells were washed three times with 300 μl of PGT buffer, and the radioactivity of each well measured in a γ-counter (LKB 1261 Multigamma). Membrane VEGF165 and KDR Binding to Heparin-Albminum—Heparin-albumin (Sigma) at concentration 17 μg/ml in 100 μl of PBS was coated on the surface of polystyrene wells (Disposable Immulon 1 Remowarell) as described above for anti-Fc IgG. The binding assays to coated heparin-albumin were performed in total volume of 90 μl overnight at 4 °C. We studied the binding of 40 pm 125I-VEGF165 (Amersham Pharmacia Biotech) in the presence or absence of CMDB7 and the binding of 125I-KDR.
CMDB7 is able to inhibit the VEGF165 signal transduction in a dose-dependent manner with an IC50 of 2 μM. The addition of a 200-fold excess of unlabeled VEGF165 prevented the formation of these bands (Fig. 4, lane 2), evidencing their specificity. CMDB7 (40 μM) decreased the intensity of two complexes (Fig. 4, lane 3), demonstrating an inhibition of VEGF165 binding to its specific receptors with apparent molecular masses of 200–210 and 130–140 kDa, calculated by subtraction of the molecular mass of VEGF165. The first protein corresponds to KDR monomer. Like Tao et al. (36), we did not detect the complexes with KDR dimers. This observation can be explained by the inefficiency of cross-linker used in our study to covalently bind KDR dimers.

CMDB7 Binds Directly to VEGF165 and Inhibits Its Binding to Heparin—In order to explore whether CMDB7 is able to interfere directly with VEGF165, we used an affinity-electrophoresis in agarose.

RESULTS

CMDB7 Inhibits the VEGF165 Mitogenic Activity—The inhibitory effect of CMDB7 was evaluated using HUV-ECs. First, we established the optimal VEGF165 concentration at which mitogenic effect on those endothelial cells can be observed. HUV-EC number was increased by VEGF165 at concentrations from 60 to 500 pM, with a maximal 3-fold augmentation observed in the presence or absence of CMDB7 at various concentrations. Cells were washed with PBS, dissociated with 0.05% trypsin-EDTA, and counted using a Coulter counter. The values represent mean of cell numbers ± S.E. (bare) obtained in triplicate in one of three independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

(1.5 × 106cpm/5 ng) in the presence or absence of CMDB7 in combination or not with unlabeled VEGF165 (R&D Systems). After washing, the radioactivity in each well was measured in a γ-counter (LKB 1281 Mulkigamma).

Indication of KDR—Radiiodination was carried out utilizing the chloramine B method. Briefly, 5 μg of carrier-free human recombinant KDR-Fc chimera (R&D Systems) was suspended in 45 μl of 10× PBS-0.1% Triton X-100. To the reaction tube, 0.5 μCi of Na125I (Amersham Pharmacia Biotech) was added, followed by one IODO-BEAD (Pierce Ferbio Science). Incubation was performed for 12 min and then stopped by addition of 20 μl of 2 mg/ml KI, followed by 420 μl of PBS-0.05% bovine serum albumin. The mixture was transferred to prequillibrated PD-10 column (Amersham Pharmacia Biotech) for separation from free iodine. The specific activity of iodinated KDR was 3 × 104cpm/μg. The radiolabeled KDR was used to perform a KDR binding to heparin-albumin and affinity electrophoresis in agarose.

CMDB7 inhibits mitogenic effects of VEGF165 on HUV-ECs. A, HUV-ECs were seated in 24-well plates in medium containing 10% FCS. After 24 h, cells were growth-arrested by serum starvation for another 24 h and then incubated for 72 h with VEGF165 at concentrations from 60 to 500 pM. B, HUV-ECs were serum-deprived for 24 h and incubated subsequently for 72 h with 250 pM VEGF165 in the presence or absence of CMDB7 at various concentrations. Cells were washed with PBS, dissociated with 0.025% trypsin-EDTA, and counted using a Coulter counter. The values represent mean of cell numbers ± S.E. (bare) obtained in triplicate in one of three independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Dextran Derivative Inhibits VEGF<sub>165</sub> Activity

Fig. 2. Inhibition of VEGF-induced tyrosine phosphorylation of HUV-ECs receptor by CMDB7. Quiescent HUV-ECs untreated (lane 1), treated with 1.2 nM VEGF<sub>165</sub> (lane 2), or 40 μM CMDB7 (lane 3), or 1.2 nM VEGF<sub>165</sub> and 40 μM CMDB7 in combination (lane 4) were lysed, analyzed by SDS-polyacrylamide gel electrophoresis (6%), and transferred to a nitrocellulose filter. A, tyrosine-phosphorylated proteins were detected by probing with an anti-phosphotyrosine antibody. B, Western blot was probed with anti-VEGFR2 (KDR). Bands were visualized with an ECL kit. Molecular sizes of markers (kDa) are shown on the left.

Fig. 3. CMDB7 inhibits VEGF<sub>165</sub> binding to endothelial cells. A, preconfluent HUV-ECs were incubated for 2 h at 4 °C in the presence of 7 pm 125I-VEGF<sub>165</sub> and CMDB7 at indicated concentrations (logarithmic scale). B, for the Scatchard analysis, the cells were incubated with 7 pm VEGF<sub>165</sub> and unlabeled VEGF<sub>165</sub> at increasing concentrations in the absence (control) or presence of 5, 10, and 20 μM CMDB7. Results are presented as the means ± S.E. (bars) of three separate experiments performed in duplicate.

Fig. 4. Inhibition of 125I-VEGF<sub>165</sub> cross-linking to receptor by CMDB7. Subconfluent cultures of HUV-ECs were incubated with 100 pm 125I-VEGF<sub>165</sub> (lane 1) in the presence of a 200-fold excess of unlabeled VEGF<sub>165</sub> (lane 2) or 40 μM CMDB7 (lane 3). After two washes in PBS, a cross-linking was performed using 0.25 mM disuccinimidyl suberate for 15 min at room temperature. Cells were lysed and then cell extracts were analyzed by SDS-polyacrylamide gel electrophoresis (6%). The gels were dried and exposed to autoradiography. The positions of molecular size standards, migrated in a parallel lane, are indicated on the left.

Fig. 5. CMDB7 increases the VEGF<sub>165</sub> electrophoretic mobility. 125I-VEGF<sub>165</sub> (10<sup>5</sup> cpm/3 ng) was mixed with CMDB7 at various concentrations in running buffer and incubated for 2 h at 4 °C. Lane 1, no CMDB7; lanes 2-5, 1, 2.5, 5, and 10 μM CMDB7, respectively. Electrophoresis in a 1% agarose gel was performed under nondenaturing and nonreducing conditions in neutral running buffer. The gel was dried and then exposed to autoradiography. The arrow indicates the direction of migration in the gel toward the anode.

Dextran derivative technique (ACE). Fig. 5, lane 1, shows that 125I-VEGF<sub>165</sub>, being weakly cationic under nondenaturing conditions, remains close to loading well. The presence of negatively charged CMDB7 increases the migration of labeled growth factor evidencing a formation of 125I-VEGF<sub>165</sub>-CMDB7 complex. This shift toward the anode is already visible in the presence of 1 μM CMDB7 (Fig. 5, lane 2), and migration distances increase with enhanced CMDB7 concentrations (Fig. 5, lanes 3–5). These results demonstrate that CMDB7 binds directly to VEGF<sub>165</sub>.

To test whether CMDB7 could alter the VEGF<sub>165</sub> binding to heparin, we have measured the binding of 125I-VEGF<sub>165</sub> to coated heparin-albumin in the presence of CMDB7 at various concentrations (Fig. 6). Specific 125I-VEGF<sub>165</sub> binding to hepa-
rin-albumin was inhibited by heparin with an IC_{50} of 5 \mu g/ml (data not shown) and in the presence of 10 \mu M CMDB7 by 44% (Fig. 6). These results show that CMDB7 competes for heparin binding to VEGF_{165}.

**CMDB7 Does Not Bind to KDR and Does Not Compete for Heparin Binding to Receptors**—To study the interactions of CMDB7 with VEGF receptors, we radioiodinated the KDR-Fc chimera. The possible direct interactions of CMDB7 and KDR were explored using an affinity-electrophoretic technique (Fig. 7). 125I-KDR-Fc remains close to the loading well (Fig. 7, lane 1), and its migration does not change in the presence of CMDB7 at concentrations 2.5 to 20 \mu M (lanes 2–5), indicating the absence of CMDB7-KDR complexes. To study the effect of CMDB7 on heparin binding to KDR, we have used coated heparin-albumin (Fig. 8). The binding of 125I-KDR to heparin was not affected by 0.1, 1, or 10 \mu M CMDB7.

**CMDB7 Competes to Heparin Binding to VEGF_{165}-KDR Complexes**—Finally, we studied the CMDB7 effects on formation of VEGF_{165}-KDR-heparin complexes. Fig. 9A shows that 125I-KDR-Fc binding to the coated heparin-albumin is enhanced by VEGF_{165} in a dose-dependent manner. This indicates that under our experimental conditions, we can reproduce, at least in part, the formation of triple complexes. Although CMDB7 has no effect on KDR-heparin interactions (Fig. 8), it does efficiently inhibit the 125I-KDR binding to heparin in the presence of 3 nM VEGF_{165} (Fig. 9B). This inhibitory effect is dose-dependent and significantly measured at CMDB7 concentration of 0.1 \mu M. Further support for CMDB7 action on VEGF_{165}-KDR-heparin complexes was supplied by results of another experimental design. This time, the KDR-Fcs were coated and the binding of 125I-VEGF_{165} was measured in the presence or absence of 0.1, 1.0, and 10 \mu M CMDB7.

**DISCUSSION**

The role of angiogenesis in tumor progression and invasiveness is well documented now (38–40), and anti-angiogenesis is becoming a promising new therapeutic approach for the treatment of cancer (41). As the high expression of VEGF and its receptors have been closely correlated to tumor vascularity, progression, and metastasis (42, 43), targeting of VEGF/VEGF receptors is an excellent anti-angiogenic strategy. Different anti-VEGF strategies have been used with success, but direct targeting of the heparin binding sites on VEGF and/or KDR by a heparin analog have never been proposed. In this study, we demonstrated that the dextran derivative CMDB7 is capable of neutralizing the VEGF_{165} activities by direct interactions with VEGF_{165} and by heparin displacement from VEGF_{165} and VEGF_{165}-KDR complexes.

We have shown here that CMDB7 efficiently inhibited the VEGF_{165}-induced proliferation of endothelial cells in a dose-dependent manner. Furthermore, CMDB7 inhibited the formation of 125I-VEGF_{165}-receptor complexes, namely, 240–250- and 170–180-kDa complexes containing KDR and NRP1 receptors, respectively. This is in agreement with the findings of Soker et al. (34), who reported that VEGF binds to endothelial cells via these two receptors. In addition, by studying a receptor autophosphorylation, we demonstrated that CMDB7 suppressed the VEGF_{165} signal transduction in endothelial cells. These findings are in agreement with our previous results showing that CMDB7 inhibited in vitro the migration and proliferation of endothelial cells and inhibited in vivo the tumor angiogenesis (24–26).

Concerning the mechanism of action, we observed that CMDB7 displaced the VEGF_{165} from its specific binding sites on HUV-ECs. It is noteworthy that CMDB7 was originally designed as a heparin analog and might therefore behave as an antagonist or partial agonist. Heparin can interfere with VEGF_{165} action either by binding to a specific domain on
growth factor β, FGF2, and FGF4, by binding them and thus altering the conformation of ligand-receptor complexes (24–26). Consequently, we did not observe any effect of CMD7 on heparin-binding growth factors, including EGF and IGF1 (24–26).

Because of structural similarity to heparin, CMD7 could bind to VEGF165 requiring the heparin binding sites. Our results support this hypothesis as we demonstrate the dose-displacement of VEGF165 from heparin-albumin by CMD7 (Fig. 6). This suggests that CMD7 and heparin can compete for the same site on VEGF165. But the fact that CMD7 inhibits the VEGF165 binding to heparin-albumin with IC₅₀ higher than heparin doses, 10 μM versus 0.3–1.7 μM, suggests that the affinity of this site is weaker for CMD7 than for heparin.

In contrast to VEGF165, KDR does not directly interact with CMD7. Indeed, we showed that CMD7 did not change the electrophoretic migration of KDR-Fc (Fig. 7), suggesting that the CMD7/KDR complexes are not formed. One can think that CMD7 negative charge is not enough strong to move a molecule as big and heavy as KDR-Fc, which is characterized by a molecular mass of 380 kDa. Our hypothesis that CMD7 does not act directly on KDR is supported by the fact that CMD7 does not displace the radiolabeled KDR from heparin-albumin (Fig. 8). The binding of heparin to KDR is well established now (45, 46). However, in the light of our observations, it seems that heparin-binding site on KDR differs from that on VEGF165 because CMD7 is able to interact with the growth factor but not with the receptor.

CMD7 binding to VEGF165 could change the growth factor conformation in a different manner than heparin does, as we previously showed for FGF2 (47). This could perturb the VEGF165 interactions with KDRs. Indeed, we observed that CMD7 inhibits the formation of VEGF165/KDR complexes on heparin-albumin (Fig. 9B). Interestingly, when the ligand-receptor complexes are formed without heparin, as in the case of VEGF165 binding to coated KDR-Fcs, CMD7 has no effect (Fig. 10). This argues for the hypothesis that the mechanism of CMD7 action involves the displacement of heparin from VEGF165 and VEGF165/KDR complexes.

In conclusion, CMD7 prevents the binding of VEGF165 to its cell surface receptors, inducing an inhibition of the receptor phosphorylation and consequently the endothelial cell proliferation arrest. It acts by displacing heparin from VEGF165 and VEGF165 receptor complexes. Being completely nontoxic and very efficient in tumor animal models, this dextran derivative could be used to increase the efficiency of conventional anticancer treatments.

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Vascular Endothelial Growth Factor 165 (VEGF$_{165}$) Activities Are Inhibited by Carboxymethyl Benzylamide Dextran That Competes for Heparin Binding to VEGF$_{165}$ and VEGF$_{165}$-KDR Complexes

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