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Systemic Activation of the Vascular Endothelial Growth Factor Receptor KDR/flk-1 Selectively Triggers Endothelial Cells with an Angiogenic Phenotype

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The hypothesis that tumor growth is angiogenesis dependent has been documented by a considerable body of direct and indirect experimental data. A prerequisite for the development of novel anti-angiogenic agents is the design of drugs that would be active only on those endothelial cells with an angiogenic phenotype. We took advantage of the anti-idiotypic strategy to obtain circulating agonists specific for the vascular endothelial growth factor receptor KDR/flk-1 (J-IgG). They induced in the absence of VEGF cell proliferation *in vitro* and angiogenesis in the corneal pocket assay either through local or systemic delivery. Intraperitoneal injections of J-IgG in nude mice grafted with a prostatic adenocarcinoma led to tumor enlargement associated with an increase in both tumor vascularization and proliferation. In contrast KDR/flk-1 overstimulation had no detectable effect on normal tissues. These data underline that KDR/flk-1 is a functional marker of the angiogenic phenotype of endothelial cells. *(Am J Pathol 1997, 151:1215–1224)*

Sprouting of new capillaries from pre-existing vessels, or angiogenesis, occurs in several physiological or pathological conditions, such as tumor progression, diabetic retinopathy, and rheumatoid arthritis.[1] This local hypervascularization is thought to result from release by the tissues of growth factors interacting with their receptors on endothelial cells, which in turn migrate, proliferate, and differentiate into new capillaries. Vascular endothelial growth factor (VEGF) is a growth factor, the mitogenic action of which is restricted *in vitro* to vascular endothelial cells,[2–4] T lymphocytes,[5–7] and retinal pigment epithelial cells.[8] *In vivo* VEGF is a potent inducer of angiogenesis[3] and vascular permeability.[4] Two membrane-spanning tyrosine kinases have so far been identified as VEGF receptors: flt-1[9] and KDR[10] or its murine homologue flk-1.[11,12] The crucial role of the VEGF system in vasculogenesis and embryonic angiogenesis has been explicitly demonstrated by the observation that targeted inactivation of any of the genes coding for VEGF,[13,14] flt-1,[15] or flk-1[16] was lethal. However, knock-out of the flt-1 or flk-1 genes led to different phenotypes, thus suggesting distinct roles for these receptors.

Several studies have shown that VEGF plays a major role in pathological neovascularization. VEGF is, for instance, among eight angiogenic factors studied, the only one constantly expressed in fibrovascular membranes from diabetic patients,[17] and it is overexpressed in many pathological states. Once expressed by tumor cells it is released and accumulates in surrounding endothelial cells, suggesting that VEGF bioavailability is restricted to the vicinity of the location of its synthesis.[18] Several studies have demonstrated that the overexpression of VEGF and its receptors is associated with tumor angiogenesis (reviewed in Ref. 19). The immunoneutralization of VEGF[20] or the expression of dominant negative flk-1 receptors[21,22] have demonstrated that tumor progression is linked to VEGF up-regulation and VEGF receptors activation.

Nevertheless, the expression of VEGF receptors in adult endothelial cells that do not proliferate[23] remains puzzling and raises serious questions about the function of their translation products in quiescent endothelial cells. However, a prerequisite to demonstrate that a growth factor activates *in vivo* a receptor is the obtainment of circulating agonists specific for this receptor. For instance, VEGF binds to proteoheparan sulfates of the vascular wall, and thus intravenous injections of VEGF would unlikely trigger tumor endothelial cells. To investigate the potential functional expression of flk-1 in quies-

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cent and proliferating endothelial cells we designed a specific circulating probe for flk-1 by eliciting anti-idiotypic antibodies of VEGF. This strategy[24] has been a powerful clue as to demonstrate the mechanisms of action of insulin[25] or neurotransmitter[26] receptors. We thus demonstrate that long-lasting activation of flk-1 does not affect quiescent endothelial cells but stimulates tumor angiogenesis and corneal angiogenesis even in the absence of VEGF.

Materials and Methods

Materials

Tissue culture trays and media were from Poly Labo Paul Block & Cie (Strasbourg, France). Chromatography media were from Pharmacia (Uppsala, Sweden) and chemicals from Sigma (Poole, UK). Anti-phosphotyrosine monoclonal (PY 69) IgG, rabbit IgG, anti-flk-1, and anti-flt-1 were from Santa Cruz Biotechnology (Santa Cruz, CA). VEGF 165 and PlGF131 (Placenta growth factor) were expressed in a baculovirus system.[27] VEGF was iodinated as previously described.[28] pSV7d expression plasmids carrying the coding sequences of flt-1 or flk-1 were generously provided by T. Quinn and L. Williams (University of California at San Francisco, San Francisco, CA).

Anti-Idiotypic Antibodies Preparation

Previously immunized New Zealand rabbit (CRL:KDL (NZW)-BR) IgG was purified on protein A Sepharose, and an affinity column was prepared by coupling the preimmune IgGs to CnBr-activated Sepharose (PI-IgG chromatography) according to the manufacturer's recommendations. Thirty micrograms of VEGF was purified from the mouse ATt20 cell line conditioned medium as described,[3] electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose. The nitrocellulose sheet containing VEGF was dissolved in 0.6 ml of dimethylsulfoxide. Rabbits were then immunized by 3-monthly subcutaneous injections of 2 ml of VEGF emulsified in complete Freund's adjuvant.[1–1] Two weeks after the last injection, the IgGs was purified on protein A Sepharose (V-IgG). These IgGs inhibited VEGF-dependent adrenal-cortex-derived capillary endothelial (ACE) cell proliferation (IC_{50} = 5 μg/ml) but not their basal proliferation. Ten micrograms of V-IgG emulsified in phosphate-buffered saline (PBS)/complete Freund's adjuvant (1:1) were injected monthly in lymph nodes from other rabbits. Blood collected between 4 and 7 months was purified by affinity chromatography for protein A Sepharose. These IgGs were removed from putative anti-allotypic antibodies by passage through the PI-IgG affinity column and used for systemic delivery experiments. The anti-idiotypic IgGs were then purified by affinity chromatography for the V-IgG conjugated to CN-Br Sepharose, hereafter referred to as J-IgG, and used for all analytical experiments.

Cell Culture and Transfections

ACE cells were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with antibiotics and 10% newborn calf serum.[29] Stock cultures received 1 ng/ml fibroblast growth factor-2 every other day.

COS cells seeded in 24 multiwell dishes were grown in DMEM/10% fetal calf serum until subconfluency and transferred to serum and antibiotic-free DMEM 24 hours before transfection, and 2 μg/ml pSV7d-flk-1 or pSV7d-flt-1 plasmids were mixed in DMEM containing 10 μg/ml lipofectin (Gibco BRL, Gaithersburg, MD) for 30 minutes and then added to the dishes for 6 hours. The medium was then replaced by fresh DMEM supplemented with 10% fetal calf serum.

Binding Assays, Immunoprecipitations, and Tyrosine Phosphorylations

Forty-eight hours after transfection, pSV7d-flk-1 and pSV7d-flt-1 plasmid-transfected COS cells were rinsed with cold binding buffer (DMEM supplemented with 2 mg/ml gelatin and 20 mmol/L HEPES, pH 7.4) and incubated with 5 ng/ml ^{125}I-labeled VEGF and various dilutions of unlabeled VEGF, J-IgG, or PI-IgG as control IgG. After 3 hours, the cells were washed three times with cold binding buffer and lysed with 0.5 ml of O.2 mol/L NaOH. Solubilized material was counted in a gamma counter. The nonspecific binding at saturating concentrations was less than 20%.

Serum-starved ACE cells (3×10^6) were lysed with RIPA buffer (0.1% SDS, 1% cacodylate, 1 mmol/L EDTA in 10 mmol/L phosphate buffer, pH 7.4, and 5 μg/ml each of aprotinin, pepstatin, and benzamidine). Lysates were clarified by centrifugation and incubated 2 hours with 2 μg/ml polyclonal antibodies against flk-1 or flt-1 (Santa Cruz Biotechnology) or 20 μg/ml J-IgG on ice. Immune complexes were adsorbed to protein A Sepharose beads (Pharmacia) at 4°C for 1 hour, washed three times with lysis buffer, and solubilized in SDS-PAGE sample buffer. Immunoprecipitates were resolved in nonreducing conditions in a 6% polyacrylamide gel, electrophoretically transferred to nitrocellulose and probed with anti-flt-1 or flk-1 antibodies diluted 1/200. Detection of immune complexes was performed by horseradish-peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG using a chemiluminescent substrate (ECL, Amersham, Little Chalfont, UK).

Subconfluent ACE cells (3×10^6) were incubated 5 minutes at 37°C in the absence or the presence of 2 nmol/L VEGF or J-IgG and then lysed with RIPA containing 40 mmol/L sodium pyrophosphate and 1 mmol/L orthovanadate. Lysates were incubated 2 hours with 2 μg of anti-phosphotyrosine PY 22 monoclonal antibody. The immune complexes adsorbed on protein A Sepharose beads at 4°C for 2 hours were then washed five times with lysis buffer and solubilized in SDS-PAGE sample buffer. The samples were then processed as above and the blots revealed with anti-P-Tyr or anti-flk-1 antibodies.

ACE Cell Proliferation and Migration Assays

Proliferation assays were performed on ACE cells seeded at 5000 cells per 12 multiwell plates in DMEM supplemented with 5% newborn calf serum, 2 mmol/L glutamine and antibiotics. Modulators were inoculated every other day and the cells were trypsinized and counted after 5 days in a Coulter counter. Migration assays were performed on confluent ACE cells seeded in 12-well cluster plates. Cell monolayers were growth arrested by exposure to serum-free DMEM for 24 hours and then wounded with a rubber policeman. The dishes were washed with medium to remove the cell debris and each well was photographed at ×100 magnification. Triplicate dishes were then incubated for 16 hours in DMEM in the presence of modulators. A second photograph of each well was taken and the cells that had migrated were counted by superposing the two photographs.

Neovascularization Assay

Slow-releasing implants of hydrogel (2 × 1 mm) were rehydrated with 2 μl of PBS containing 50 μg of bovine serum albumin supplemented or not with 200 ng of VEGF or 1 μg of immunopurified J-IgG or control PI-IgG. These implants were inserted in New Zealand rabbit corneal stroma 2 mm away from the limbus. The neovascularization was assessed on day 12 by direct examination with a slit lamp and scored according to a four-grade scale.[30] The score was the mean of the area neovascularized for each condition. In another set of experiments, 1 × 1 mm slow-releasing implants were inserted in Wistar rat corneal stroma 1 mm away from the limbus. The rats received intravenous injections of 2 mg of protein-A-purified J-IgG or PI-IgG on days 3, 6, and 9, and the neovascularization was assessed on day 12 as described above. The Mann-Whitney test was used for comparison between different treatments. A P value of <0.05 was considered significant.

Tumor Xenograft and Treatments

A prostatic adenocarcinoma corresponding to a Gleason's score IX was serially transplanted in nude mice. The fragments (3 × 3 × 3 × mm) were implanted subcutaneously in mouse flank, and 1 month later, after the onset of tumor enlargement, 8 to 10 mice per group were injected intraperitoneally twice weekly with 200 μl of PBS or protein-A-purified J-IgG, PI-IgG, or V-IgG (1 mg/ml). The largest (L) and lowest (l) dimensions of the tumors were measured twice weekly with a caliper and the volume calculated by the formula $L \times l^2 \times 0.52$. Statistical analysis was monitored by the Mann-Whitney U test.

Histological Examinations

Tissue fragments were fixed in formaldehyde acetic acid ethanol and embedded in paraffin, and sections were stained with hematoxylin and eosin (H&E). Fixed sections were stained with the endothelial cell specific marker *Ulex europaeus* (Dako, Glostrup, Denmark) or anti-flt-1 antiserum (Santa Cruz Biotechnology). Vessel counts was determined in flt-1-stained sections photographed at ×70 magnification by image analysis with Adobe Photoshop software. Cell proliferation was assessed by incubating paraffin-embedded tissue sections with monoclonal Mib-1 antibody (Immunotech, Marseilles, France), which labels only proliferating cells.[31] Positive staining was detected by incubating sequentially with biotin-labeled secondary antibodies and avidin-biotin peroxidase complex and developed under a microscope with diaminobenzidine peroxidase substrate. Sections were washed three times for 5 minutes in PBS at room temperature between incubations. Mib-1-positive cells were counted in each tumor or kidney on 10 fields randomly chosen under a light microscope at a ×400 magnification. Selected tissues, such as frontal and occipital lobes, choroid plexus, eyes, kidneys, and aorta, were observed by electron microscopy. The samples were immediately fixed for 2 hours in 2.5% glutaraldehyde and transferred to 1% osmium tetroxide in 0.1 mol/L phosphate buffer, pH 7.2. The samples were dehydrated in alcohol/acetone and embedded in Epon. Thick sections (1.0 mm) were stained with toluidine blue and examined under light microscopy to select the most interesting areas. Ultrathin sections were cut and mounted on copper grids, stained with uranyl acetate/lead citrate, and observed with an electron microscope (Zeiss EM 10).

Results

Anti-VEGF Idiotypic J-IgG Is a KDR/flk-1 Ligand

Sera collected from rabbits immunized with V-IgG were subjected to protein A chromatography, and 15% of the purified IgGs were able to compete with iodinated VEGF for its binding to ACE cells. One preparation (J-IgG) was loaded on a PI-IgG affinity chromatography column to remove the putative anti-allotypic IgG and further purified by V-IgG affinity chromatography. To determine whether J-IgG recognized actual VEGF receptors, COS cells transiently transfected with expression vectors carrying the flt-1 or the flk-1 coding sequences were incubated with iodinated VEGF in the presence or absence of J-IgG, with VEGF as a positive control and PI-IgG as a negative control. Preliminary experiments showed that untransfected COS cells did not bind iodinated VEGF, but COS cells transfected with pSV7d-flt-1 or pSV7d-flk-1 bound iodinated VEGF with dissociation constants corresponding to 24 and 380 pmol/L, respectively. Unlabeled VEGF more efficiently prevented the binding of iodinated VEGF to flt-1-expressing (Figure 1A) than to flk-1-expressing (Figure 1B) COS cells (IC_{50} values of 90 pmol/L and 2 nmol/L, respectively). In contrast, J-IgG inhibited the binding of iodinated VEGF only to flk-1-expressing cells (IC_{50} = 70 nmol/L). The inability of J-IgG to inhibit the binding of VEGF to COS cells expressing flt-1 ruled out the possibility of a direct association between iodinated VEGF and J-IgG. Furthermore, PI-IgG was unable to

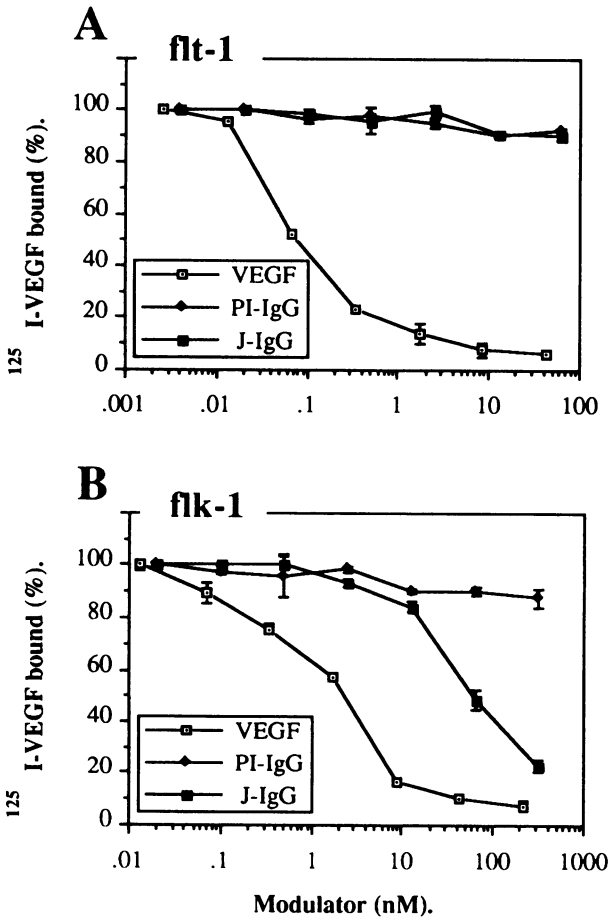


Figure 1. Anti-idiotypic J-IgG binds to COS cells expressing flk-1 (**B**) and not flt-1 (**A**). COS cells were transfected with 2 μg/ml pSV7d-flt-1 (**A**) or pSV7d-flk-1 (**B**). Two days later the cells were incubated with the indicated concentrations of VEGF, J-IgG, or PI-IgG at 4°C and 5 ng/ml iodinated VEGF for 3 hours. Nonspecific binding was measured in the presence of 4 μg of VEGF. Results are expressed as the percentage of specific binding. Binding curves are the results of triplicate assays.

compete with iodinated VEGF binding to flk-1 or flt-1 receptors.

To confirm the direct interaction of J-IgG and KDR/flk-1, ACE cell lysates were immunoprecipitated with anti-flt-1, anti-flk-1, or J-IgG and the immune complexes electrophoresed and transferred to nitrocellulose. Anti-flk-1 revelation showed that anti-flk-1 and J-IgG precipitated a single molecular species migrating at the very same M_r (220,000). Conversely, anti-flt-1 revelation showed that J-IgG did not precipitate the 185-kd molecular species corresponding to the flt-1 receptor (Figure 2). In both cases, PI-IgG did not precipitate any immune complexes.

The blots prepared from ACE cells challenged or not with VEGF or J-IgG were probed with anti-phosphotyrosine IgG. As shown in Figure 3, the intensity of several bands corresponding to 205, 140, and 90 kd was increased by VEGF and to a greater extent by J-IgG exposure. The identity of the 205-kd band with flk-1 was confirmed by reprobing the Western blot with anti-flk-1 IgG.

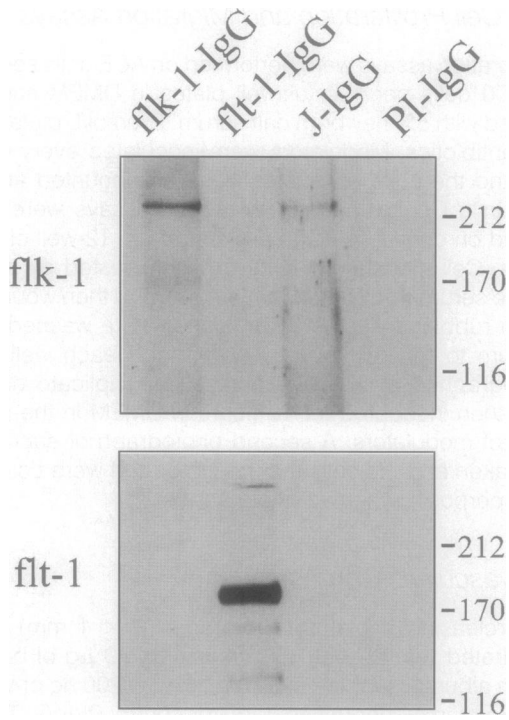


Figure 2. J-IgG immunoprecipitates KDR/flk-1 in ACE cells. ACE cells were lysed in RIPA buffer and immunoprecipitated with 2 μg/ml anti-flt-1 or anti-flk-1 or 20 μg/ml J-IgG or PI-IgG. The immune complexes were collected on protein A Sepharose beads, electrophoresed, transferred to nitrocellulose, and further probed with anti-flk-1 or anti-flt-1.

J-IgG Induces ACE Cell Proliferation but Not Migration

To characterize the biological functions mediated by KDR/flk-1 activation, J-IgG was tested for its ability to

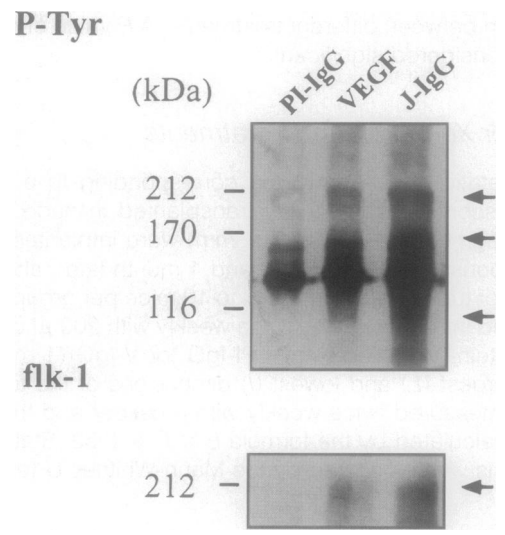


Figure 3. VEGF and J-IgG induce tyrosine phosphorylation of KDR/flk-1 in ACE cells. ACE cells were stimulated or not for 5 minutes at 37°C with 2 nmol/L VEGF or J-IgG, lysed in RIPA buffer, and immunoprecipitated with anti-phosphotyrosine monoclonal IgG. The immune complexes were collected on protein A Sepharose beads, electrophoresed, transferred to nitrocellulose, and further probed with anti-phosphotyrosine IgG (**upper panel**) or anti-flk-1 IgG (**lower panel**).

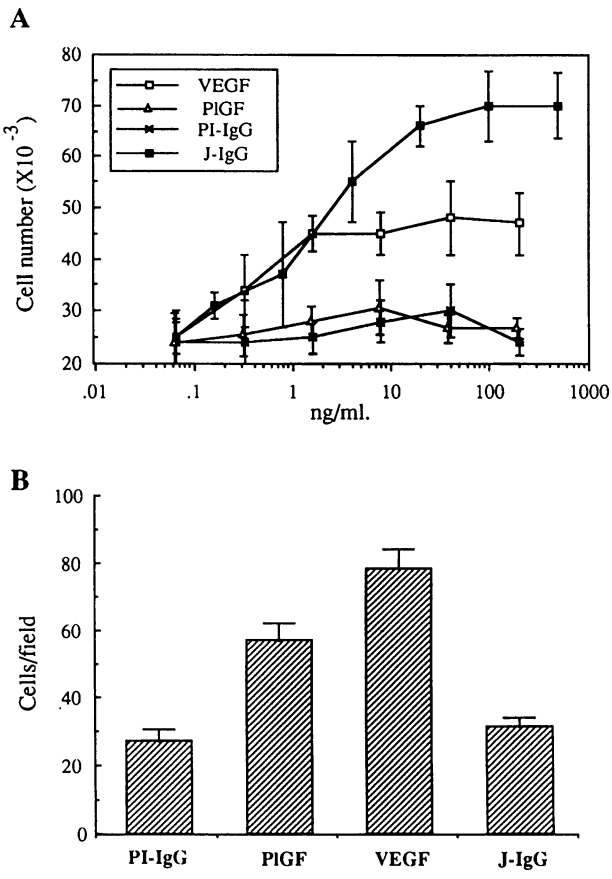
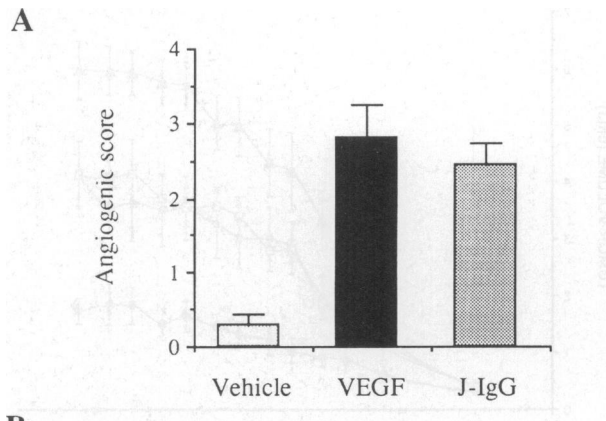


Figure 4. J-IgG stimulates ACE cell proliferation (**A**) but not migration (**B**). **A:** ACE cells were seeded at 5000 cells per well in 12 multiwell plates. Every other day the indicated concentrations of VEGF, J-IgG, or PI-IgG were added to cultures and the cells were trypsinized and counted on day 5. **B:** Confluent ACE monolayers were transferred to serum-free conditions for 24 hours after which the cells were scraped and 100 pmol/L VEGF, PlGF, or J-IgG added. Twenty-four hours later the cells that had migrated were counted as indicated in the text. Data are presented as the mean ± SEM of triplicate dishes. The results are representative of four distinct experiments.

promote ACE cell proliferation. After 5 days, the cell number of cultures that had received up to 200 ng/ml PI-IgG or PlGF were not statistically different from control cultures. J-IgG was as potent as VEGF on a molar basis (ED_{50} of 1.4 and 0.4 ng/ml, respectively, which corresponds to 10 pmol/L), but its mitogenic response reached a significantly higher level than that obtained in the presence of VEGF (Figure 4A). ACE cell migration was assessed in a wound assay. Dose-response curves of VEGF and PlGF induced a strong chemotactic activity (Figure 4B), thus confirming that flt-1 activation was sufficient to induce cell migration as observed for macrophages.[32,33] Accordingly KDR/flk-1 activation by J-IgG remained inefficient even at higher concentrations (0.02 to 10 nmol/L).

J-IgG Induces Angiogenesis Even in the Absence of VEGF

When subjected to the corneal pocket assay, J-IgG elicited an angiogenic response similar to that obtained with



B

systemic / local	PI-IgG	J-IgG
vehicle	< 0.1	1.22 ± 0.54
VEGF	1.21 ± 0.41	2.48 ± 0.35

Figure 5. Activation of KDR/flk-1 induces angiogenesis. **A:** Slow-releasing implants of hydrogel containing 50 μg of BSA without growth factors (vehicle; n = 24) or 200 ng of VEGF (n = 16) or 1 μg of J-IgG (n = 11) were inserted in New Zealand rabbit corneal stroma. After 12 days the neovascularization was assessed by a four-grade scale as described in the text. **B:** Slow-releasing implants of hydrogel containing 20 μg of BSA without growth factors (vehicle) or 50 ng of VEGF were inserted in Wistar rat corneal stroma, and 2 mg of J-IgG or PI-IgG was injected intravenously on days 3, 6, and 9. The neovascularization was assessed on day 12 as described above.

a similar dose of VEGF (Figure 5A). The average angiogenic scores were 2.82 and 2.45 compared with 0.28 for the vehicle alone.

In a parallel set of experiments, rats grafted with corneal implants containing the vehicle alone or 100 ng of VEGF were challenged with PI-IgG or J-IgG every 3 days. As expected, the angiogenic score of VEGF-induced angiogenesis increased on flk-1 activation (Figure 5B; 1.21 ± 0.41 *versus* 2.48 ± 0.35; $P < 0.05$). More surprisingly, the systemic delivery of J-IgG induced an angiogenesis in implants containing the vehicle alone (<0.1 *versus* 1.22 ± 0.54; $P < 0.05$).

Systemic Injections of J-IgG Increase Prostatic Adenocarcinoma Xenograft Volume in Nude Mice but Not the Proliferation of Resting Endothelial Cells

To investigate the physiopathological functions of VEGF and VEGF receptors in tumor angiogenesis, the effects of VEGF immunoneutralization and KDR/flk-1 overstimulation were tested on a poorly differentiated prostatic adenocarcinoma (Gleason's score IX). Tumor fragments were grafted in nude mice, and 1 month later, when the tumors were clinically palpable, the mice received twice weekly 200 μg of either V-IgG, PI-IgG, or J-IgG partially purified on protein A affinity chromatography columns.

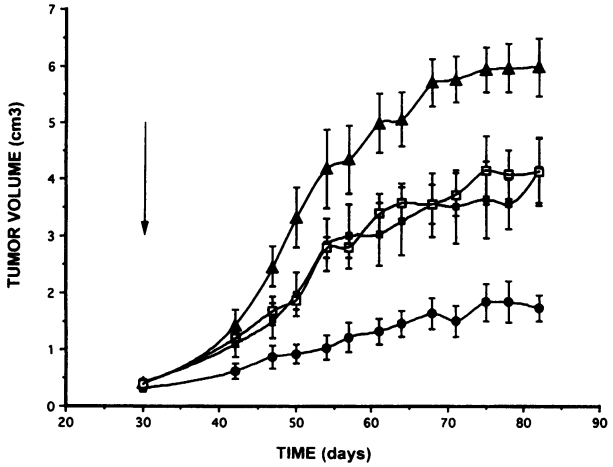


Figure 6. Systemic injections of J-IgG increase the volume of a prostatic adenocarcinoma grafted in nude mice. Fragments (3 × 3 × 3 mm) of a prostatic adenocarcinoma were grafted in nude mice. One month later, after the onset of tumor enlargement, animals (n = 8 to 10 for each group) received twice weekly 200 μg of neutralizing V-IgG (●), PI-IgG (□), J-IgG (▲), or vehicle alone (■). The tumor burden was estimated twice weekly by measuring the largest and the lowest tumor dimensions with a caliper.

We found a significant reduction in the kinetics of tumor growth and the final volume (P = 0.0016) in animals treated with V-IgG as compared with PI-IgG, whereas J-IgG treatment resulted in an increase of both (Figure 6).

Tumor sections immunostained with anti-flt-1 antibodies demonstrated an increase of vascularization on J-IgG treatment. Image analysis of low-magnification photomicrographs (Figure 7) demonstrated striking differences in the percentages of endothelial cells in tumor sections (2.3 ± 1.15, 6.5 ± 0.8, and 12.8 ± 1.54 for V-IgG, PI-IgG, and J-IgG, respectively). Histopathological examination of tumor sections stained with H&E showed that J-IgG-treated cells were smaller and more basophilic (Figure 8) and had a higher nuclear/cytoplasmic ratio. Histochemical staining with *Ulex europaeus* showed that V-IgG-treated tumors were less labeled than the PI-IgG controls. In contrast, J-IgG-treated tumors showed an increase in the number of vessel lumina. Immunolabeling serial sections with anti-flt-1 antibodies provided similar results. Tumor sections were immunostained with Mib-1 monoclonal antibody. A change in Mib-1 nuclei staining occurred on treatment. V-IgG-treated tumors showed fewer positive cells than their PI-IgG-treated counterparts. In contrast, a dramatic increase in immunostaining was observed in J-IgG-treated tumors. As shown in Figure 9, the difference in the percentages of positive cells measured in 8 to 12 high-power fields for each tumor was highly significant (6.3 ± 1.2%, 14,6 ± 2.2%, and 37.4 ± 2.1% for systemic injection of V-IgG, PI-IgG, and J-IgG, respectively).

Electron microscopy of tumors from PI-IgG- or V-IgG-treated animals showed an almost identical ultrastructure (Figure 10). Conversely, in tumors from J-IgG-treated animals, the nucleoli appeared less coiled and dense than in the PI-IgG counterparts. Although KDR/FLK-1 mRNA is expressed in adult mouse glomeruli,[34] the electron microscopy analysis failed to detect any change

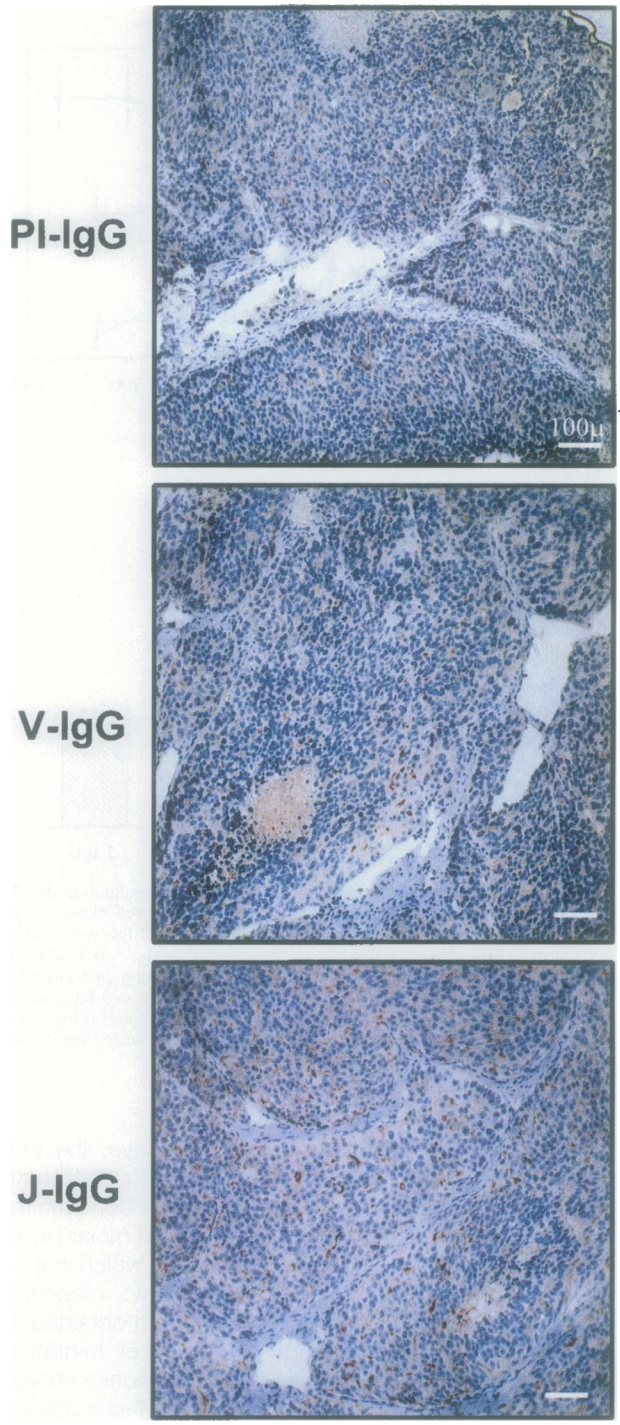


Figure 7. Systemic injections of J-IgG increase flt-1 expression of a prostatic adenocarcinoma grafted in nude mice. After 2 months of treatments (described in Figure 6) the tumor sections were immunostained with anti-flt-1 antibody and examined at low magnification (×70).

whatever the treatment. Histological examination of kidneys, liver, lungs, and brain did not demonstrate any vascular modification. Although cultured retinal pigment epithelial and capillary endothelial cells express VEGF and KDR,[8] no sign of proliferation could be detected in the retina (not shown).

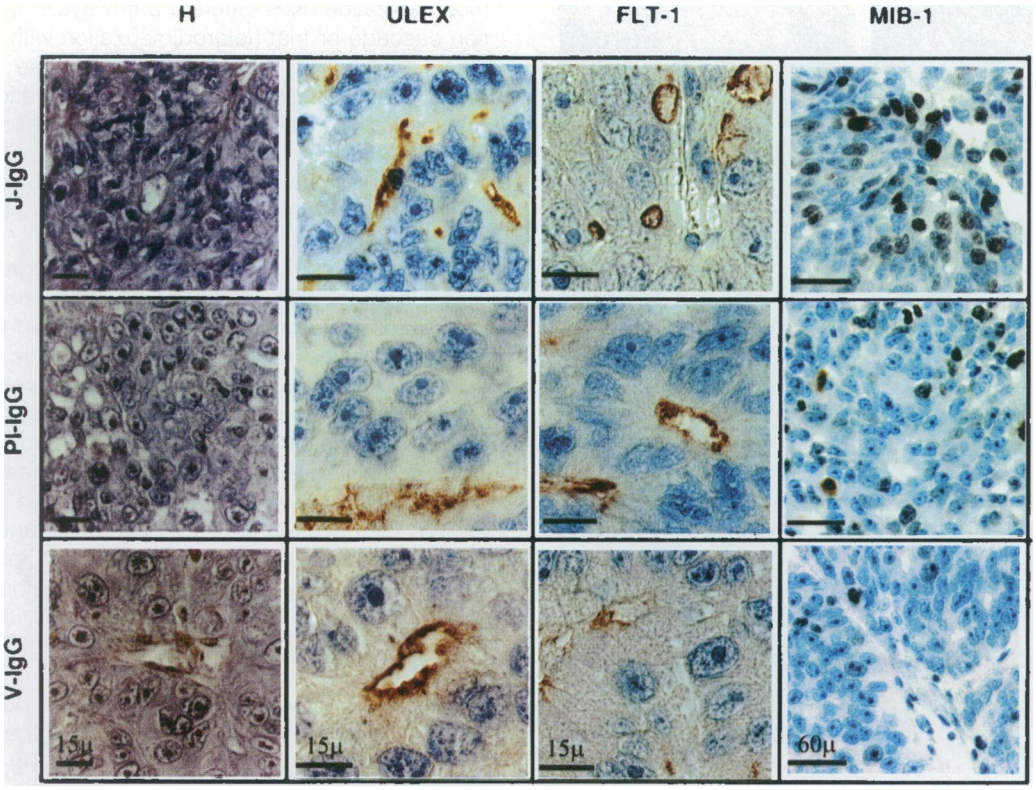


Figure 8. Systemic injections of J-IgG increase the vascularization and proliferation of a prostatic adenocarcinoma grafted in nude mice. After 2 months of treatments (described in Figure 6) the tumor sections were stained with H&E, *Ulex europaeus*, anti-flt-1 antibody, and Mib-1. Few vessels were visible in V-IgG-treated sections, whereas intense staining was detected in J-IgG-treated samples. Similar immunostaining was obtained with anti-flt-1 antibody. Proliferation was analyzed by immunostaining with the Mib-1 monoclonal antibody.

Discussion

In this study we show that systemic activation of only one VEGF receptor is sufficient to elicit both controlled and tumor angiogenesis without affecting quiescent endothelial cells located in the normal vasculature.

The straightforward demonstration that VEGF acts by activating a VEGF receptor and not by releasing other growth factors from the extracellular matrix raises several methodological points. The systemic delivery of growth factors might be impaired by their inability to reach their targets as a result of being sequestered in the extracellular matrix. We never observed any modification of the tumor volume by injecting systematically VEGF. The large number of heparin-binding growth factor receptors makes screening of the distinct functions mediated *in vivo* by each receptor a difficult task. We had to construct circulating agonists mimicking the distinct domains of VEGF interactions with its receptors and therefore relied on the anti-idiotypic strategy.[24–26] We raised several anti-idiotypic antibodies by priming lymphocytes in the lymph nodes with neutralizing V-IgG. Almost 15% of these sera contained IgGs able to compete with VEGF for its binding to vascular endothelial cells. To ascertain the specificity of these anti-idiotypic antibodies, the purified IgGs were similarly tested on COS cells transfected with expression vectors carrying one of the cognate VEGF receptors. J-IgG was highly specific for flk-1 as it immunoprecipitated flk-1 but not flt-1 and did not bind to flt-1-expressing cells. The acquisition of internal images selective for only one VEGF receptor confirms that the VEGF-binding domains of VEGF for its receptors are different. Site-directed mutagenesis of VEGF has recently demonstrated that Asp^{63}, Glu^{64}, and Glu^{67} are associated with flt-1 binding whereas Arg^{82}, Lys^{84}, and His^{86} are associated with KDR binding.[35] Anti-idiotypic J and VEGF induced the tyrosine phosphorylation of a set of proteins (205, 140, and 90 kd) with molecular masses consistent with those described by D'Angelo, namely, flk-1, phospho-

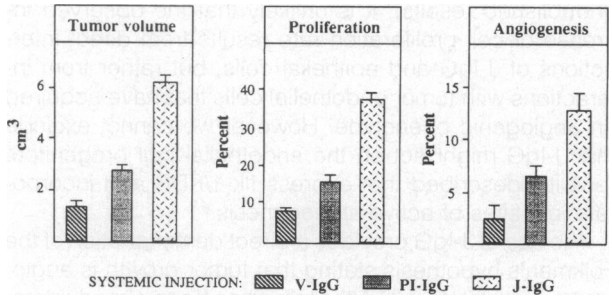


Figure 9. Systemic injections of J-IgG increase the cell proliferation in tumors. Tumor sections of V-IgG-, PI-IgG-, or J-IgG-treated mice were analyzed for angiogenesis (flt-1) and proliferation (Mib-1). Mib-1-positive cells were counted in each tumor in 10 fields representing at least 300 cells randomly chosen under a light microscope at a ×400 magnification. The flt-1-positive cells were counted by image analysis at a ×70 magnification. Values are represented as means ± SEM.

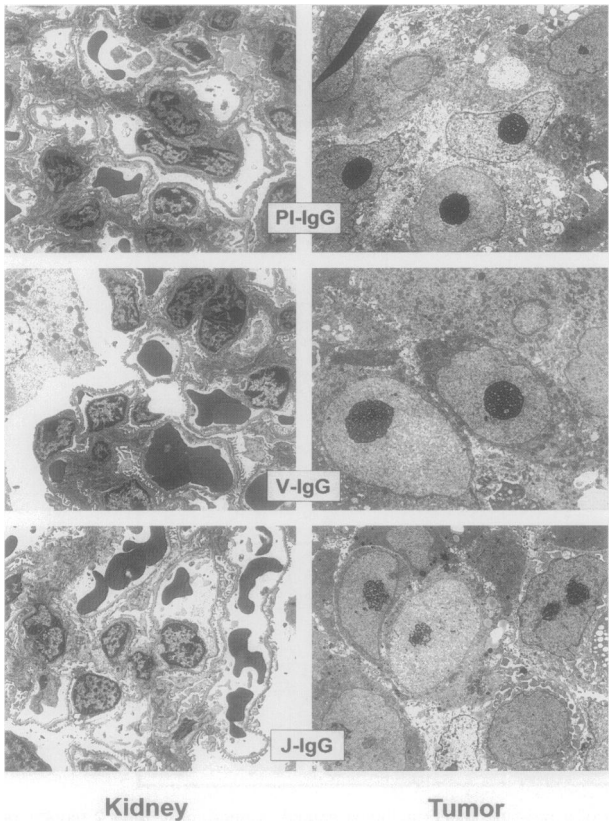


Figure 10. Ultrastructural analysis of tumors and kidneys. Cross-sectional views of kidneys and tumors as visualized by transmission electron microscopy (×3700). Note that nucleoli of J-IgG-treated tumors are less dense and wound whereas no difference is observed in kidneys.

lipase Cγ, and MAPK.[36] We confirmed by Western blot that the 205-kd band represented the KDR/flk-1 receptor. Saturating concentrations of J-IgG promoted more efficiently than VEGF the tyrosine phosphorylation of KDR/flk-1, suggesting that homodimerization and transphosphorylation of KDR/flk-1 might be more efficient than when KDR/flk-1 heterodimerizes with flt-1. Ligand-independent receptor activation by anti-receptor antibodies has been demonstrated in the receptor tyrosine kinase family. For example, monoclonal antibodies to insulin growth factor 1 receptor or epidermal growth factor receptor[37] cause increases in receptor kinase activity and stimulation of DNA synthesis in serum-starved NIH 3T3 cells. IgG directed against the nerve growth factor high affinity receptor TrkA[38] can promote neuron survival. It was shown in such cases that bivalent IgGs are required for antibody-dependent activation of the epidermal growth factor receptor or TrkA, probably because they mimic the ligand-induced dimerization. Although J-IgG was 35-fold less potent than VEGF in VEGF-binding experiments at 4°C, we observed that J-IgG induced a mitogenic effect on ACE cells higher than that achieved with VEGF although their ED_{50} values were in the same range (O.3 and 1.6 ng/ml for VEGF and J-IgG) corresponding to 10 pmol/L. The greater effect of J-IgG on KDR/flk-1 tyrosine phosphorylation and transduction of the mitogenic signal suggest either that KDR/flk-1 homodimerization uses different pathways of the transduction cascade or that heterodimerization with flt-1 attenuates its activity. In contrast to the report of Waltenberger[39] stating that KDR/flk-1 activation mediates both endothelial cell proliferation and migration, we found that J-IgG did not promote cell migration. Our observation that flt-1 homodimerization, achieved by PlGF, was sufficient to mediate cell migration thus confirms the results described by other groups.[32,33]

J-IgG, but not PI-IgG, stimulated the outgrowth of capillaries from the limbus vessels in rabbit and rat corneal pocket assays, demonstrating that KDR/flk-1 homodimerization was sufficient to induce angiogenesis and was not species dependent. Histology of the corneas demonstrated that VEGF led to the formation of corneal edema surrounding the pellet whereas J-IgG did not (not shown). This edema resulted probably from an increase of VEGF-driven permeability, which we have shown to be mediated by the activation of flt-1.[40] Internal images of murine or human VEGF also provided similar binding affinity (data not shown), thus demonstrating that this idiotope is highly conserved. For unknown reasons, this strategy never generated flt-1 agonists. It was not totally unexpected that systemic injections of J-IgG enhanced the VEGF-driven corneal neovascularization, but more surprisingly, it also induced angiogenesis in the absence of local delivery of growth factor, suggesting that in this animal model of controlled angiogenesis, the proliferating phenotype switch of the endothelial cells is not linked to the presence of angiogenic growth factors. It is tempting to speculate that the functional expression of KDR/flk-1 on limbus endothelial cells is triggered by corneal cytokines released after the local trauma occurring during the graft of the pellet.

Vessel counts have been proposed as a prognosis marker of prostatic adenocarcinoma,[41] so we examined the putative role of VEGF in this pathology. VEGF immunoneutralization reduced the tumor volume, thus indirectly confirming this hypothesis. Direct evidence showing that KDR/flk-1 activation is sufficient to increase tumor size was obtained by the use of systemic delivery of KDR/flk-1 agonists, which increased the vascularization as evidenced by *Ulex europaeus* and flt-1 staining. As normal or tumor human prostate epithelial cell lines do not express KDR or proliferate on VEGF addition (our unpublished results), it is unlikely that the observed increase of cell proliferation rate results from direct interactions of J-IgG and epithelial cells, but rather from interactions with tumor endothelial cells that have acquired an angiogenic phenotype. However we cannot exclude that J-IgG might act on the endothelial cell progenitors recently described that express flk-1/KDR and incorporate into sites of active angiogenesis.[42]

The use of J-IgG provides a direct demonstration of the Folkman's hypothesis stating that tumor growth is angiogenesis dependent.[43] Although it has been already demonstrated that the disruption of the VEGF/KDR-activating pathway led to an inhibition of tumor growth,[20–22] these data are indirect. For instance, we recently reported that VEGF overexpression could induce cell transformation through the expression of fibroblast growth factor recep-

tor 1.[44] Despite the fact that KDR/flk-1 mRNAs are expressed in adult glomeruli[34] and retinal pigment epithelial cells,[8] no microscopic modification could be detected by photonic or electronic examination in kidneys or retinas, thus indicating that KDR/flk-1 gene translation products are not functional in healthy organs. J-IgG appears, as integrins $\alpha v\beta 5$ or $\alpha v\beta 3$,[45] as a functional marker of the endothelial cells that have switched to the angiogenic phenotype occurring in controlled and uncontrolled angiogenesis. VEGF has been used to restore the blood flow in patients with ischemic limbs.[46] However, patients can develop edemas that might not happen if therapeutic angiogenesis was stimulated by these VEGF anti-idiotypic antibodies. Investigations are underway to demonstrate the possible anti-angiogenic interest of systemic delivery of immunotoxins targeted on KDR/flk-1.

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