Antiangiogenic Properties of Gold Nanoparticles

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Abstract Here, we report an intrinsic property of gold nanoparticles (nanogold): they can interact selectively with heparin-binding glycoproteins and inhibit their activity. Gold nanoparticles specifically bound vascular permeability factor/vascular endothelial growth factor (VPF/VEGF)-165 and basic fibroblast growth factor, two endothelial cell mitogens and mediators of angiogenesis resulting in inhibition of endothelial/fibroblast cell proliferation *in vitro* and VEGF-induced permeability as well as angiogenesis *in vivo*. In contrast, nanogold did not inhibit VEGF-121 or epidermal growth factor, two non–heparin-binding growth factors, mediated cell proliferation. Gold nanoparticles significantly inhibited VEGF receptor-2 phosphorylation, intracellular calcium release, and migration and RhoA activation *in vitro*. These results report for the first time a novel property of gold nanoparticles to bind heparin-binding proteins and thereby inhibit their subsequent signaling events.

Angiogenesis, the formation of new blood vessels is essential for the growth and progression of tumors (1, 2). This process is also important for the promotion and maintenance of other diseases like neoplasia and rheumatoid arthritis (3). As there are several reports that indicate that gold salts can retard the progression of rheumatoid arthritis (4), we reasoned that gold nanoparticles might also inhibit angiogenesis. Because vascular endothelial growth factor/vascular permeability factor (VEGF/VPF; refs. 5, 6) and basic fibroblast growth factor (bFGF; ref. 7) are two critical cytokines for the induction of angiogenesis, we investigated whether nontoxic novel gold nanoparticles (8) being used at present in several biomedical applications could inhibit the functions of these two important proangiogenic growth factors.

Here, we report for the first time that nanogold binds to heparin-binding growth factors like VEGF165 and bFGF and inhibit their activity, whereas it does not inhibit the activity of non–heparin-binding growth factors like VEGF121 and endothelial growth factor (EGF). We have found excellent correlation between our *in vitro* results and *in vivo* mouse ear and mouse ovarian tumor model systems, where VEGF/VPF-induced permeability was inhibited by nanogold.

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Note: P. Mukherjee and R. Bhattacharya contributed equally to this work.

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Materials and Methods

Reagents. VEGF165, bFGF, VEGF121, and EGF were obtained from R&D Systems, Minneapolis, MN. Tetrachloroauric acid trihydrate and sodium borohydride were from Sigma-Aldrich, St. Louis, MO. [^{3}H]Thymidine was from Amersham Biosciences, Piscataway, NJ. Anti-VEGF, VEGF receptor (VEGFR)-2, and RhoA antibody were from Santa Cruz Biotechnology, Santa Cruz, CA. Phosphotyrosine antibody was from Upstate Biotechnology, Lake Placid, NY.

Preparation of gold nanoparticles. In a typical experiment, 50 mL of aqueous solution containing 4.3 mg of solid sodium borohydride was added to 100 mL of 100 μmol/L aqueous solution of tetracholoroauric acid under vigorous stirring that was continued overnight (9). Nanogold thus formed were filtered through a 0.22-μm filter and used for experiments.

Cell proliferation assay. Human umbilical vascular endothelial cells (HUVEC; 2×10^3) were seeded in 24-well plates, cultured for 2 days in endothelial basal medium, serum-starved (0.1% serum) for 24 hours, and then treated with VEGF165 or VEGF121 (10 ng/mL) that was first preincubated with gold nanoparticles overnight at 4°C. After culture for 20 hours, 1 μCi [^{3}H]thymidine was added. Four hours later, cells were washed with cold PBS, fixed with 100% cold methanol, and collected for the measurement of trichloroacetic acid–precipitable radioactivity (10). NIH3T3 fibroblasts (2×10^4) were seeded in 24-well plates, cultured for 1 day in DMEM, serum-starved for 24 hours (11), and then treated with 10 ng/mL bFGF or EGF that was first preincubated with gold nanoparticles overnight at 4°C and proceeded as above. Experiments were repeated at least thrice.

Immunoprecipitation and Western blot analysis. HUVECs were serum-starved for 24 hours and stimulated using different concentrations of gold nanoparticles preincubated with 10 ng/mL VEGF. After 5 minutes, chilled PBS was added and cell lysates in radioimmunoprecipitation assay buffer were collected after centrifugation at 14,000 × *g* for 10 minutes at 4°C. Lysate protein (500 μg) was incubated with 1 μg rabbit polyclonal VEGFR-2 antibody for 1 hour and 50 μL of protein A-conjugated agarose beads for an additional hour at 4°C. Beads were washed and boiled in 2× SDS sample buffer. Experiments were repeated at least thrice.

Intracellular Ca^{2+} release. Serum-starved HUVECs were loaded with Fura-2 AM dye and stimulated with 10 ng/mL

VEGF165 preincubated with or without gold nanoparticles for 30 minutes at 4°C. The assay was carried out as described previously (12). Experiments were repeated at least thrice.

Cell migration assay. VEGF165 preincubated with nanogold was used in these experiments. Cell migration was carried out as described previously (13).

RhoA activation assay. Briefly, 24-hour serum-starved HUVEC cells were stimulated for 5 minutes with 10 ng/mL of VEGF165 preincubated with nanogold (670, 335, and 67 nmol/L concentrations). Chilled PBS was added and cells were lysed with lysis buffer and RhoA activation was carried out as described previously (14).

Nude mouse ear experiments and mouse ovarian tumor experiments. Six to eight-week-old male nude mice were purchased from the National Cancer Institute. A nonreplicating adenoviral vector was engineered to express the predominant (164–amino acid) murine isoform of VEGF-A as previously described. Immediately prior to use, the adenovirus was desalted using Quick Spin, High-Capacity G-50 Sephadex columns (Boehringer Mannheim, Indianapolis, IN) and diluted in PBS-3% glycerol. 0.2 to 0.5 $\times$ 10^8 plaque-forming units of Ad-VEGF virus in a volume of 10 μL was injected intradermally into the mouse ears. The experimental group received two additional injections of 670 nmol/L, 10 μL nanogold on the 2nd and 4th day post-Ad-VEGF administration. Animals were sacrificed on day 7 post-Ad-VEGF administration and ears were collected and processed for 1 μm/L Epon sections. For mouse ovarian tumor (MOT) experiments, 1 $\times$ 10^6 MOT cells were injected in the peritoneal cavity of C3H mice (15) and treatment was started 2 days after the injection. Nanogold (1.3 mg) was injected to each mouse everyday over a period of 7 days. Mice were then sacrificed and ascites fluids collected. At least five animals were used in each set. All experiments were done under protocols approved by the Beth Israel Deaconess Medical Center and the Mayo Clinic Institutional Animal Care and Use Committee.

Statistical analysis. Statistical analysis was done using ANOVA.

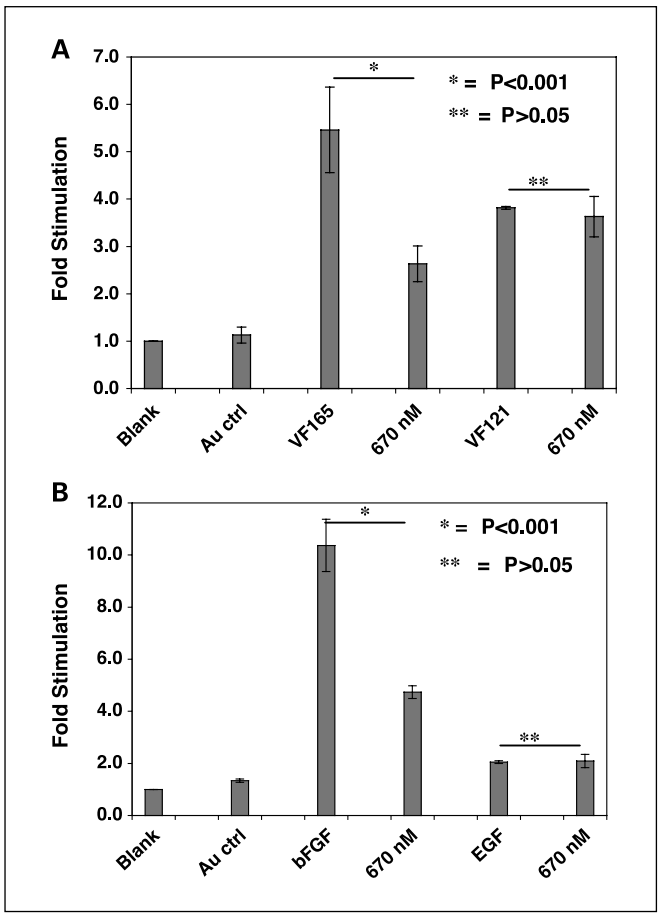

Fig. 1. Effect of nanogold on growth factor – induced proliferation. [^{3}H]Thymidine incorporation is represented as fold stimulation. *A,* serum-starved HUVECs were stimulated with VEGF165/VEGF121 (10 ng/mL) that was preincubated with nanogold (see Materials and Methods). *B,* serum-starved NIH3T3 cells were stimulated with 10 ng/mL bFGF/EGF preincubated gold (see Materials and Methods). Average of three independent experiments, each one done in triplicate.

Results and Discussion

Characterization of nanogold. Gold nanoparticles were prepared by the reduction of aqueous chloroaurate ions with sodium borohydride (see Materials and Methods). The presence of characteristic surface plasmon resonance band at ~512 nm in the UV-Visible spectrum of the solution confirmed the formation of spheroid nanogold in solution (16), which was further confirmed by transmission electron microscope analysis showing ~5 nm nanogold with a nearly uniform particle size distribution.

Effect of nanogold on the proliferation of human umbilical vascular endothelial cells and NIH3T3 cells. We first examined whether nanogold had any effect on VEGF165- and VEGF121-induced HUVEC proliferation. Figure 1A clearly shows that nanogold inhibited VEGF165-induced proliferation of HUVECs but did not inhibit VEGF121-induced proliferation. We also tested the effect of nanogold on bFGF, another heparin-binding growth factor, and EGF, a non–heparin-binding growth factor, induced proliferation of NIH3T3 cells. Like VEGF165, nanogold also inhibited bFGF-induced proliferation of NIH3T3 cells (Fig. 1B) but did not interfere with the activity of EGF. Importantly, nanogold was not toxic to either cell line as evidenced in Fig. 1A and B, where no inhibition in proliferation was observed in the only nanogold-treated samples compared with the control. The inhibition of proliferative activity of two heparin-binding growth factors, namely VEGF165 and bFGF, by nanogold and its inability to inactivate non–heparin-binding growth factors, namely VEGF121 and EGF, clearly suggest that the heparin-binding domain of VEGF165 and bFGF plays the crucial role for their interactions with nanogold and hence inactivation.

Effect of nanogold on VEGF165-induced phosphorylation of VEGFR-2. To find out the mechanism of the inhibitory effect of gold nanoparticles, we next investigated the effect of nanogold on downstream signaling induced by VEGF165. With the addition of nanogold at a concentration of 335 to 670 nmol/L, VEGF165-induced phosphorylation of VEGFR-2 was profoundly inhibited (Fig. 2A). However, at 67 nmol/L concentration of nanogold, ~40% inhibition of phosphorylation was evident (from densitometry quantitation using NIH Image software; Fig. 2B; $P < 0.001$). Almost complete inhibition of VEGFR-2 phosphorylation was observed at 335

[5] Bhattacharya R, Mukherjee P, Xiong Z, et al., unpublished observation.

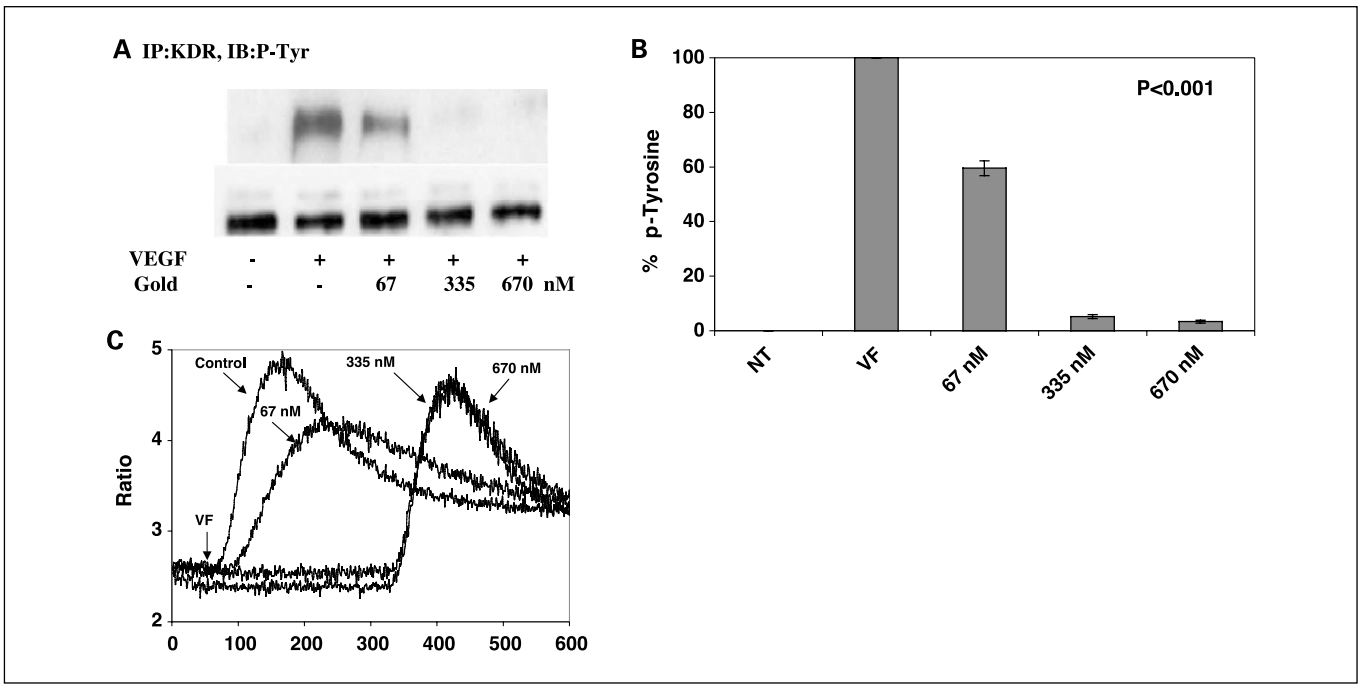

Fig. 2. Effect of nanogold on receptor phosphorylation. *A*, serum-starved HUVECs were stimulated for 5 minutes with VEGF165 (10 ng/mL) that was preincubated with or without gold nanoparticles, immunoprecipitated with antibodies to VEGFR-2, and immunoblotted with antibodies to phosphotyrosine. The same membrane was stripped and reprobed to show total VEGFR-2 levels in the cell extracts. *B*, densitometry scanning of phosphotyrosine blots using NIH Image, expressed in percentage. *C*, serum-starved HUVECs were suspended in the Ca2+ buffer containing Fura-2AM dye and were stimulated with VEGF165 (10 ng/mL) that was preincubated with or without gold. Fresh VEGF was added 300 seconds later to the 335 and 670 nmol/L VEGF-conjugated gold samples.

and 670 nmol/L concentrations of nanogold. The results of receptor phosphorylation clearly suggest that nanogold binds directly to VEGF165 and inhibits its interaction with cell surface receptors hence inhibiting phosphorylation. To prove the direct binding of gold nanoparticles to the heparin-binding domain of VEGF165, we carried out heparin Sepharose precipitation of VEGF165 preincubated with nanogold. We found that with an increase in nanogold concentrations, there is a decrease in binding of VEGF165 to heparin Sepharose.[5] These observations clearly verify our hypothesis that gold nanoparticles bind to heparin-binding growth factor through its heparin-binding domain.

Effect of nanogold on intracellular calcium release. To further support our hypothesis that nanogold binds to VEGF165 and inactivate its signaling capability, we did an intracellular calcium release experiment in HUVECs. Figure 2C clearly shows a decrease in calcium release at a gold concentration of 67 nmol/L (~34%, determined by comparing the length of the upstroke of VEGF165-induced sample to the nanogold-treated samples) and complete inhibition at 335 to 670 nmol/L as detected by the change in the fluorescence ratio of the Fura-2 AM dye ($P < 0.05$). The VEGF receptors on the HUVECs were still functional as evidenced by an increase in calcium release comparable to the control when VEGF was added after 300 seconds. These observations also clearly show that gold nanoparticles bind directly to VEGF165 and inhibit its signaling events but do not perturb the receptor functions.

Effect of nanogold on VEGF165-induced migration of human umbilical vascular endothelial cells. We also tested the effect of nanogold on VEGF165-induced migration of HUVECs. Figure 3A clearly shows that nanogold inhibited VEGF165-induced migration of HUVECs. RhoA activity was also completely inhibited at 670 nmol/L nanogold concentration (Fig. 3B); further confirming our hypothesis that nanogold binds directly with the heparin-binding growth factor

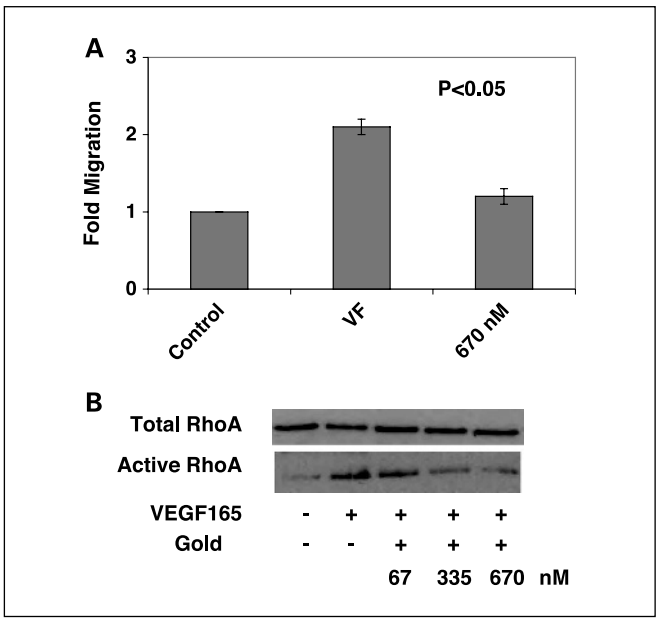

Fig. 3. Effect of nanogold on migration of HUVECs. *A*, serum-starved HUVECs were stimulated for 2 hours with VEGF165 (10 ng/mL) that was preincubated with or without nanogold. *B*, VEGF165-induced RhoA activation of serum-starved HUVECs in the presence or absence of nanogold.

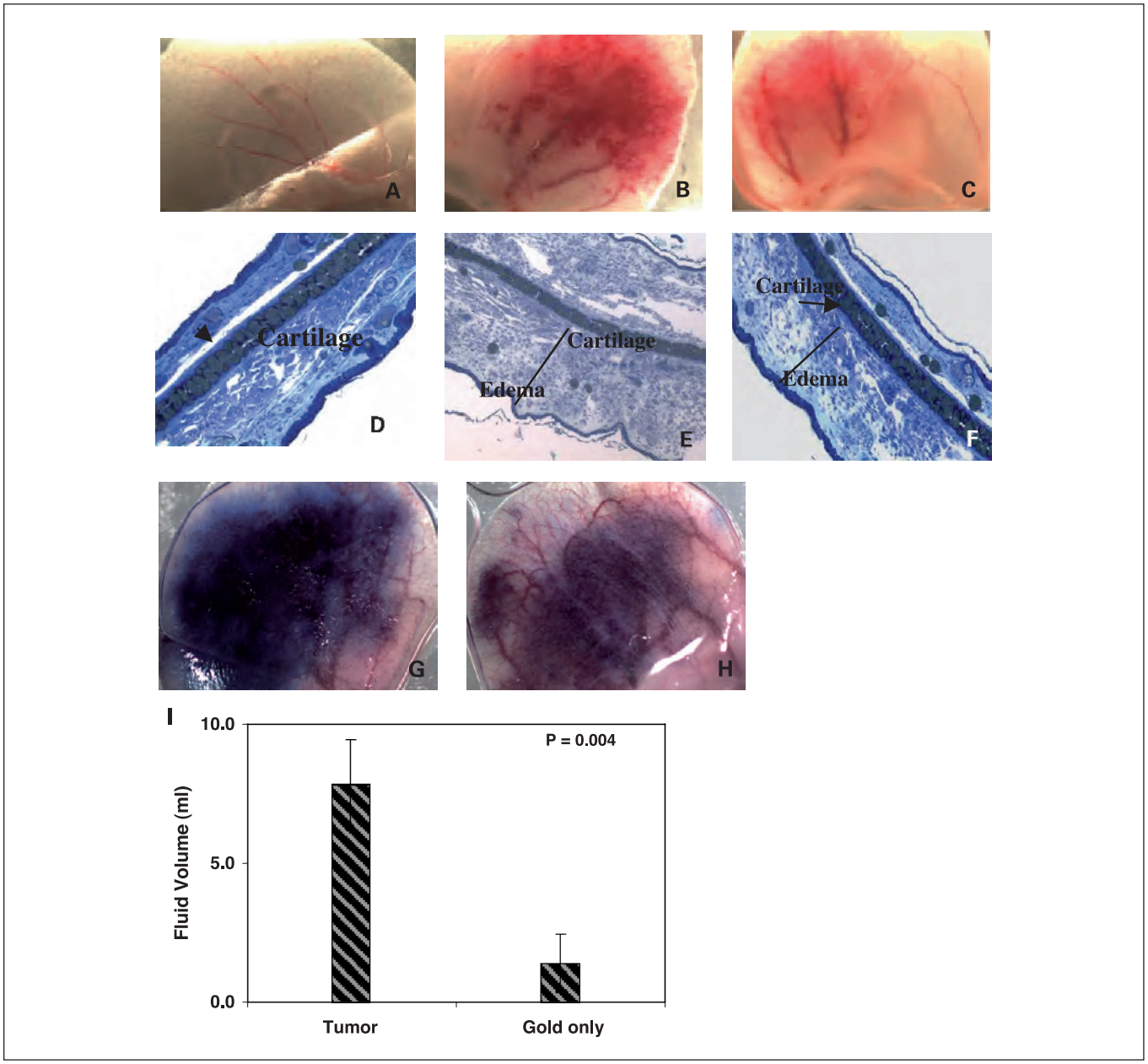

Fig. 4. Effect of nanogold on angiogenesis *in vivo* in the ears of nude mice. Gross appearance of angiogenesis 7 days (see Materials and Methods) after injection of nanogold only (*A*), Ad-VEGF only (*B*), nanogold and Ad-VEGF (*C*). Giemsa stained 1 μmol/L epon sections of the ears were photographed at 10× magnifications, nanogold only (*D*), Ad-VEGF only (*E*), nanogold and Ad-VEGF (*F*). Effect of nanogold on permeability, Ad-VEGF only (*G*), nanogold and Ad-VEGF (*H*), ascites fluid accumulation in the peritoneal cavity (*I*).

through the heparin-binding domain and inhibits its signaling activity.

***Effect of nanogold on angiogenesis* in vivo.** We tested the efficacy of nanogold to inhibit VEGF165-induced permeability and angiogenesis *in vivo* in a nude mouse ear model. Figure 4A-C show whole mount of ears 7 days after administration of Ad-VEGF with or without nanogold treatment. A reduction in angiogenesis was observed (Fig. 4A and C). Ad-VEGF-injected mice treated with nanogold developed less edema than mice treated with Ad-VEGF only (Fig. 4D and F). As shown, 30 minutes after injection of Evan's blue dye into the tail vein of these treated mice, a decrease in permeability was also observed (Fig. 4G and H). In the MOT model, less fluid accumulation in the peritoneal cavity was observed in nanogold-treated samples compared with the controls (Fig. 4I).

Taken together, our results indicate that gold nanoparticles selectively inhibit VEGF165- and bFGF-induced proliferation of HUVECs and fibroblasts, respectively. In a similar fashion, nanogold also inhibited the activity of placental growth factor (data not shown). Gold nanoparticles directly bind heparin-binding growth factors presumably through cysteine residues of the heparin-binding domain and inhibit growth factor–mediated signaling. It is well documented in the literature that gold binds strongly with thiols and amines (17–19). Such an ionic/pseudo-covalent reaction may explain the interaction between the gold nanoparticles and

VEGF that led to a decrease in its proliferative activity. Gold nanoparticles also inhibit VEGF-induced angiogenesis and permeability *in vivo* as well as cause lesser ascites fluid accumulation in a MOT model where VEGF/VPF activity is primarily responsible for fluid accumulation in the peritoneal cavity. Here we would like to mention briefly about toxicity of nanogold. Because metal poisoning is associated with renal and hepatic toxicities, we therefore determined the effect of nanogold administration on liver and renal function tests. Normal mice were given the same dose of nanogold as in MOT model i.p. for 7 consecutive days. On day 8, the mice were sacrificed and serum was collected. There were no significant differences between serum levels of creatinine, blood urea nitrogen, bilirubin alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase between the nanogold treated and untreated control animals (data not shown). This finding is significant because growth factor–mediated proliferation and angiogenesis has a central role in many pathologic conditions including neoplasia, rheumatoid arthritis, and chronic inflammation. The low production cost and relative ease to modify nanogold make them a feasible choice in future biomedical applications.

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