

Carbon inhibits vascular endothelial growth factor- and fibroblast growth factor-promoted angiogenesis

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Abstract Angiogenesis is important for normal growth and wound healing processes. An imbalance of the growth factors involved in this process, however, causes the acceleration of several diseases including malignant, ocular, and inflammatory diseases. Inhibiting angiogenesis through interfering with its pathway is a promising methodology to hinder the progression of these diseases. Herein, we studied the anti-angiogenic effects of various carbon materials such as graphite, multiwalled carbon nanotubes and fullerenes in vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF2)-induced angiogenesis evaluated in the chick chorioallantoic membrane (CAM) model. All the carbon materials tested showed substantial anti-angiogenic activity against either FGF2- or VEGF-induced angiogenesis in the CAM model. Those carbon materials did not have any significant effects on basal angiogenesis in the absence of the added growth factors.

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1. Introduction

Angiogenesis (the formation of new blood vessels) is an essential event for normal growth processes including embryonic development, wound healing and the menstrual cycle [1]. Various growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factors (acidic and basic FGFs) and angiopoietin are involved in neovascularization. Growth factors such as basic FGF (or FGF2) and VEGF induce endothelial cells to secrete proteases and plasminogen activators that degrade the vessel basement membrane, leading to cell invasion into the surrounding matrix and the formation of new vessels. Thus, VEGF and FGF2 constitute the most important growth factors that induce angiogenesis [2]. An

imbalance in the levels of these factors will cause many serious diseases including malignant cell growth.

Angiogenesis is required for cancer tumor survival and metastasis [1,3,4]. Inhibiting angiogenesis can both eliminate the blood supply to the primary cancer cells and prevent cancer metastasis [5]. The current strategy of inhibiting angiogenesis relies mainly on inhibiting the activities of the growth factors and/or interfering in the angiogenesis pathways. Various angiogenesis inhibitors such as angiostatin, endostatin and interferons have been found to effectively arrest the cancer metastasis [5–7]. There is still active research being carried out to find less expensive, more potent, easily administrable and more common inhibitors.

Nanomaterials such as liposomes, polymeric nanoparticles, metallic and non-metallic nanoparticles, carbon nanotubes, nanowires, viral nanoparticles and hybrids, quantum dots and dendrimers are currently being proposed, with appropriate surface modifications, as tools for drug delivery and imaging and in cancer treatment and diagnosis [8,9]. The numerous potential applications of nanomaterials in cancer research diagnosis and treatment have given rise to the new field of “nanoncology”. Carbon-based nanomaterials such as carbon nanotubes, fullerenes and carbon nanofibers are preferred over other nanomaterials owing to their high aspect ratio, mechanical strength, ease of chemical/physical conjugation with small and macromolecules, high surface area, easy manufacturability and light weight. Biocompatibility, blood compatibility, bioclearance and toxicology are some of the many issues that need to be addressed when proposing the use of nanomaterials in biomedical applications [10]. Surface modified hydrophilic carbon nanotubes were recently reported to have excellent bioclearance through the kidney when administered *in vivo* in mice [11]. In addition, our laboratory has recently demonstrated that multiwalled carbon nanotubes (MWNTs), modified with heparin, show excellent blood compatibility [12]. These studies suggest the possibility of using carbon nanotubes (CNTs) in various *in vivo* biomedical applications including the treatment of cancer, with appropriate surface modifications [13].

Here we report the anti-angiogenic efficacy of pristine (unmodified) MWNTs, C₆₀ fullerenes and graphite by using VEGF/FGF2 induced angiogenesis in chick chorioallantoic membrane (CAM). All three carbon materials showed significant anti-angiogenic activity, with efficacy differing with respect to the promoters (VEGF/FGF2).

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2. Materials and methods

2.1. Materials

High purity MWNTs and fullerenes were purchased from Carbon Nanotechnologies Inc., and used without any purification. Human recombinant VEGF and human recombinant FGF2 were purchased from R&D Systems (Minneapolis, MN). Ten-day-old chick embryos were purchased from SPAFAS (Preston, CT). Sterile filter paper disks were purchased from Whatman International (Florham Park, NJ).

2.2. Methods

2.2.1. Physical binding studies. MWNTs, fullerenes and graphite were evaluated for any physical binding that might occur with the growth factors. MWNTs, fullerenes and graphite (100 µg) were incubated with VEGF (2 µg/ml) or FGF2 (1.5 µg/ml) in phosphate buffered saline (PBS) (1 ml) at 37 °C for 24 h. After incubation, the solution was filtered from the carbon-based materials and tested for VEGF/FGF2 by using UV at 280 nm. A standard curve of growth factor concentration versus UV absorbance was used for calibration.

2.2.2. CAM assay. Angiogenesis was examined by CAM model that has been described in detail previously [14–16]. The 10-day-old chick embryos were incubated at 37 °C with 55% relative humidity. A small hole was made in the shell concealing the air sac by using a hypodermic needle. A second hole was made on the broad side of the egg, directly over an avascular portion of the embryonic membrane that was identified by candling. By applying negative pressure at the first hole, a false air sac was created beneath the second hole, causing the CAM to separate from the shell. A window 2.0 cm² was cut in the shell over the dropped CAM with a small-crafts grinding wheel (Dremel, Emerson Electric Co.), allowing direct access to the underlying CAM. FGF2 (1.5 µg/mL) was used as a standard pro-angiogenic agent to induce new blood vessel branches on the CAM of 10-day-old embryos. Sterile filter paper disks were pretreated with 3 mg/mL cortisone acetate and air dried under sterile conditions. PBS (control), FGF2 or VEGF and the MWNTs, fullerenes or graphite were then applied to the cortisone acetate pretreated disks (1.0 cm, diameter) and dried. The disks were suspended in PBS, and placed on growing CAMs.

2.2.3. Microscopic analysis of CAM sections. After incubation with 55% relative humidity at 37 °C for 3 days, the CAM tissue directly below the filter disk was cut off and washed three times with PBS. The washed tissues were then placed in 35-mm Petridishes, and examined under a Karl Zeiss SV6 stereomicroscope (Thornwood, NY) at 50-fold magnification. Digital images of CAM tissues were

collected using a 3-CCD color video camera system from Toshiba America (New York, NY), and analyzed with Image-Pro software from Media Cybernetics (Silver Spring, MD). The number of vessel branch points contained in a circular region equal to the area of each CAM preparation (1.5 cm²), and findings from 7 to 8 CAM preparations were analyzed for each treatment condition. The resulting angiogenesis index is the mean ± S.E.M. of new branch points in each set of samples. The % inhibition was calculated using the following equation:

$$\begin{aligned} \% \text{Inhibition} = & 100 - \left[\frac{\text{(Test agent plus VEGF or FGF2)} \right. \\ & \left. - \text{PBS(control)} \right] / \left[\frac{\text{(VEGF or FGF2)} \right. \\ & \left. - \text{PBS(control)} \right] \times 100 \end{aligned}$$

** Mean % inhibition was then calculated based on the different (*n*) carried out

2.2.4. Statistical analysis. Statistical analyses of blood vessel branching patterns were performed by one-way analysis of variance (ANOVA) comparing experimental data with control groups.

3. Results

The anti-angiogenic activities were evaluated by using either VEGF or FGF2 stimulated angiogenesis in the CAM model. This assay is a well established, fast and simple, real time *in vivo* assay, and is suitable for the evaluation of potential angiogenesis inhibitors or stimulators in a living organism [17]. The carbon materials used did not have any significant effects on basal angiogenesis as compared to control group (Fig. 1 and Table 1). In VEGF induced angiogenesis, new blood vessels were stimulated by the addition of VEGF as shown in the representative illustration in Fig. 2b. Increased neovascularization is observed in comparison to CAM treated with PBS buffer alone – Fig. 2a. When CAM was treated with MWNTs, fullerenes or graphite, angiogenesis was inhibited to a significant extent (Table 2). Graphite at 100 µg showed the strongest inhibition of angiogenesis (64.3 ± 7.5%), followed by fullerenes (49.6 ± 7.6%) and MWNTs (37.1 ± 21.1%).

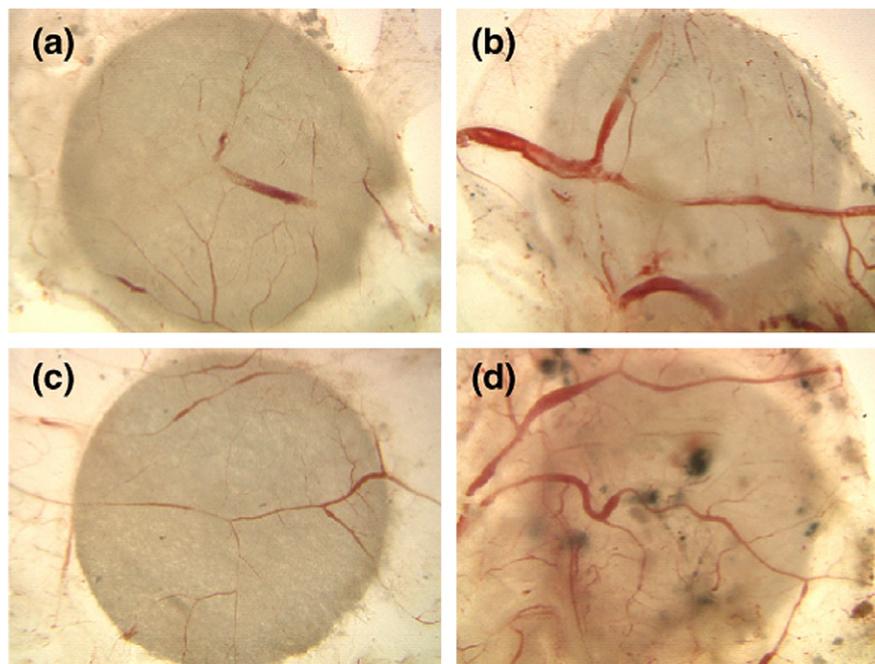


Fig. 1. Representative microscopic images of (a) PBS control; (b) graphite; (c) fullerene; (d) MWNTs – treated CAM models.

Table 1
Effect of MWNTs, fullerenes and graphite on basal angiogenesis in the CAM model

Treatment	Mean branch points \pm S.E.M.
PBS (control)	71.0 \pm 5
FGF2 (1 μ g/ml)	155.5 \pm 8**
VEGF (2 μ g/ml)	161.2 \pm 11**
MWNTs (100 μ g)	81.3 \pm 8
Fullerene (100 μ g)	72.0 \pm 5
Graphite (100 μ g)	75.3 \pm 5

Data represent mean \pm S.E.M., $n = 7$ per group, $P < 0.001$ as compared to control.

FGF2 induced angiogenesis is shown in Fig. 3b, and the PBS control in Fig. 3a. Graphite and MWNTs showed approximately equal levels of inhibition ($\sim 36\%$) of FGF2 induced angiogenesis, followed by fullerenes ($\sim 20\%$). A dose response relationship in the FGF2 system is clearly illustrated by an increase from $36 \pm 15.8\%$ inhibition to $76.4 \pm 9.6\%$ inhibition with 100 μ g versus 1 mg of MWNTs, respectively. Binding studies, performed by adding growth factors to carbon-based materials in PBS, removing these materials by filtration and measuring residual growth factors, showed that the growth factors were not physically adsorbed to the carbon materials.

4. Discussion

To our knowledge, this is the first report of the anti-angiogenesis activity associated with carbon materials such as MWNTs, fullerenes and graphite. Next, we performed adsorption experiments to assess whether VEGF or FGF2 bound to these carbon materials physically. Neither VEGF nor FGF2 adsorbed on to any of the carbon materials studied. Gold nanoparticles reportedly inhibit VEGF165-induced proliferation of HUVEC cells by binding the sulfur/amines present in

Table 2
Percentage inhibition of VEGF/FGF2 induced angiogenesis up on treating with MWNTs, fullerenes and graphite

Treatment groups	Branch points \pm S.E.M.	%Inhibition \pm S.E.M.
PBS	64.5 \pm 8.6	
VEGF (2 μ g/ml)	163.6 \pm 12.6	
VEGF + MWNTs (100 μ g)	129.8 \pm 21.9	37.1 \pm 21.1*
VEGF + fullerenes (100 μ g)	116.8 \pm 7.9	49.6 \pm 7.6**
VEGF + graphite (100 μ g)	101.5 \pm 7.8	64.3 \pm 7.5**
FGF2 (1.5 μ g/ml)	168.3 \pm 9.3	
FGF2 + MWNTs (100 μ g)	130.8 \pm 16.4	36.1 \pm 15.8*
FGF2 + fullerenes (100 μ g)	147.3 \pm 21.7	20.2 \pm 12.9*
FGF2 + graphite (100 μ g)	130.3 \pm 12.3	36.6 \pm 11.8*
FGF2 + MWNTs (1 mg)	91.5 \pm 8.2	76.4 \pm 9.6**

Data represents mean \pm S.E.M., $n = 8$ per group.

* $P < 0.05$.

** $P < 0.01$.

the heparin binding domains of VEGF165 [18]. While it is unclear how carbon inhibits angiogenesis, it is not the result of the physical adsorption of the growth factors on carbon materials. Carbon materials might block the binding of VEGF and FGF2 with their receptors interfering with signal transduction. No toxicity of these materials was observed in the fertilized eggs used in these experiments. Upon the termination of the study and after 3 days of exposure to the carbon materials, the embryos were examined and compared in all groups. There was no effect on the survival of the embryos or any apparent adverse effects based on gross examination. More detailed mechanistic studies are currently underway in our laboratories. MWNTs can also be loaded with anti-cancer agents (small or macromolecular drug) through either by chemical or physical attachment methods. The intrinsic anti-angiogenic activity of MWNTs might afford a synergistic cancer treating strategy.

Carbon based nanomaterials including nanotubes and fullerenes are widely proposed for various applications of

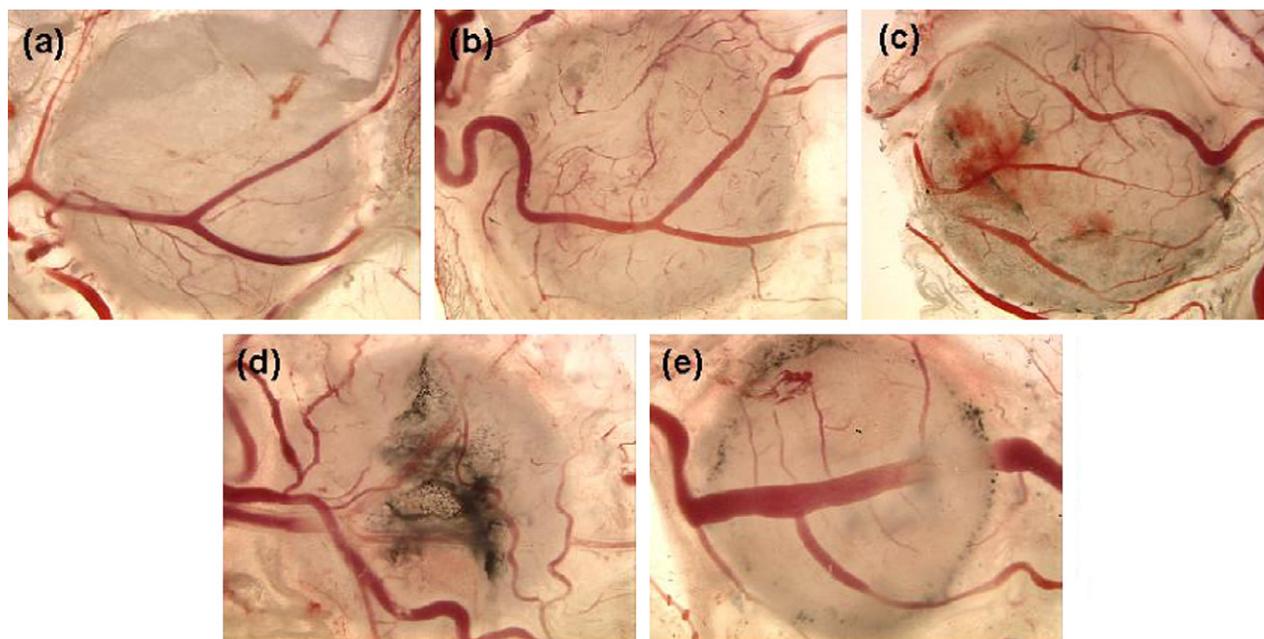


Fig. 2. Representative microscopic images of (a) PBS control; (b) VEGF; (c) VEGF – MWNTs; (d) VEGF – graphite; (e) VEGF – fullerene treated CAM models.

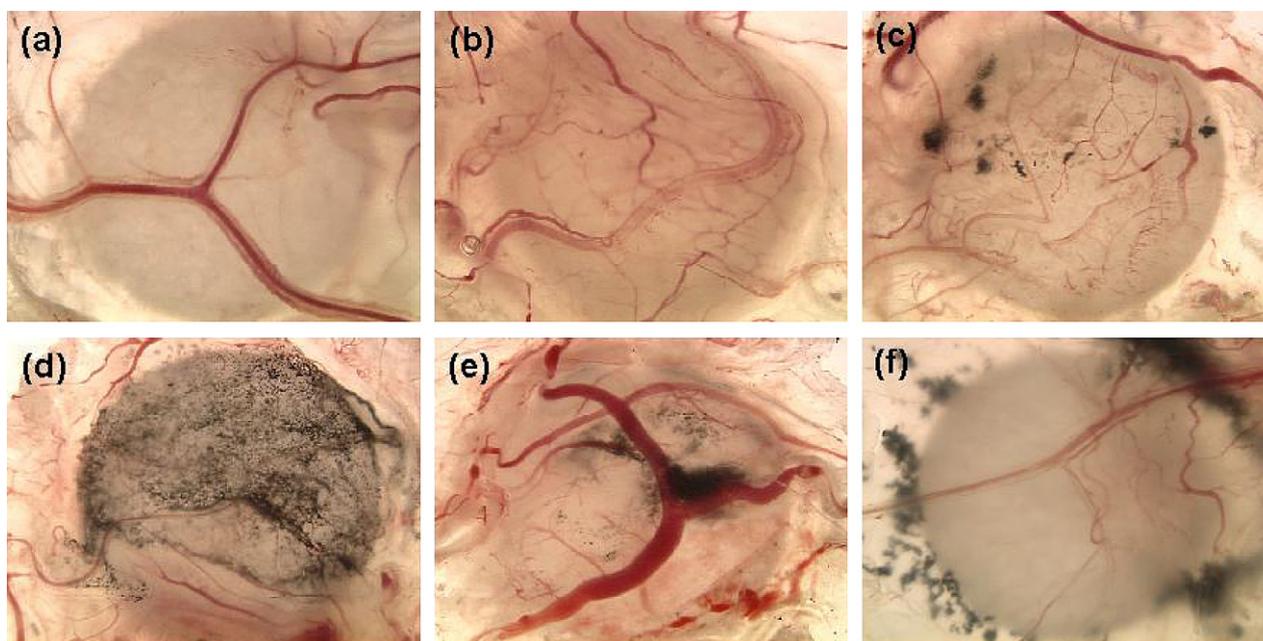


Fig. 3. Representative microscopic images of (a) PBS control; (b) FGF2; (c) FGF2 – MWNTs (100 µg); (d) FGF2 – graphite; (e) FGF2 – fullerene; (f) FGF2 – MWNTs (1 mg) treated CAM models.

biotechnology and biomedicine. In this report, we have evaluated the anti-angiogenic activity of pristine MWNTs, fullerenes and graphite. In both VEGF and FGF2 induced angiogenesis in the CAM model, carbon materials showed significant inhibition of angiogenesis. To the extent of our knowledge, this is the first observation of the anti-angiogenic activity of carbon materials. There is a relatively greater potency in inhibiting VEGF *versus* FGF2 in the CAM model by the various carbon materials.

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