Journal of the American College of Cardiology
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Published by Elsevier Science Inc.

Vol. 35, No. 6, 2000
ISSN 0735-1097/00/$20.00
PII S0735-1097(00)00575-1

Ten-fold Augmentation of Endothelial Uptake of Vascular Endothelial Growth Factor With Ultrasound After Systemic Administration

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OBJECTIVES In this study, the feasibility of delivering and enhancing the uptake of vascular endothelial growth factor (VEGF) into the intact endothelium by using ultrasound (US) facilitation was determined.

BACKGROUND A limitation of tissue-targeted drug delivery is the need for direct arterial cannulation. We postulate a mechanism by which agents injected intravenously may be targeted to a tissue using US and ultrasonic contrast agents.

METHODS We used a rat model to test the ability of US and an ultrasonic contrast agent perflurocarbon exposed sonicated dextrose albumin (PESDA) to increase uptake of VEGF in the myocardium. Continuous wave Doppler US (0.6 W/cm^2 at 1 MHz for 15 min) was applied to the chest wall overlying the myocardium during intravenous injection with either VEGF (100 μg/kg) alone or a combination of VEGF and PESDA (0.1%). Control rats had VEGF infused without US or PESDA. The VEGF uptake was measured quantitatively in the heart, lung, liver and kidneys by enzyme-linked immunosorbent assay (ng/g of tissue) and morphologically by fluorescence microscopy.

RESULTS There was an eight-fold increase in VEGF uptake in the heart by US alone (16.86 $\pm$ 1.56 vs. 2.11 $\pm$ 0.953 ng/g of tissue, $p < 0.0001$) and a 13-fold increase with US + PESDA (26.78 $\pm$ 2.88 vs. 2.11 $\pm$ 0.953 ng/g of tissue, $p < 0.0001$) compared with control rats. Fluorescence microscopy revealed deposition of VEGF in the endothelium of small intramyocardial arterioles.

CONCLUSIONS These results show a marked increase in endothelial VEGF uptake with US and US + PESDA. Thus, US may be used to augment endothelial VEGF uptake 10-fold to 13-fold. (J Am Coll Cardiol 2000;35:1678–86) © 2000 by the American College of Cardiology

Ischemic heart disease is the major cause of morbidity and mortality in the U.S. With the aging of the population and with the increased survival of patients with coronary artery disease, increasing numbers of patients have diffuse coronary artery disease that is not amenable to traditional methods of revascularization, that is, bypass surgery or angioplasty (1). Newer methods of therapy are being investigated in these patients, including transmyocardial revascularization, percutaneous transmyocardial revascularization and infusion of growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor to promote angiogenesis.

Vascular endothelial growth factor is a 46-kDa glycoprotein that is a mitogen for endothelial cells (2). A number of observations point to the importance of VEGF in angiogenesis. Vascular endothelial growth factor and its receptors are expressed in cells surrounding the expanding vasculature during embryonic development, and VEGF is predominantly produced in tissues acquiring new capillary networks (3,4). Furthermore, the ability of VEGF to induce formation of collateral circulation has been demonstrated in a rabbit ischemic leg model (5) and human peripheral vascular disease (6). More recently, VEGF was administered by intracoronary infusion in a group of patients with reversible thallium defects who were not candidates for coronary artery bypass grafting or percutaneous transluminal coronary angioplasty (CABG or PTCA) (7). These approaches may usher in a new era of treating vascular disease both coronary and peripheral by improving blood supply with formation of new blood vessels.

A major limitation of current methods of tissue-targeted drug delivery involves invasive arterial cannulation. We hypothesized that drugs injected via a peripheral vein may be targeted to a particular tissue using ultrasound (US) and

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Manuscript received February 19, 1999; revised manuscript received November 18, 1999, accepted January 12, 2000.

Abbreviations and Acronyms

CABG = coronary artery bypass grafting
ELISA = enzyme-linked immunosorbent assay
FITC = fluorescein isothiocyanate
KHB = Krebs-Henseleit bicarbonate
PBS = phosphate buffered saline
PCNA = proliferating cells
PESDA = perfluorocarbon-exposed sonicated dextrose albumin
PTCA = percutaneous transluminal coronary angioplasty
rhVEGF = recombinant human VEGF
US = ultrasound
VEGF = vascular endothelial growth factor
vWF = von Willibrand factor

ultrasonic contrast agents. We sought to test the hypothesis that US with or without contrast microbubbles could enhance uptake of VEGF to the myocardium using both Langendorff and intact rat models. We also studied varying power levels of US to determine the relationship of US acoustic power to uptake of VEGF.

METHODS

In vitro studies. A Langendorff rat heart preparation was used to test the effect of US with and without contrast agent on endothelial uptake of VEGF.

LANGENDORFF RAT HEART PREPARATION. Sixteen week-old male Wistar-Kyoto rats (Taconic Farm; Germantown, New York) were anesthetized with sodium pentobarbital (20 mg/kg intraperitoneally). After intravenous administration of sodium heparin (2,000 IU), the hearts were removed and rapidly immersed in ice-cold Krebs-Henseleit bicarbonate (KHB) solution. After trimming and weighing, the hearts were mounted on a non recirculating Langendorff apparatus and perfused at 80 mm Hg (coronary perfusion pressure) at flow rates of 2.5 ml/min while the temperature was maintained at 37°C. Perfusion media (KHB) was pH balanced and iso-osmolar to plasma. The medium was equilibrated by continual gassing with 95% O_2 and 5% CO_2, and adjusted to pH of 7.35 to 7.40 for the duration of the experiment. All animal experiments were approved by the Animal Review Committee and the Research and Programs committee of the Institution.

PERFLUOROCARBON-EXPOSED SONICATED DEXTROSE ALBUMIN. Perfluorocarbon-exposed sonicated dextrose albumin (PESDA), a solution of microbubbles containing perflurocarbon (<6 μm in diameter) enveloped in an albumin shell, is produced by sonicating a solution of dextrose containing albumin and perflurocarbon gases (8). Three parts of 5% dextrose and one part of 5% human serum albumin (total 16 ml) were drawn into a 35-ml syringe (Monoject). Each dextrose albumin sample was hand agitated with 8 ± 2 ml of a fluorocarbon gas (decafluorobutane), and the sample was then exposed to electromechanical sonication at 20 kHz for 80 ± 5 s. The maximal energy output from the sonicating horn was 550 W. Energy output was manually adjusted using a digital scale to achieve 25% ± 3% of the maximum output during the entire sonication time (124 ± 15 W). During this process, the albumin formed bubbles creating a relatively rigid shell. The mean size of the PESDA microbubbles produced in this manner was 4.7 ± 0.2 μm. The PESDA is in the form of microbubbles both at room temperature and at 37°C in the body (8). The PESDA was obtained from one of the coauthors (T.P.). The safety of PESDA has already been demonstrated in humans (8).

FLUORESCEIN LABELING OF VEGF. One hundred micrograms of fluorescein isothiocyanate (FITC) mixture (Calbiochem) was mixed with 500 μg of rhVEGF (recombinant human VEGF) to achieve a conjugation ratio of 5:1 (protein:FITC) and incubated at room temperature for 2 h. The mixture was dialyzed overnight with a dispodialyzer (Spectrapor) to remove free FITC molecules. Fluorescein-labeled VEGF was mixed with PESDA at room temperature for 60 min prior to injection into animals.

STUDY PROTOCOL. Recombinant human vascular endothelial growth factor ($rhVEGF_{165}$; Genentech Inc.; San Francisco, California) alone or in combination with PESDA (0.1%) was infused into the aorta via a cannula. In the group receiving VEGF with PESDA, VEGF was preincubated with PESDA for 60 min at room temperature. The perfusion rate (precalibrated before each use) was maintained at 2.5 ml/min using a perfusion pump (Pharmacia & Upjohn; Bridgewater, New Jersey). Animals were randomized into six groups. Then 100 μg/kg of VEGF labeled with fluorescein (FITC labeling kit; Calbiochem; La Jolla, California) was infused over 15 min in appropriate groups. The dose of

Table 1. Relationship Between Ultrasound Power and Endothelial Vascular Endothelial Growth Factor Uptake With a Plateau Beyond 0.6 to 0.8 W/cm^2

	Group 1 (n = 2)	Group 2 (n = 2)	Group 3 (n = 2)	Group 4 (n = 4)	Group 5 (n = 2)	Group 6 (n = 2)
Ultrasound (W/cm^2)	—	0.2	0.4	0.6	0.8	1.0
MPa	—	0.164	0.194	0.328	0.394	0.419
VEGF (ELISA)	2.35 ± 0.67	9.37 ± 1.98	18.58 ± 2.46	23.12 ± 3.95	25.46 ± 2.78	26.48 ± 3.98

ELISA = enzyme-linked immunosorbent assay; Mpa = peak negative pressure; VEGF = vascular endothelial growth factor.

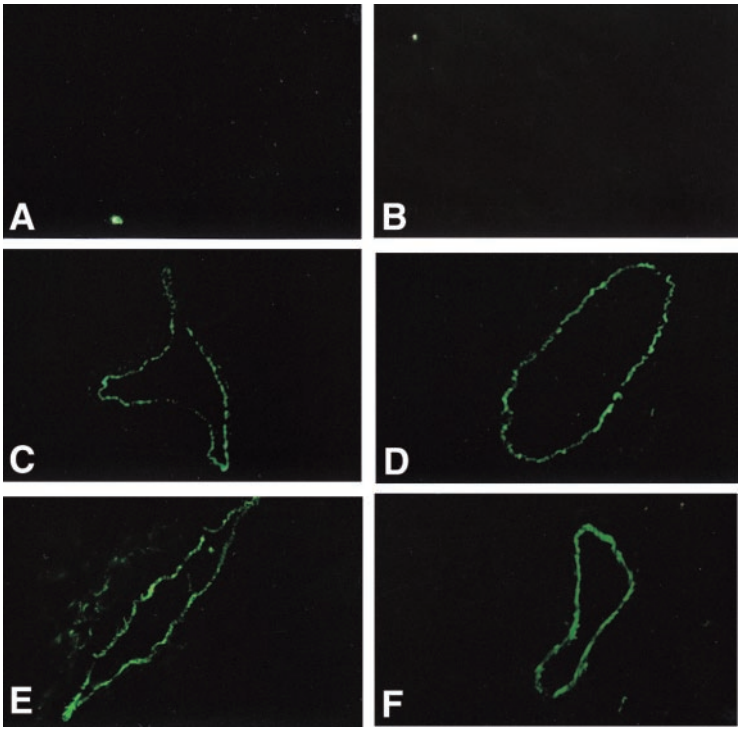

Figure 1. Fluorescent microscopy (100×) revealed marked enhancement of VEGF uptake with US and PESDA. **A)** reveals minimal endothelial uptake of VEGF after intravenous injection of VEGF without ultrasound. **B)** reveals minimal fluorescence in the endothelium after injection of buffer solution without VEGF. **C)** reveals significant deposition of VEGF in the endothelial cells with PESDA and US, using a Langendorff preparation. **D)** reveals significant deposition of VEGF in the endothelial cells with PESDA and US, after intravenous injection in short-term in vivo experiments. **E)** reveals significant deposition of VEGF in the endothelial cells with US alone, using a Langendorff preparation. **F)** reveals significant deposition of VEGF in the endothelial cells with US alone, after intravenous injection in short-term in vivo experiments.

VEGF is based on previous animal experiments (9–13). During the infusion of VEGF, US energy (1.0 MHz at 0.6 W/cm^2 power or 0.328 peak negative pressure [MPa]) was delivered to the myocardium directly with a sonicator US generator, 1.9 cm in diameter (model ME 720; Mettler Electronics; Anaheim, California) for 15 min. This is the expected time that PESDA remains in circulation after an intravenous injection (8). The myocardial area covered by the US probe was approximately 0.5 to 0.6 cm^2. The peak negative pressure generated was measured with a hydrophone (Sonic Technologies, model S/N804-077; Grass Valley, California) in our laboratory. The acoustic pressure measurement was made by placing a calibrated membrane hydrophone (spot size approximately 0.5 mm) in front of the transducer and recording the amplitude of the received signal. A table was then used to calculate the acoustic pressure from the received voltage. After the treatment period, the myocardium was perfused with fresh KHB solution for 5 min to clear any unbound intraluminal collections of PESDA or VEGF. Uptake of VEGF was assessed quantitatively using enzyme-linked immunosorbent assay (ELISA) and fluorometry, and morphologically by fluorescence microscopy (Fig. 1). The effect of US on endothelium was assessed by electron microscopy.

To determine the optimum ultrasonic acoustic power for enhancement of VEGF uptake, US was applied with various acoustical outputs (0.2, 0.4, 0.6, 0.8, and 1.0 W/cm^2) and VEGF uptake measured in the myocardium (Table 1). For the dose response experiments, VEGF was coadministered with PESDA.

TISSUE COLLECTION, PREPARATION AND HISTOLOGY. After perfusion was discontinued, the heart was removed and a coronal section of the midventricular region was excised, mounted in Tissue Tek optimal cutting temperature compound (Miles Inc.; Elkhart, Indiana), immediately frozen under liquid nitrogen and stored at −80°C. Frozen sections were cut at 5 μm for fluorescence microscopy while an adjacent section (2.5 μm) was stained with hematoxylin-eosin. One piece was preserved in phosphate buffered saline (PBS) while the other was immediately submerged into liquid nitrogen and stored for frozen sections for microscopy. Frozen sections were viewed immediately using a fluorescence microscope (Dialux 22; Leitz, Germany), and morphologic localization of FITC-labeled VEGF was examined. Photographs of the fluorescent sections were taken (Kodak Elite II 400 ASA film).

ELISA. For ELISA, a section of the heart muscle was homogenized in lysis buffer (0.1 M sodium carbonate at a pH of 9.6, 0.1% NP-40, 1 μM leupeptin, 1 mM phenyl-

Table 2. Enhanced Endothelial VEGF Uptake With US and Contrast Agent After Intravenous Injection*

	Group 1 (n = 4)	Group 2 (n = 4)	Group 3 (n = 4)	Group 4 (n = 4)
PESDA	−	−	+	+
Ultrasound	−	+	−	+
VEGF	+	+	+	+
Heart (VEGF ng/g)	2.11 ± 0.953	**16.86 ± 1.564***	2.01 ± 0.895	**26.78 ± 2.880***
Lung (VEGF ng/g)	2.10 ± 0.094	4.14 ± 0.186	2.21 ± 0.078	4.89 ± 0.102
Liver (VEGF ng/g)	2.52 ± 0.056	2.56 ± 0.065	2.39 ± 0.049	2.47 ± 0.067
Kidney (VEGF ng/g)	2.36 ± 0.198	2.96 ± 0.112	2.03 ± 0.137	2.71 ± 0.239

*As measured by enzyme-linked immunosorbent assay in an in vivo model.
PESDA = perfluorocarbon-exposed sonicated dextrose albumin; VEGF = vascular endothelial growth factor.

methane sulphonyl fluoride, and 10 μl/ml ethylenediamine tetraacetic acid). The homogenate was then centrifuged at 750*g*, at 4°C for 6 min. Protein G microtiter plates (Pierce Immunochemicals; Rockford, Illinois) were coated with monoclonal antibody to VEGF (Genentech, Inc.). Superblock buffer (Pierce) was added to these plates and incubated for 60 min, to reduce nonspecific binding. The samples and VEGF standards (R & D Systems) were diluted in carbonate buffer and added to these plates. The samples were incubated at 37°C for 2 h. Standards consisted of two-fold dilutions of $rhVEGF_{165}$, starting at 0.3 ng per well to 38.4 ng per well. The samples were removed and the wells were washed in washing buffer (PBS at a pH of 7.4, 0.05% polysorbate 20 [Tween 20]). A specific polyclonal antibody to VEGF (R & D Systems; Minneapolis, Minnesota) labeled with horseradish peroxidase was added to the wells and incubated for 1 h. The wells were again washed with washing buffer. One hundred microliters of a 50:50 mixture of hydrogen peroxide and tetramethylbenzidine was added to the wells. To this, 100 μl of 0.01 N HCl was added, and optical density was determined by spectrophotometry at 450 nm using a spectrophotometer (Beckman DU-64). A standard curve was plotted using known concentrations of standard VEGF and the amount of VEGF in the samples was extrapolated from this standard curve using a polynomial regression equation. All ELISA results are expressed as nanograms of VEGF per gram tissue. The accuracy of the assay was ascertained by adding known amounts of VEGF to the wells as control.

ELECTRON MICROSCOPY. Transmission electron microscopy was performed at magnifications between 2,800× and 10,000×, on sections of the left ventricle adjacent to the US transducer. After treatment with US and perfusion of KHB solution, the hearts were randomized into three fixation protocols. Control hearts (n = 2) were fixed without application of US or PESDA. In treated groups (n = 2 for each group), hearts were fixed during application of US (0.6, 0.8, 1.0 W/cm^2) and with or without 0.1% PESDA added to the buffer. In all groups, 0.1 M phosphate-buffered 2.5% glutaraldehyde in sucrose was infused for 5 min at 2.5 ml/min to fix the myocardium. Twelve sections from each ventricle were analyzed.

In vivo experiments (short-term). Sixteen week-old Wistar-Kyoto rats were anesthetized with 0.2-ml intraperitoneal pentobarbital. Appropriate amounts of VEGF (100 μg/kg) and PESDA were injected in 0.2-ml volume using a 25-gauge needle into the femoral vein. The dose of PESDA was based on a 15-ml blood volume, 10 ml/min cardiac output and an expected in vivo PESDA dose to reach 0.1% concentration in circulation. Ultrasound (0.1 MHz at 0.6 W/cm^2 power or 0.328 peak negative pressure amplitude [MPa]) was applied directly to the chest wall for 15 min. The animals were killed with 0.8 ml intraperitonial pentobarbital sodium (Nembutal), and the heart, liver, kidneys and the lungs were removed. The heart was perfused with Krebs buffer in a Langendorff preparation for 5 min to clear the intravascular space of blood and unbound VEGF. The amount of VEGF taken up by the myocardium, liver, kidneys, and lung was measured quantitatively by ELISA and morphologically by fluorescence microscopy.

In vivo experiments (long-term). Sixteen week-old spontaneously hypertensive rats (Taconic Farm; Germantown, New York), fed high-cholesterol diet, were anesthetized with 0.2-ml intraperitoneal pentobarbital. Appropriate amounts of VEGF (100 μg/kg) and PESDA were injected in 0.2-ml volume using a 25-gauge needle into the tail vein, and US was applied to the chest wall for 15 min as described above. Vascular endothelial growth factor was injected once a week for four weeks. The animals were euthanasized with 0.8-ml intraperitonial pentobarbital (Nembutal), and the hearts were removed. The hearts were fixed in formalin for immunohistochemistry. The different groups are shown in Table 2.

Immunohistochemistry. SAMPLE PREPARATION. The formalin-fixed rat hearts were taken and a 2-mm-thick cross section was taken through the mid portion of the heart, allowing visualization of the right ventricle, left ventricle, coronary arteries and septum. The tissue was then processed

and paraffin embedded according to standard laboratory procedures. Five sections were cut from each block at 4 μm, collected onto electrostatically charged slides and baked at 60°C for 60 min. Two sections were stained with hematoxylin-eosin and Movat pentachrome, while the remaining three were stained immunohistochemically for PCNA (proliferating cells), von Willibrand factor (vWF) for endothelial cells and alpha-smooth muscle actin for smooth muscle cells as described (14).

IMMUNOHISTOCHEMICAL STAINING PROCEDURE. The paraffin was removed from the baked tissue sections in three changes of xylene, and the tissue was hydrated through graded alcohols before rinsing in PBS. Immunohistochemical staining was performed (Jung Histostainer; Leica, Illinois), with processing occurring at 30°C. The first stage of the procedure involved the application of 1% hydrogen peroxide solution in methanol for 5 min to remove any endogenous peroxidase present in the tissue section. For mouse monoclonal primary antibodies (PCNA and alpha-smooth muscle actin), a blocking solution comprising a 1:10 dilution of normal rabbit serum (Dako; Carpenteria, California) in PBS was then added for 10 min before application of the primary antibody. The required dilutions of antibody were prepared using 1% bovine serum albumin in PBS. Incubation occurred at 30°C for 60 min, and a 1:200 dilution of biotinylated rabbit antimouse polyclonal antibody (Dako) was added for a further 30 min. The antibody was labeled using an Elite avidin/biotin/peroxidase complex (Vector Laboratories; Burlingame, California) applied for 30 min. The final stage comprised the addition of 3,3-diaminobenzidine as a chromogen (DAB Kit; Vector Laboratories). Between each step, the slides were rinsed twice for 2 min in PBS. For the polyclonal antibody (vWF), a similar procedure was followed except that normal swine serum instead of rabbit serum was used as a blocking agent, and biotinylated swine antirabbit polyclonal antibody was used as the link. Following staining, the sections were rinsed in distilled water, counterstained with hematoxylin, dehydrated and cleared in xylene; a coverslip was placed on the section. The negative control sections were treated in an identical manner to the test tissue except that 1% BSA in PBS was added instead of primary antibody. Cell counts are expressed as number of positively stained cells per 200× magnification field. Ten fields were counted on each slide. Isolated endothelial and smooth muscle cells (not associated with a vascular lumen) were counted to determine migrating cells.

Statistics. Data are expressed as mean ± SD. Groups were compared using an analysis of variance and contrasts for pairwise comparisons were made using Sheffe's post hoc test (Statistica for Windows 4.0; Statsoft, Inc.). A $p < 0.01$ was used to determine statistical significance.

RESULTS

In vitro experiments. Combinations of VEGF, PESDA and US were used and results are summarized. Using ELISA, there was an eight-fold increase in VEGF taken up by the myocardium using US alone (19.23 ± 1.78 vs. 2.35 ± 0.67 ng/g of tissue, $p < 0.0001$) and a ten-fold increase with the combination of PESDA and US (23.12 ± 2.95 vs. 2.35 ± 0.67 ng/g of tissue, $p < 0.00001$). Fluorescent microscopy revealed a marked increase in deposition in VEGF with US alone (Fig. 1E) and with the combination of US and PESDA (Fig. 1C). Vascular endothelial growth factor is predominantly detected lining the endothelium of intramyocardial arterioles as demonstrated by hematoxylin-eosin staining of an adjacent section (Fig. 2).

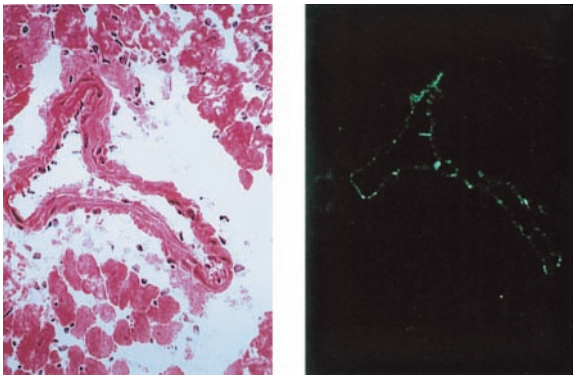

Figure 2. Hematoxylin-eosin staining of an adjacent section revealed that the fluorescein-labeled VEGF is predominantly deposited in the endothelium of intramyocardial arterioles (100×).

Determination of optimum power. Table 1 shows the response of incremental US power on uptake of VEGF. There was a significant increase in VEGF uptake with combination of US and PESDA at all power levels of US energy compared with controls. There was a dose-dependent increase in the amount of VEGF uptake with increasing power until 0.8 W/cm^2, with a subsequent plateau. There was no significant increment in VEGF uptake beyond a power level of 0.6 W/cm^2 (Table 1).

Electron microscopy. As cavitational and thermal effects of US are more evident when US power is raised, it was necessary to determine whether deleterious effects were produced at power levels that enhanced the uptake of VEGF with or without PESDA. When US was applied at 1.0 W/cm^2 (0.419 MPa) for 15 min with or without PESDA, severe coagulative necrosis (swollen cells, decreased optical density of the cytoplasm, fatty changes in cytoplasm, condensation of nuclear chromatin and dissolution of the nucleus by karyolysis) of the small capillaries was detected (Fig. 3A). In addition, large gaps appeared between endothelial cells, exposing the subendothelial matrix. These changes were less pronounced with a power of 0.8 W/cm^2 (0.394 MPa) as seen in Figure 3B, and no such changes were seen in hearts exposed to 0.6 W/cm^2 (0.328 MPa) (Fig. 3C). Figure 3D shows a section from a control rat heart.

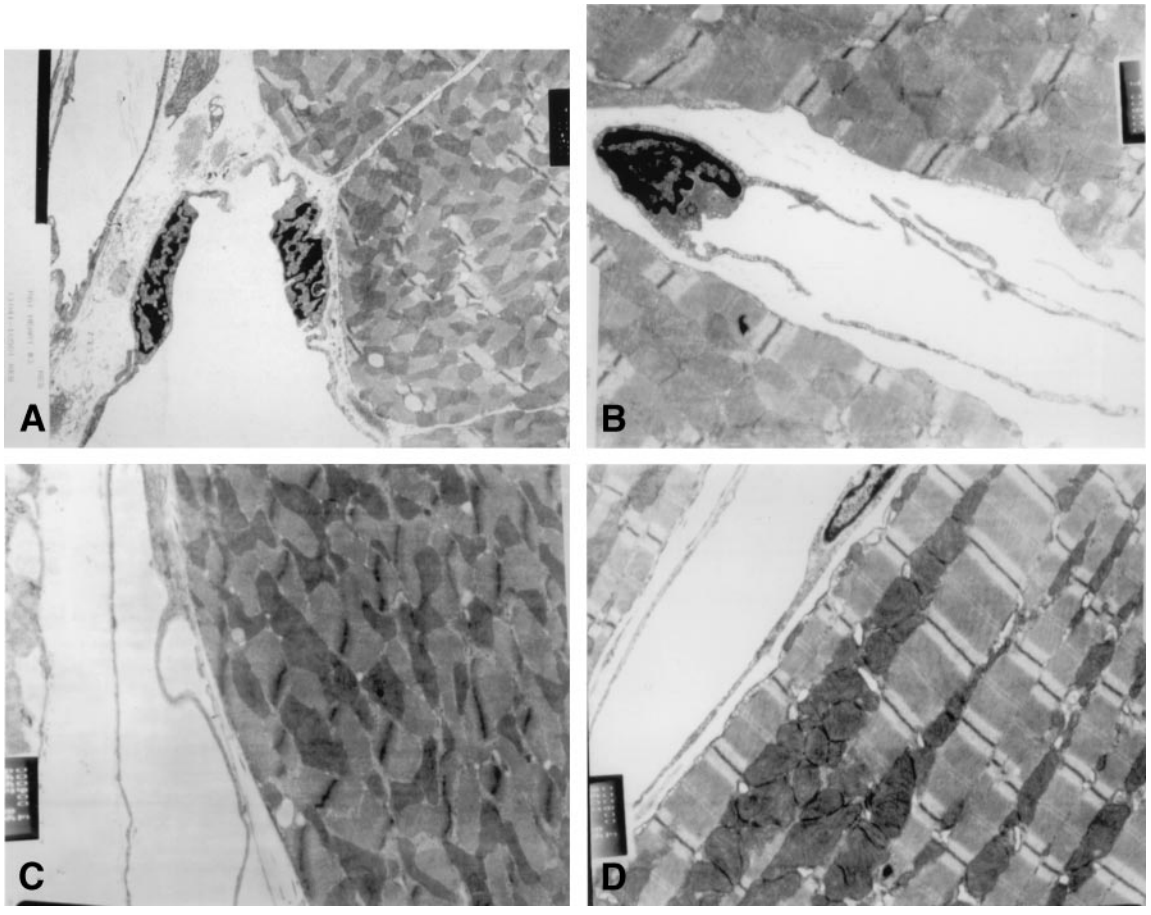

Figure 3. **A)** shows extensive coagulative necrosis and endothelial disruption with US power of 1.0 W/cm^2 without PESDA (4,800×). **B)** shows endothelial pore formation with US power of 0.8 W/cm^2 without PESDA (5,400×). **C)** (8,000×) shows no significant endothelial changes with US power of 0.6 W/cm^2 as compared with control rat heart sections. **D)** shows a control rat heart section (4,800×).

In vivo experiments (short-term). The different groups for in vivo studies are summarized in Table 2. There was approximately an eight-fold increase in VEGF uptake by US alone (16.68 ± 1.56 vs. 2.11 ± 0.953 ng/g of tissue, $p < 0.0001$) and a 13-fold increase in uptake with PESDA and US (26.78 ± 2.88 vs. 2.11 ± 0.95 ng/g of tissue, $p < 0.00001$). Fluorescent microscopy revealed that most VEGF is taken up by the arterial wall. Figure 1D shows enhancement of VEGF uptake by US and PESDA, and Figure 1f shows enhancement with US alone in the endothelium by intramyocardial arterioles.

In vivo experiments (long-term). There was approximately a four-fold increase in endothelial cell count (17.91 ± 1.63 vs. 4.83 ± 0.89, $p < 0.0001$) in the VEGF + US group as compared with VEGF administration alone, as measured by vWF immunohistochemistry and three-fold increase in smooth muscle cell count (12.70 ± 2.47 vs. 3.41 ± 0.67, $p < 0.001$), as measured by smooth muscle cell actin antibody. There was no significant increment in endothelial or smooth muscle cell count with the combination of US and PESDA compared with US alone. Figure 4 shows the vWF and smooth muscle cell immunohistochemistry in the control and US groups. There was no difference in PCNA count between the groups.

DISCUSSION

Summary of results. This study shows a significant increase in endothelial uptake of VEGF with US alone or a combination of US and PESDA. The optimum acoustic power for enhancement of VEGF uptake was 0.6 to 0.8 W/cm^2. Commercial echocardiography machines in clinical use deliver an acoustic output up to 0.44 W/cm^2 in the pulsed and color Doppler mode (15). In our study, even with an acoustical output within the limits of clinical echocardiography machines, there was an eight-fold increase in VEGF uptake. There was some increase in uptake in the lung with US related probably to US scatter while delivering US to the heart.

Review of the literature. A number of therapeutic modalities currently involve the delivery of medications directly to the diseased tissue rather than systemically. Takeshita et al.

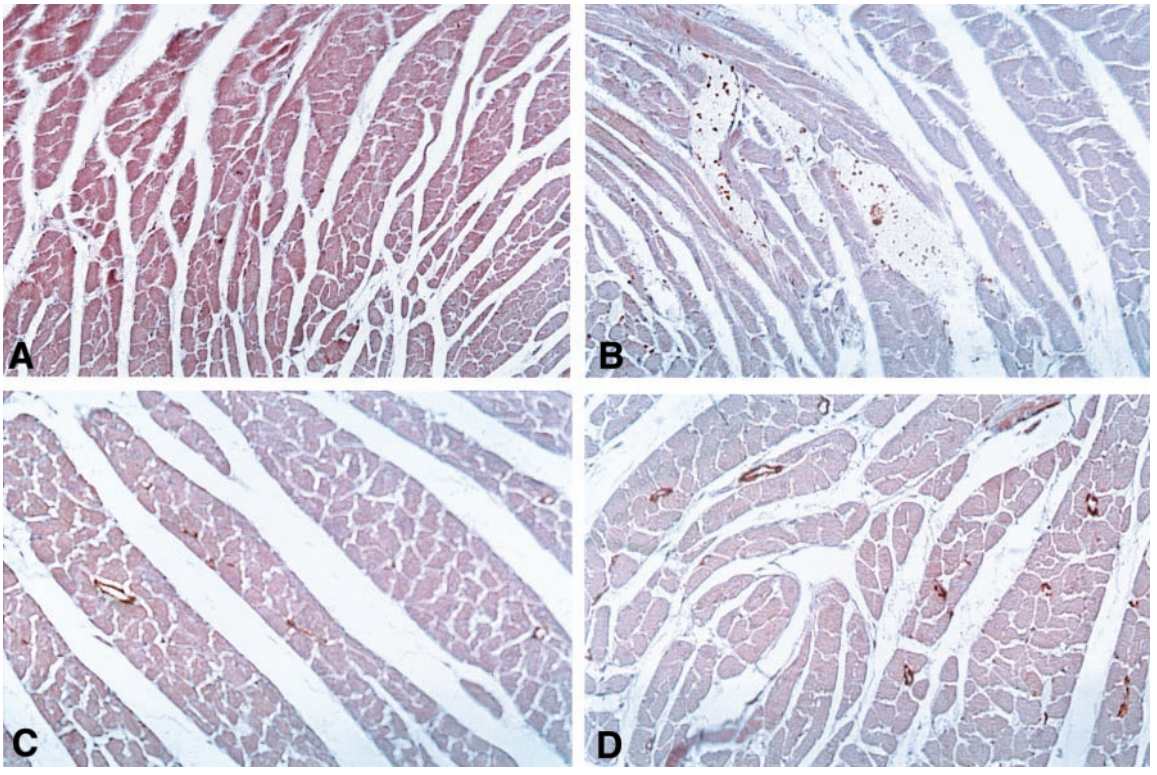

Figure 4. Immunohistochemistry showed marked increase in endothelial cell and smooth muscle cell count (not associated with a vascular lumen) in groups treated with US during administration of VEGF compared with groups treated with VEGF alone. **A)** shows endothelial cells as shown by vWF staining in a rat heart from an animal treated with VEGF alone and **B)** shows endothelial cells in a rat heart treated with VEGF + US. **C)** shows smooth muscle cells in a rat heart from an animal treated with VEGF alone and **D)** shows a rat heart from an animal treated with VEGF + US. Positively stained cells were counted at 200×.

(5,16) have shown that VEGF administered locally to ischemic muscle in patients with peripheral vascular disease via the artery perfusing that area results in angiogenesis and improvement in blood supply. Recently, Schumaker et al. (17) applied VEGF locally to ischemic muscle during coronary artery bypass surgery with significant improvement in perfusion. The ability of VEGF to induce formation of collateral circulation has been demonstrated in a rabbit ischemic leg model (4) and human peripheral vascular disease (5). More recently, VEGF was administered by intracoronary infusion in a group of patients with reversible thallium defects who were not candidates for CABG or PTCA. There was overall improvement in nuclear perfusion scans in these patients (6). However, one of the major limitations of this approach is the necessity of either invasive arterial cannulation of a diseased artery with all the risks involved or open heart surgery as done by the German group (17). Peripheral intravenous injection without myocardial targeting may induce undesirable angiogenesis and vascularity in other organs, as angiogenesis with VEGF has also been described in the absence of ischemia (18). Also there is an approximately six-fold increase in VEGF receptor up-regulation with hypoxia-ischemia (19) and this may not be enough to increase VEGF uptake sufficiently by itself after systemic administration to cause adequate angiogenesis. By targeting VEGF to specific tissue, lower doses of this agent may be used with decreased risk of side effects such as hypotension or angiogenesis in nonischemic tissue and with increased uptake to the ischemic site. The approach discussed in this article is a noninvasive method to direct delivery of a therapeutic agent to a specific tissue.

Mechanism of US enhancement of VEGF uptake. Vascular endothelial growth factor is known to bind serum proteins (8) and we postulated that one of the binding proteins is albumin. It has previously been demonstrated that the ability of albumin to bind oligonucleotides remains intact in PESDA (20). The basic hypothesis of the study was that by destroying microbubbles containing VEGF in the coronary circulation, a large amount of VEGF would be released into the coronaries and taken up by the heart. However, this hypothesis failed to explain the phenomenon of significant increase in VEGF uptake with US alone that we observed. Perfluorocarbon-exposed sonicated dextrose albumin had minimal incremental effect on enhancing VEGF uptake. Most of the effect appears to be due to US energy, which may be locally enhanced by the presence of "acoustically active" microbubbles. Ultrasound-mediated enhancement of transfection has been reported in the literature (21–24) and is attributed to ultrasonic cavitation

of cells. Since the transfected cells went on to express the transfected gene, it appears that US at 1 to 2.25 MHz did not cause any irreversible damage to the cellular machinery. We have previously reported more than 10-fold increase in endothelial uptake of oligonucleotides with US alone (25). Reversible membrane damage with resealing of the holes (or pores) has been reported with US (23). Electron microscopy of the rat heart during application of US revealed disruption or pore formation of the membrane of the endothelial cells as well as increased gap between endothelial cells with an acoustic power of 0.8 to 1.0 W/cm^2. However no structural changes were evident with a power of 0.6 W/cm^2, which was the power used for experiments in our study. It is conceivable that there is micropore formation or increased permeability with a power of 0.6 W/cm^2 that could not be detected by TEM at the magnification used by us and that facilitated endothelial VEGF uptake. The mechanism of US-facilitated enhancement is not simply the bursting of microbubbles to deposit therapeutic agents. Instead US is likely to be actively involved in a more complex interplay among the therapeutic substance, echocontrast agent, the nature of the US energy and the target tissue characteristics. It is also possible that VEGF is combining with the albumin in the echocontrast mixture, which then binds to the endothelial surface. It has been shown previously that albumin is incorporated into the luminal glycoprotein surface of vascular endothelium as a means to augment resistance to solute flux (26). The exact mechanism, however, remains to be determined. Skyba et al. (27) have demonstrated rupture of $\leq$7-μm microvessels (mostly capillaries) with microbubble destruction during US exposure. Based on this, Price et al. (28) have devised an elegant model of tissue-targeted delivery of colloidal particles and red blood cells by targeted microbubble destruction with US.

Conclusions. It should be noted that we have not demonstrated actual binding of VEGF to PESDA and it is possible that an alternative mechanism may explain the enhancement of VEGF uptake with PESDA. A previous report showed that there was marked enhancement of transfection of a plasmid DNA into cultured cells by a combination of microbubble (albunex) and US, suggesting that microbubbles may play a role in reversible pore formation in endothelial cells during the process of cavitation (sonoporation) (23). It is conceivable that the incremental effect of PESDA with US seen in these studies is due to more efficient sonoporation rather than binding of VEGF.

Study Limitations. We used spontaneously hypertensive rats for the long-term experiments rather than the Wistar-Kyoto rats, as myocardial ischemia has been reported in spontaneously hypertensive rats. Studies in a variety of animal models using VEGF have shown angiogenesis only in the presence of ischemia (29,30). To demonstrate the biological effects of VEGF, we wanted to use an animal with some degree of myocardial ischemia. Our weekly injections of VEGF for four weeks in a spontaneously hypertensive model revealed approximately a four-fold increase in endothelial and smooth muscle cell count without a change in PCNA, suggesting that the increased cell count is due to cell migration rather than proliferation. During angiogenesis, migration always precedes proliferation by approximately 24 h (31,32), and it is possible that we killed the animals prior to the stage of proliferation. This does demonstrate that the VEGF delivered using this technique is biologically active. Although ischemia has been demonstrated in SHR hearts (33), it is not a well characterized model of ischemia. A longer-term in vivo experiment in a well developed ischemic model needs to be performed to evaluate the efficacy of delivered VEGF. Another potential limitation is the need to use a 25-gauge needle for intravenous injections in a rat model as most microbubbles may have been destroyed and minimized the benefits of PESDA in the in vivo studies.

Clinical applications. This study needs to be validated in a larger animal model and eventually in humans. Ultrasound with or without contrast agents promises to be a feasible way to target drug delivery to specific organs noninvasively. This technique may be used not only to deliver drugs to the myocardium but also may be applied to any organ accessible to US. The technique described may have a special role in giving high does chemotherapeutic agents to specific organs in malignancies to reduce toxic reactions.

Acknowledgments

Recombinant human VEGF$_{165}$ and VEGF monoclonal antibody was a gift from Genentech, Inc., South San Francisco, California.

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