Short Communication

Nicotine and Cotinine Up-Regulate Vascular Endothelial Growth Factor Expression in Endothelial Cells

Brian S. Conklin,*† Weidong Zhao,* Dian-Sheng Zhong,* and Changyi Chen*

From the Department of Surgery,* Emory University School of Medicine, Atlanta; and the School of Mechanical Engineering,† Georgia Institute of Technology, Atlanta, Georgia

Cigarette smoking is an important risk factor for both vascular disease and various forms of cancer. Vascular endothelial growth factor (VEGF) is an endothelial-specific mitogen that is normally expressed only in low levels in normal arteries but may be involved in the progression of both vascular disease and cancer. Some clinical evidence suggests that cigarette smoking may increase plasma VEGF levels, but there is a lack of basic science studies investigating this possibility. We show here, using an intact porcine common carotid artery perfusion culture model, that nicotine and cotinine, the major product of nicotine metabolism, cause a significant increase in endothelial cell VEGF expression. VEGF mRNA levels were compared between groups using reverse transcriptase-polymerase chain reaction, whereas protein level changes were demonstrated with Western blotting and immunohistochemistry. Our results showed significant increases in endothelial cell VEGF mRNA and protein levels because of nicotine and cotinine at concentrations representative of plasma concentrations seen in habitual smokers. VEGF immunostaining also paralleled these results. These findings may give a clue as to the mechanisms by which nicotine and cotinine from cigarette smoking increase vascular disease progression and tumor growth and metastasis. (Am J Pathol 2002, 160:413–418)

Cigarette smoking is one of the leading causes of preventable morbidity and premature mortality in the United States. It is the single most important risk factor for peripheral vascular disease and abdominal aortic aneurysm, and greatly increases the risk of stroke and heart attack. More than 85% of lung cancers are attributed to smoking, and several other cancers are also associated with smoking. However, the mechanisms by which cigarette smoking contributes to vascular disease and cancer are not yet completely understood.

The major active component of cigarette smoke is nicotine. Nicotine has been shown to have a variety of effects on vascular biology that may contribute to atherosclerosis. At levels similar to those in the blood plasma of habitual smokers, nicotine has been shown to induce changes in expression of various atherosclerosis-related genes in endothelial cells including endothelial nitric oxide synthase, angiotensin I-converting enzyme, tissue-type plasminogen activator, platelet-derived growth factor, and basic fibroblast growth factor. Nicotine has also been shown to cause morphological changes in endothelial cells, increased endothelial cell death, and enhanced transendothelial transport of plasma macromolecules. However, the mechanisms of increased endothelial turnover and permeability because of nicotine have not yet been completely elucidated.

The role of nicotine in tumorigenesis has not been as clearly defined as its role in vascular disease. It has been demonstrated that nicotine may enhance proliferation and inhibit apoptosis of certain types of human cancer cell lines. Another important mechanism by which nicotine may contribute to tumor growth is by the enhancement of angiogenesis, a process necessary for tumor growth and metastasis. It has been demonstrated that nicotine produces a proliferative response in endothelial cells and thus it is possible that it may also enhance angiogenesis and metastasis, although this has not directly been shown.

One common factor that could contribute to both increased endothelial permeability and turnover related to
vascular disease and increased angiogenesis and tumor growth in cancer is vascular endothelial growth factor (VEGF). It has been demonstrated that VEGF is expressed in high levels in human atherosclerotic lesions whereas it is expressed in very low levels in nondiseased arteries. Furthermore, VEGF is expressed in the majority of cancers and blocking its activity has been shown to inhibit growth of experimental tumors in vivo.

Despite the known correlations between cigarette smoking and vascular disease and cancer, there is little research on the effects of nicotine on VEGF expression, an important factor in the progression of both diseases. This study was therefore undertaken to determine the effects of nicotine and the major product of its metabolism, cotinine, on the expression of VEGF in endothelial cells. To this end, we used an intact porcine artery ex vivo perfusion culture model. Our results demonstrated, for the first time, that nicotine and cotinine, in doses similar to those seen in the plasma of habitual smokers, significantly increase endothelial cell VEGF mRNA and protein expression.

Materials and Methods

Reagents

Dextran, phosphate-buffered saline solution, Tris-buffered saline solution, Tri-Reagent, β-actin monoclonal antibodies, nicotine, cotinine, and Tween 20 were obtained from Sigma Chemical Co. (St. Louis, MO). Dulbecco’s modified Eagle’s medium was from Life Technologies, Inc. (Grand Island, NY), antibiotic-anti-mycotic was from Mediatech Inc. (Herndon, VA), and the one-step reverse transcriptase-polymerase chain reaction (RT-PCR) kit was from Promega Corp. (Madison, WI). The protein assay kit and precast polyacrylamide gels were obtained from Bio-Rad Laboratories (Hercules, CA). Antibodies against VEGF were from Santa Cruz Biotechnology (Santa Cruz, CA), the horseradish peroxidase-conjugated anti-rabbit secondary antibodies and the Enhanced Chemiluminescence kit were from Amersham Life Sciences (Buckinghamshire, England). VEGF and β-actin primers were synthesized by Operon Technologies Inc. (Alameda, CA).

Vascular Perfusion Culture

The vascular perfusion culture system and methods have been described in detail previously. Porcine common carotid arteries were harvested from 6- to 8-month-old male and female domestic pigs and mounted on an adjustable cannula. Branches were then ligated and the vessels were stretched to their physiological length. The perfusion culture systems were assembled, filled with culture medium (Dulbecco’s modified Eagle’s medium with 5% 207-kd dextran to increase the viscosity) and placed in standard cell culture incubators. Lastly, the flow loop tubing was connected to the pump, the pressure transducers were connected to the monitors, and the flow was initiated. Vessels were cultured for 24 hours with 100 mmHg of pressure, 150 ml/minute flow, and as controls, with $10^{-7}$ mol/L nicotine, or with $10^{-7}$ mol/L cotinine.

After culturing, vessels were removed from the systems and a ring was cut from the middle of each vessel for histological analysis. The endothelial cells were removed from the remaining portions of each vessel for mRNA and protein analysis as described previously by gently scraping the luminal surface with a scalpel blade and resuspending the cells in Tri-Reagent. Great care was taken to not disrupt the internal elastic lamina during scraping to avoid smooth muscle contamination. Purity of endothelial cells insulated in this manner was determined to be ~95 to 100% by culturing cells for 1 week after removal from vessels and examining for smooth muscle cell and fibroblast contamination morphologically (data not shown).

RT-PCR

Total RNA was isolated from endothelial cells from cultured vessels using Tri-Reagent. RNA concentrations were determined by absorption at 260 nm. A one-step RT-PCR kit was used according to the manufacturer’s instructions. The same amount of total RNA (0.2 µg) was used from each sample. The upstream VEGF primer was 5’-ATGCGCATCAAAACCTCACC-3’ and downstream primer was 5’-ATCTGGTTCGGAAACGCTG-3’. β-actin was used as an internal RNA loading control for each sample with an upstream primer of 5’-CTTCCTGGGCGATGATCCCTCCT-3’ and a downstream primer of 5’-GATCTTTGATCTTCATCGT-3’. The thermal cycle conditions used were as follows: 45 minutes at 48°C (reverse transcription), 2 minutes at 94°C (denaturation), followed by PCR cycling of 94°C for 30 seconds (denaturation), 60°C for 1 minute (annealing), and 68°C for 2 minutes (extension), and lastly a final extension for 7 minutes at 68°C. Using these conditions, the linear ranges of amplification for VEGF and β-actin products were determined by performing RT-PCR for both with 0, 8, 15, 20, 24, 26, 28, 30, 32, 36, and 38 cycles. The middle of the linear range for both VEGF and β-actin was determined to be ~24 cycles (data not shown). PCR was thus performed for 24 cycles for all experiments. Controls were performed with no RT-enzyme for both VEGF and β-actin to demonstrate the lack of DNA contamination in samples. VEGF and β-actin RT-PCR products were combined and were resolved on a 2% agarose gel stained with ethidium bromide. Semi-quantitative analysis was performed using an Alpha-Imager gel documentation system and software (Alpha Innotech Co., San Leandro, CA). The density of each VEGF band was divided by the density of its respective β-actin band to account for variations in loading.

Western Blotting

Endothelial cell protein from vessels after culturing was isolated using Tri-Reagent and resuspended in 50 µl of 10 mol/L urea. Protein concentrations were determined using a protein assay kit. The same amount of endothelial protein (3 µg) from each sample was resolved by sodium
The VEGF/1B. These results showed a significant 30% increase in cotinine. Equal amounts of endothelial cell protein from changes in VEGF expression because of nicotine and cotinine were gently washed and then re-blotted for β-actin using a monoclonal β-actin antibody and a horseradish peroxidase-conjugated secondary antibody. Blots were developed using the avidin-biotin complex immunoperoxidase procedure as described previously.13 Sections were counterstained with hematoxylin.

Immunohistochemistry

After culturing, a 4-mm long ring was cut from the middle of each vessel and fixed overnight in 10% neutral buffered formalin. Sections were paraffin-processed, embedded, and 5-μm thick sections were cut. Immunohistochemical staining was performed for VEGF using a monoclonal anti-VEGF antibody. Color was developed using the avidin-biotin complex immunoperoxidase procedure as described previously.13 Sections were counterstained with hematoxylin.

Statistics

Statistical differences were determined using the two-tailed Student’s t-test and analysis of variance, with significance considered to be P < 0.05 unless otherwise noted. Results are reported as mean ± SEM.

Results

The effects of nicotine and cotinine on VEGF mRNA expression were investigated. Porcine common carotid arteries were perfusion cultured for 24 hours as controls, with 10−7 mol/L nicotine, or with 10−7 mol/L cotinine. Subsequently, endothelial RNA was isolated and RT-PCR was performed for VEGF and β-actin for each sample (Figure 1A). The endothelial VEGF/β-actin optical density ratio for control vessels (n = 6) was 0.583 ± 0.042, 0.758 ± 0.034 (n = 6) for nicotine-treated vessels, and 0.790 ± 0.033 (n = 6) for cotinine-treated vessels (Figure 1B). These results showed a significant 30% increase in the VEGF/β-actin optical density ratio for endothelial cells from vessels treated with nicotine relative to controls (P < 0.01), and a significant 36% increase for endothelial cells from vessels treated with cotinine relative to controls (P < 0.01), thus demonstrating that both nicotine and cotinine increase VEGF mRNA expression in endothelial cells at levels representative of those seen in the plasma of habitual smokers.

We next performed Western blot analysis on protein from endothelial cells after perfusion culture to determine changes in VEGF expression because of nicotine and cotinine. Equal amounts of endothelial cell protein from each sample were loaded and β-actin was used as an internal loading control for each sample (Figure 2A). The resulting VEGF protein bands were ~20 to 24 kd in molecular mass, corresponding to the correct size as stated by the antibody manufacturer. The average VEGF/β-actin optical density ratio for endothelial cells from control vessels was 0.744 ± 0.040 whereas for nicotine-treated vessels the ratio was 1.128 ± 0.095 (n = 4) and for endothelial cells from cotinine-treated vessels the ratio was 1.239 ± 0.16 (n = 4) (Figure 2B). The changes in the VEGF/β-actin ratio because of treatment of vessels with nicotine and cotinine were significantly different from control vessels (analysis of variance, P < 0.05). These changes corresponded to a 52% increase (P < 0.05, t-test) in endothelial cell VEGF expression in vessels treated with nicotine and a 66% increase (P < 0.05, one-tailed t-test) in endothelial cell VEGF expression in vessels treated with cotinine. These results agree well with the VEGF mRNA results, demonstrating that both nicotine and cotinine significantly increase VEGF protein expression in endothelial cells.

Changes in VEGF protein expression because of nicotine and cotinine were also demonstrated with immuno-
hitochemical staining of vessel segments after perfusion culture. After culturing, vessel sections were cut and stained with an anti-VEGF antibody. Results showed very low endothelial VEGF staining intensity in control vessels (Figure 3A), whereas nicotine-treated (Figure 3B) and cotinine-treated (Figure 3C) vessels showed much more positive, intense staining of VEGF in the endothelium.

Discussion

These results demonstrate, for the first time to our knowledge, that nicotine and cotinine cause an increase in VEGF expression in endothelial cells in concentrations representative of those seen in the blood plasma of habitual smokers. At the same concentration, nicotine caused a 52% increase and cotinine caused a 66% increase in endothelial VEGF protein expression, indicating that cotinine may have an even more pronounced effect than nicotine. The nicotine concentration in the plasma of habitual smokers is in the range of 6.2 to 19 × 10⁻⁵ mol/L, whereas plasma concentrations of cotinine range from 5.9 to 15 × 10⁻⁷ mol/L.²⁰,²¹ Plasma cotinine concentrations are generally ~10 times higher than nic-
otine concentrations because of the long half-life of cotinine compared to that of nicotine.\textsuperscript{22} In this study we used an ex vivo perfusion culture system with intact porcine common carotid arteries cultured with physiological pressure and flow levels. This model was chosen because it is more physiological than cell culture models because of retention of different cell types, and allows nicotine and cotinine to be studied separately, which cannot be done in vivo because cotinine is produced by nicotine metabolism in the liver. Deeper studies are warranted including combining nicotine and cotinine to investigate possible synergisms in their effects as well as dose-response studies. Such studies are currently underway in our laboratory. The increased endothelial VEGF expression because of cotinine and cotinine shown here could have important implications in both vascular disease and cancer.

Previous clinical studies on the effects of smoking on VEGF expression have been inconsistent. One study showed no difference in plasma VEGF levels between smokers and non-smokers.\textsuperscript{23} However, in another study, the majority of the control patients with detectable plasma VEGF levels were smokers.\textsuperscript{24} Furthermore, it was shown in this same study that smoking acutely raises VEGF levels in 22.6% of habitual smokers. In another study, patients with lung cancer that smoked had an average of 33% more plasma VEGF compared to patients with lung cancer that did not smoke.\textsuperscript{25} Likewise, a recent study demonstrated that nicotine caused a significant increase in serum VEGF levels in a mouse model as well as an increase in tumor growth and atherosclerosis.\textsuperscript{26}

Nicotine has been shown to alter the expression of other growth factors in vascular cells. It has been shown, for example, that nicotine induces basic fibroblast growth factor and platelet-derived growth factor release in endothelial cells and increases proliferation.\textsuperscript{7,8} Cotinine has also been shown to stimulate basic fibroblast growth factor production in smooth muscle cells.\textsuperscript{27} In fact, cotinine has also been shown to be a more powerful mitogen for smooth muscle cells than nicotine.\textsuperscript{28} Likewise, the results presented here demonstrated that at the same concentration, cotinine caused a larger increase in endothelial cell VEGF expression than nicotine. Taken together, these data may suggest that cotinine is even more harmful than nicotine and should be studied more widely.

The exact mechanisms by which nicotine and cotinine may up-regulate VEGF expression in endothelial cells is not known at this time. However, it seems likely that it may involve interactions between nicotine and the nicotine-sensitive acetylcholine receptors (nAChR), which have recently been shown to be present on endothelial cells.\textsuperscript{29} Cotinine may also have interacted through nAChR because it has been shown to bind to the \( \alpha_7 \) nAChR subtype in Xenopus oocytes.\textsuperscript{30} Additionally, it has been demonstrated \textit{in vitro} that blocking of the nAChR with hexamethonium in endothelial cells treated with nicotine abolished the increase in DNA synthesis seen in cells treated with nicotine alone.\textsuperscript{13} It was also shown \textit{in vivo} that blocking nAChR with hexamethonium in a nicotine-treated mouse hind-limb ischemia model eliminated the increase in capillary formation seen with nicotine treatment alone, possibly indicating a decrease in nicotine-stimulated VEGF secretion.\textsuperscript{26} Nicotine has been shown in cultured bovine adrenal medullary chromaffin cells to activate the fibroblast growth factor-2 (FGF-2) gene through nAChR via tyrosine phosphorylation of cytoplasmic and nuclear proteins including 50- to 55-kd promoter-binding factors.\textsuperscript{31} It is possible that nicotine and cotinine stimulation of VEGF production in endothelial cells may use a similar pathway. This is a current area of investigation in our laboratory.

VEGF was only recently shown to be present in human atherosclerotic plaques.\textsuperscript{15} Since then, studies have shown it to be involved in the genesis of vascular lesions in research models. Recently, it was demonstrated in hypercholesterolemic mice and rabbits that low VEGF doses cause an increase in plaque macrophage levels and endothelial cell content relative to controls without VEGF treatment.\textsuperscript{32} VEGF also increases the permeability of endothelial cells and may thus contribute to the atherosclerotic process by increasing transport into the vessel wall of low-density lipoprotein, fibrin, and other atherogenic macromolecules. One mechanism by which VEGF may increase vascular permeability is by increasing endothelial turnover.\textsuperscript{15,33} VEGF may also exert its influence on vascular permeability by regulating endothelial tight junction molecules ZO-1 and occludin.\textsuperscript{34,35} Increased endothelial VEGF expression because of nicotine and cotinine from cigarette smoking may thus contribute vascular disease progression in part by increasing endothelial permeability and turnover.

In addition to its role in atherogenesis, VEGF has been shown to be important in tumor growth and metastasis. Elevated plasma VEGF levels have been shown in patients with lung cancer and prostate cancer, and VEGF is expressed in high levels in most tumors.\textsuperscript{16,25,36} VEGF is known to be a powerful stimulator of angiogenesis, a process necessary for the growth of tumors.\textsuperscript{16} Blocking of VEGF in animal models of cancer with monoclonal VEGF antibodies has been demonstrated to suppress tumor growth.\textsuperscript{37} Clinical studies have also shown a positive correlation between tumor VEGF expression and metastasis in several forms of carcinomas.\textsuperscript{38,39} It has also been demonstrated in mice that inhibitors of the VEGF-receptor tyrosine kinases significantly reduced lung metastasis in a renal cell carcinoma model.\textsuperscript{40} The increase in endothelial VEGF expression shown in the present study could therefore be an important mechanism by which nicotine and cotinine increase cancer growth and metastasis.

In conclusion, we have demonstrated that nicotine and cotinine cause a significant increase in endothelial cell VEGF expression. This was demonstrated in intact porcine common carotid arteries using a vascular perfusion culture model that retains \textit{in vivo} cell types and structure and is thus more physiological than cell culture models. Increased VEGF expression because of nicotine and cotinine may have important implications in vascular disease by increasing endothelial turnover and permeability to atherogenic macromolecules such as low-density lipoprotein as well as increasing tumor growth and metastasis because of increased angiogenesis.
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References