Published in final edited form as:

Life Sci. 2007 May 30; 80(24-25): 2347–2351.

Angiogenesis and the role of the endothelial nicotinic acetylcholine receptor

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Abstract

An endothelial nicotinic acetylcholine receptor (nAChR) mediates endothelial proliferation, survival, migration and tube formation in vitro, and angiogenesis in vivo. Exogenous nicotine stimulates this angiogenic pathway. This action of nicotine may contribute to tumor angiogenesis and tumor growth; atherosclerotic plaque neovascularization and progression; and other tobacco-related diseases. The endothelial nAChR mediates an angiogenic pathway that is interdependent with growth factor mediated pathways, as shown by pharmacological and molecular studies. The characterization of this new angiogenic pathway may provide a new therapeutic avenue for disorders of insufficient or pathological angiogenesis.

A serendipitous observation

In 1999, my laboratory group was examining factors that interfere with therapeutic angiogenesis. As a specialist in vascular medicine, I was interested to know why some individuals are not capable of generating an adequate angiogenic response to coronary or peripheral arterial obstruction. I asked one of the students in the laboratory (James Jang) to help me test the hypothesis that nicotine could interfere with angiogenesis. This hypothesis was based on the observation that smokers have an impairment of endothelial vasodilator function, possibly related to decreased bioactivity or synthesis of nitric oxide (NO) and prostacyclin (PGI₂) (Celermajer et al, 1993; Reinders et al, 1986). This is significant because endothelium derived NO and PGI₂ are critically involved in a number of angiogenic processes including endothelial cell survival, proliferation and migration. Indeed, we had previously shown that the endogenous NO synthase inhibitor, asymmetric dimethylarginine (ADMA) was anti-angiogenic (Jang et al, 2000). Notably, ADMA is elevated in disease states associated with impaired angiogenesis such as hypercholesterolemia (Cooke, 2004). Thus, we hypothesized that tobacco smoking may elevate plasma ADMA levels, and thus impair endothelium dependent vasodilation and angiogenesis.

There are over 4,000 different chemicals in tobacco smoke (U.S. Department of Health and Human Services, 1989), which makes it difficult to determine the individual effects of these agents on angiogenesis. Accordingly, we focused on nicotine, the major bioactive component of tobacco. We hypothesized that nicotine would impair the NO synthase pathway, and inhibit angiogenesis. Indeed, other workers had suggested that nicotine could be toxic to endothelial cells (Suzuki et al, 1994). However, these observations were made using doses of nicotine that were above clinically relevant concentrations.

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To determine the effect of nicotine on angiogenesis, we first used a disc angiogenesis assay. A pellet of polyvinyl alcohol sponge was loaded with nicotine solution, coated with a copolymer for slow elution, and placed into a sponge disc, with filter paper backing on each side. When the disk is placed subcutaneously in mice, vessels enter through the rim of the disk. To our great surprise, in the nicotine-treated disk, fibrovascular growth was accelerated. The angiogenic effect of nicotine was equipotent to basic fibroblast growth factor (FGF) in this model (Heeschen et al, 2001). These observations caused us to consider what role nicotine-induced angiogenesis might play in tobacco-related disease.

Role of angiogenesis in tobacco-related diseases

Cigarette smoking is a major cause of preventable disease, disability, and premature death. It is estimated that more than 400,000 Americans die of smoking-related causes annually, primarily due to cardiovascular disease and cancer (Gray et al, 2001). There are multiple mechanisms by which tobacco promotes disease, and these interact with other environmental factors, as well as with diverse genetic determinants. However, neovascularization is a common pathological feature of both cardiovascular disease and cancer.

Role of Angiogenesis

Tumor angiogenesis plays a major role in tumor growth and metastasis. In order to grow beyond a critical size, tumors must recruit endothelial cells from the surrounding stroma to form their own endogenous microcirculation. Tumor progression occurs in two phases: a prevascular, and a vascular phase. The transition between the two phases is termed the “angiogenic switch” (Hanahan and Folkman, 1996). The angiogenic switch involves either the induction of positive regulators and/or the loss of negative regulators (Carmeliet and Jain, 2000). Among these, members of the vascular endothelial growth factor (VEGF) and angiopoietin (Ang) families appear to have a predominant role (Yancopoulos et al, 2000). The list of molecules that have been shown to be active as pro- and anti-angiogenic factors contains cytokines, chemokines, enzymes and their inhibitors, extracellular matrix components, coagulation factors, soluble cytokine receptors, prostaglandins, adipocyte lipids, and some inorganic ions. The primary target of pro- and anti-angiogenic stimuli is the endothelial cell. These stimuli induce precise sequential alterations in endothelial cell functions, including proliferation, migration, cell-cell and cell-matrix interactions that lead to the sprouting of new blood vessels. There is convincing evidence that inhibition of angiogenesis halts tumor growth and metastatic spread (Folkman, 2006). An antibody directed against VEGF has been approved for use in patients with colon cancer, after clinical trials showed improved survival in treated patients (Hurwitz et al, 2004).

Neovascularization and atherosclerotic cardiovascular disease

Intriguingly, pathological angiogenesis also appears to be involved in growth of atherosclerotic plaque. Advanced plaque is associated with an abundant plexus of microvessels originating from the vasa vasorum of the affected artery (Kwon et al, 1998). Neovascularization of plaque has been implicated in intra-plaque hemorrhage. Furthermore, recent data indicates that plaque neovascularization may contribute directly to plaque growth. In this regard, Folkman’s group showed that endostatin and other anti-angiogenic agents could block the progression of plaque growth in Apo E deficient mice (Moulton et al, 1999). In the same animal model, Celletti et al (Celletti et al, 2001) found that vascular endothelial growth factor (VEGF) promotes plaque neovascularization and growth. Notably, there is a strong correlation between macrophage infiltrate, and the number of plaque vessels, in aortic atheroma of the apo E deficient hypercholesterolemic mouse (Moulton et al, 2003). Furthermore, inhibition of plaque angiogenesis reduces macrophage accumulation in the atheroma.

Accordingly, there is increased interest to image plaque neovascularization in humans. Carotid and coronary artery plaque neovascularization have been imaged in patients using duplex ultrasonography and magnetic resonance imaging techniques (Moreno et al, 2006). Studies using these imaging techniques are underway to assess the prevalence of plaque neovascularization; its contribution to plaque progression and instability; and its response to therapeutic interventions.

To summarize, pathological angiogenesis plays a critical role in the growth and progression of cancer and atherosclerosis. We propose that nicotine mediates pathological angiogenesis in tobacco-related diseases.

A role for cholinergic angiogenesis in malignancy

To determine if nicotine could enhance tumor angiogenesis, we used the Lewis lung cancer model. Systemic administration of nicotine (100 g/ml in drinking water) achieved plasma nicotine levels in the mice (200–300 ng/ml) similar to those observed in moderate smokers. Sixteen days after subcutaneous implantation of the cancer cells, and treatment with nicotine or vehicle, tumor growth in the nicotine group markedly exceeded that in the vehicle treated group (Heeschen et al, 2001). This acceleration of tumor growth in the nicotine group corresponded with a 5-fold increase in capillary density within the tumor tissue. The effects of nicotine on tumor growth was not due to a direct effect on tumor cell proliferation, as nicotine had no effect on tumor cell number in vitro. The effects of nicotine on tumor growth could be blocked by nAChR antagonists, indicating that nicotine was exerting its effects via this cholinergic pathway (Heeschen et al, 2001).

In a later study, we investigated the effects of second hand smoke (SHS) on tumor growth and angiogenesis, and determined if these effects were mediated by nicotine. Mice were exposed to clean room air or SHS for 17 days after subcutaneous implantation of Lewis lung cancer cells (Zhu et al, 2003). In addition, some animals were administered mecamylamine by osmotic minipumps. Tumor size and weight were increased by SHS. Tumor capillary density was increased about 2-fold in mice exposed to SHS, in association with higher levels of plasma VEGF and circulating endothelial progenitor cells. Mecamylamine partially inhibited the effects of SHS on these angiogenic processes, and nearly abolished the effect of SHS on tumor capillary density. These studies indicated that nicotine mediated the effects of second hand smoke to increase tumor angiogenesis in this model.

Contribution of nicotine to plaque neovascularization

We used a hypercholesterolemic murine model of atherosclerosis (ApoE^{-/-} mice) to determine if nicotine affected plaque progression and neovascularization (Heeschen et al, 2001). In hypercholesterolemic mice, atheromatous lesions form in the thoracic aorta. In comparison to vehicle, treatment with nicotine (100ug/ml in the drinking water) for 20 weeks increased by twofold the number of vascularized plaques, and doubled plaque area in the apo E deficient mice. The effect of nicotine was independent of plasma lipid values, and was blocked by rofecoxib, a known inhibitor of angiogenesis.

In subsequent studies, we investigated the mechanisms by which nicotine accelerated pathological angiogenesis. Pioneering work by the groups of Grando, Wessler and Kawashima had highlighted the physiological importance of the nAChR on non-neuronal cells (Wessler et al, 1999; Grando et al, 1995; Kawashima et al, 1989). Activation of these receptors in non-neuronal cells can induce mitosis, differentiation, organization of the cytoskeleton, cell-cell interactions, locomotion, and migration. Of course, acetylcholine (not nicotine) is the endogenous agonist of these receptors. Intriguingly, acetylcholine is synthesized and stored in endothelial cells indicating that it might act as an autocrine factor in the vascular system

(Wessler et al, 1999;Grando et al, 1995). Indeed, we find that inhibition of acetylcholinesterase accelerates endothelial cell migration *in vitro* (Figure 1). The experiment illustrated by this figure indicates that acetylcholine is being produced by endothelial cells, and is stimulating migration. Notably, neostigmine (by increasing the bioavailability of acetylcholine) augments the migragenic effect of low dose VEGF. This study suggests that endogenous VEGF and acetylcholine have additive effects on angiogenic processes.

Molecular mechanisms of cholinergic angiogenesis

Indeed, endothelial nAChRs modulate blood vessel formation and remodeling, and mediate the effect of nicotine (or endogenous acetylcholine) on angiogenesis (Heeschen et al, 2002). In an *in vitro* angiogenesis model, we observed that increasing concentrations of the non-selective antagonist mecamylamine completely and reversibly inhibited endothelial network formation (Heeschen et al, 2002). The selective $\alpha 7$ -nAChR antagonist α -bungarotoxin also inhibited endothelial tube formation. An important role for this receptor subtype is also suggested by the finding that $\alpha 7$ -nAChR is upregulated during proliferation of subconfluent endothelial cells, or by hypoxia *in vitro*. In a murine model of hindlimb ischemia, we found that the $\alpha 7$ -nAChR was upregulated on endothelial cells in vessels of the ischemic limb (Heeschen et al, 2001). Pharmacological inhibition of this nAChR, or genetic disruption of $\alpha 7$ -nAChR expression, significantly inhibited angiogenesis in a number of animal models, including angiogenesis in response to inflammation, ischemia, and tumor growth (Heeschen et al, 2001;Heeschen et al, 2002). These findings indicate that there is an endogenous pathway for angiogenesis, which is modulated by ischemia, and which is activated by endogenous acetylcholine (or exogenous nicotine). The $\alpha 7$ -nAChR plays an important role in cholinergic angiogenesis. However, it is important to note that other nAChR subtypes are expressed in endothelial cells, and are most recent studies with siRNA knockdown technology indicate that these subtypes may have reinforcing or modulating effects in cholinergic angiogenesis.

Notably, there appears to be interdependence between angiogenic pathways mediated by growth factor receptors, and the pathway mediated by endothelial nAChRs. In the wounded endothelial monolayer, activation of nicotinic acetylcholine receptors (nAChRs) accelerates endothelial cell migration (Ng et al, 2006). Furthermore, the effect of growth factor (VEGF or bFGF) to accelerate endothelial cell migration in this model involves nAChR activation; specifically, nAChR antagonists blunt growth factor induced EC migration. Furthermore, by transcriptional profiling we have identified convergent genomic responses of ECs to nicotine, VEGF and bFGF. Identification of concordantly regulated genes may provide novel insights into molecular processes mediating EC migration and angiogenesis (Ng et al, 2006). Indeed, using this approach we found that thioredoxin inhibitory protein is centrally involved in cholinergic and growth factor-mediated EC migration. The nAChRs may play an important role in growth factor-induced angiogenesis, and thus may be a target for therapeutic modulation in disorders of pathological or insufficient angiogenesis.

Our findings are consistent with previous observations that nicotine stimulates endothelial cell proliferation, and promotes the synthesis of growth factors (eg. FGF) and autocoids (eg. NO, endothelin, prostacyclin) that may have angiogenic effects (Villablanca, 1998;Zhang et al, 2001;Carty et al, 1996;Lee and Wright, 1999;Boutherin-Falson and Bales, 1990). Nicotine increases the expression of matrix metalloproteinases (Carty et al, 1996) that facilitate migration of vascular cells through the extracellular matrix. Moreover, we have found that nicotine potentiates endothelial-monocyte interactions that contribute to arteriogenesis (Heeschen et al, 2003). Most intriguingly, using a parabiotic animal model, as well as fluorescent activated cell sorting, we have observed evidence that nicotine increases the incorporation of endothelial progenitor cells into newly forming vessels (Heeschen et al, in press JACC).

Clinical Ramifications

These data indicate that nicotine is an angiogenic factor via its stimulation of an endogenous cholinergic pathway that regulates angiogenesis. The maximal effect of nicotine was observed at concentrations equivalent to plasma levels observed in moderate smokers. Nicotine has similar effects as VEGF or FGF, two well characterized angiogenic peptides. Pathological angiogenesis plays a significant role in tumor growth and the progression of atherosclerosis. By promoting pathological angiogenesis, nicotine sustains the abnormal cell growth characteristic of tobacco-related diseases. This insight has clinical implications. Manipulation of cholinergic angiogenesis may have therapeutic utility. Inhibition of cholinergic angiogenesis may be useful in disorders characterized by pathological angiogenesis, such as tumor, atherosclerosis, or retinopathy. On the other hand, short-term and localized activation of this pathway may ameliorate disorders characterized by inadequate angiogenesis. Indeed, work from our laboratory reveals that therapeutic stimulation of the nicotinic receptors enhances wound healing in a murine model of wound healing (Jacobi et al, 2002). This effect is associated with an increase in angiogenesis in the wound bed of the animals treated with nAChR agonists. Accordingly, it appears that nicotinic agonists and antagonists may become useful additions to the growing list of candidates for therapeutic modulation of angiogenesis.

Acknowledgements

This study was supported by grants from the National Institutes of Health (RO1 HL63685; RO1 AT/HL00204; PO1 AI50153; PO1 AG18784); from Philip Morris Incorporated; and the Tobacco Related Disease Research Program (7RT-0128). Stanford University owns patents on the use of nACh receptor agonists and antagonists for disorders of inadequate or pathological angiogenesis. Drs. Cooke is an inventor on these patents, and receives royalties from the licenses. Dr. Cooke is a co-founder of, and has equity in Athenagen Inc. which has licensed this technology.

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Endothelial Cell Migration assay. Effect of Neostigmine

Fig 1.

Histogram showing average counts of migrating cells in wounded endothelial monolayer model. The endothelial cell (EC) migration assay begins with a confluent EC monolayer which is injured by scraping away half of the cells (see insert). EC migrating into the denuded region (ie. across the dashed line, see insert), are counted. Angiogenic agents accelerate this migration. In this experiment we have treated EC with vehicle or growth factors, in the presence or absence of neostigmine (NEO), a cholinesterase inhibitor. In this histogram, the number of migrating cells are expressed as a percentage of the number of EC migrating under unstimulated conditions. VEGF (1 ng/ml; black bar) increases EC migration to the same degree as FGF or Nicotine; high dose VEGF (10ng/ml) gives the greatest response. Neostigmine (NEO) increases EC migration over 50%, a similar effect as that with nicotine, FGF or low dose VEGF. The combination of neostigmine and low dose VEGF (3rd black bar) gives a greater response than high dose VEGF.