◆ REVIEW ARTICLE ◆

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Nicotine and angiogenesis: a new paradigm for tobacco-related diseases

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The pathophysiology of tobacco-related diseases is complex and multfactorial. Among the approximately 4,000 compounds in tobacco smoke are carcinogens such as nitrosamines, irritants such as a variety of phenolic compounds, volatiles such as carbon monoxide, and of course nicotine. Nicotine itself has quite complex actions, mediated in part by nicotinic cholinergic receptors that may have extraneuronal, as well as neuronal distribution. This review discusses the mechanisms by which nicotine contributes to tobacco-related disease, with a focus on the surprising new finding that nicotine is a potent angiogenic agent. Nicotine hijacks an endogenous nicotinic cholinergic pathway present in endothelial cells that is involved in physiological, as well as pathological angiogenesis.

Keywords: atherosclerosis; coronary artery disease; endothelium

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Introduction

In this report, we review current understanding of pathophysiological mechanisms underlying tobacco-related diseases. We introduce intriguing new information regarding the potent angiogenic effects of nicotine. By promoting pathological angiogenesis, nicotine accelerates the growth of tumor, and the progression of atherosclerotic plaque. Recent insights into the mechanisms of tobacco-related diseases may lead to new therapeutic avenues.

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Tobacco's toll

Cigarette smoking is an intractable public health problem and a principal cause of preventable disease, disability, and premature death (1, 2). Atherosclerotic cardiovascular disease, cancer, and chronic obstructive pulmonary disease account for most of the excess morbidity and mortality due to smoking. The worldwide number of smokers is estimated at 1.1 billion. In high-income countries about half of long term regular smokers are killed by tobacco, of these, about half die at middle age (35–69 years old) (3). It is estimated that more than more than 400,000 Americans die of smoking-related causes annually (4). Although the prevalence of cigarette smoking has decreased in the United States (from approximately 65% of men and 30% of women in 1965 to 24% of the population in 1996) and although similar changes have occurred in much of the developed world during the past two decades, smoking prevalence is increasing in many developing countries (4). Worldwide, about four million people died of tobacco related disease in 1998 (5). This figure is expected to rise to 10 million annual deaths by 2030. It is also estimated that about 100 million people were killed by tobacco in the 20th century. An exponential rise in this cumulative number is expected to occur throughout the 21st century if current smoking patterns continue (3, 4).

Smoking increases the risk for myocardial infarction (MI), sudden cardiac death, stroke, peripheral arterial disease, and abdominal aortic aneurysm (6). The incidence of coronary artery disease (CAD), including sudden cardiac death is more than doubled in cigarette smokers. A large body of data links smoking to the development of atherosclerosis showing that the relative risk for atherosclerotic coronary stenosis associated with smoking is greatest in the youngest age group, suggesting an acceleration of the process by smoking (7, 8). Tobacco use also increases the risk of bypass graft failure in the coronary and in the peripheral circulation (9, 10) Smoking triggers the acute ischemic events of MI, unstable angina and

sudden cardiac death (11). In this regard, data from the Framingham study indicate a 10-fold greater risk for sudden cardiac death and a 3.6-fold increase in MI in smokers (12). Altogether, of all the coronary risk factors, cigarette smoking is the strongest predictor of sudden cardiac death. In addition, smoking markedly accelerates atherosclerosis in the abdominal aorta and its branches and thus increases the risk of aortic aneurysm, peripheral arterial disease, and renal artery stenosis (13). In the cerebral circulation, two types of stroke are increased in cigarette smokers – cerebral infarction and subarachnoid hemorrhage (6, 14).

Smoking is also the single largest risk factor for a variety of malignancies and the single most important cause of cancer mortality in the United States accounting for 39% of all cancer deaths. The causal relation between tobacco smoking and lung cancer was established by large-scale epidemiological studies (2, 15, 16). Overall the relative risk of developing lung cancer is increased about 13-fold by active smoking and is quantitatively related to cigarette smoke exposure (17). The gender gap in smoking prevalence has narrowed considerably (4) and most probably as a result of that lung cancer has become the leading cause of cancer death among American women exceeding even that of breast cancer (17). Cigarette smoking is also a cause of laryngeal, oral, pharyngeal, esophageal and bladder cancer in men and women. It is a contributory factor in the development of kidney and pancreatic cancer. It is associated with cancer of the stomach and the uterine cervix and may be associated with other types of cancer as well (18).

Key messages

- There are nicotinic cholinergic receptors (nAChR) on endothelial cells.
- The endogenous ligand for these receptors is acetylcholine.
- Stimulation of the nAChR causes endothelial cells to proliferate, migrate and form capillaries.
- Nicotine can stimulate this pathway to induce angiogenesis.
- Nicotine-induced angiogenesis plays a role in neovascularization and growth of atheromatous plaque.
- Nicotine-induced angiogenesis plays a role in tumor angiogenesis and tumor growth.
- Therapeutic manipulation of the angiogenic pathway mediated by the nAChR may represent a new treatment strategy for tobacco-related diseases.

Mechanisms of tobacco-related disease

There are multiple mechanisms by which tobacco promotes disease, and these interact with other environmental factors, as well as with diverse genetic determinants. These interactions influence the particular manifestations of tobacco-related disease in any individual.

Atherosclerosis

Accumulating evidence indicates that oxidative stress plays a major role in the progression of atherosclerosis. Most of the known or suspected risk factors (including smoking, hypercholesterolemia, hyperglycemia, hypertension, hyperhomocysteinemia) are associated with increased vascular generation of superoxide anion (19, 20). Smoking, and the other risk factors, activate oxidative enzymes in the vessel wall (e.g., xanthine oxidase or NADPH oxidase), perhaps *via* protein kinase C mediated pathways (21). The subsequent production of superoxide anion activates oxidant-sensitive transcriptional proteins (e.g., nuclear factor kappa B; NFkB) that induce the expression of adhesion molecules and chemokines involved in vascular inflammation and atherogenesis (21–24). Tobacco-induced oxidative stress also generates PAF (platelet activating factor)-like lipids and leukotrienes that contribute to leukocyte adhesion (25, 26). As a result, the endothelium becomes more adhesive for monocytes and T lymphocytes, which infiltrate the vessel wall. This vascular inflammation is also involved in plaque rupture, a major cause of acute vascular events (27).

Tobacco exposure also decreases the activity of endothelial nitric oxide (NO), and reduces endothelial prostacycline (PGI_2) biosynthesis (28). These effects of tobacco on NO and PGI_2 are another manifestation of oxidative stress, which increases the degradation, and reduces the synthesis, of these molecules (29–31). Each of these vascular autocoids are potent vasodilators, and loss of their activity promotes vasoconstriction. Furthermore, these substances are endogenous anti-atherogenic molecules. They each inhibit the adherence and infiltration of inflammatory cells; the aggregation of platelets; and the proliferation of vascular smooth muscle cells (32–35). By impairing their activity and synthesis, tobacco removes a potent self-defense against atherosclerosis. These effects of smoking on the endothelium can be appreciated as impairment in endothelium-dependent vasodilatation in both forearm and coronary vascular beds. This endothelial dysfunction precedes and promotes changes in vascular structure (36, 37).

Smoking may also promote atherogenesis by its adverse effects on the lipid profile. Tobacco use

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increases plasma levels of oxidized low density lipopoprotein, very low density lipoprotein, and triglycerides; and decreases high density lipoprotein cholesterol (38, 39). Furthermore, exposure to tobacco promotes thrombosis by increasing plasma fibrinogen, red cell mass, blood viscosity, platelet activation and thromboxane A_2 release, and by impairing the release of tissue plasminogen activator from endothelial cells (40, 41).

Finally, inhalation of tobacco smoke increases myocardial oxygen demands and reduces coronary blood flow by an adrenergically mediated increase in blood pressure and coronary artery tone, thereby lowering both angina and arrhythmia thresholds (6). The exposure to carbon monoxide in cigarette smoke impairs myocardial oxygen delivery even further. Repeated episodes of coronary vasoconstriction and elevations of systemic arterial pressure in response to smoking may increase cyclic strain at the site of an atherosclerotic plaque to increase the likelihood of plaque rupture (6).

Malignancy

The major identified carcinogens in cigarette smoke are polynuclear aromatic hydrocarbons, aromatic amines, and nitrosamines and other organic (e.g., benzene, acrilonitrile) and inorganic (e.g., arsenic, acetaldehyde, cadmium) compounds. Certain metabolites of nicotine might be carcinogenic (42). There are over 40 agents in tobacco smoke that have mitogenic potential, and over 4,000 different chemicals in tobacco smoke, which makes it difficult to determine the individual contribution of these agents to carcinogenesis (17). Nicotine or nicotine derived nitrosamines such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) may directly stimulate nicotine acetylcholine receptors (nAChR) to promote oncogenesis. Recent works suggests that nicotine and NNK stimulate nAChR on human airway epithelial cells to activate the protein kinase Akt (43). This effect is associated with a partially transformed phenotype (i.e., loss of normal contact inhibition and dependence on exogenous growth factors). Furthermore, active Akt was detected in airway epithelial cells of human lung cancers derived from smokers. Notably, human lung cancer cells may express nAChRs, and nicotine inhibits apoptosis of these tumor cells (44) Thus, excessive Akt activation by nicotine and NNK could contribute to tobacco-related carcinogenesis by altering cell growth and apoptosis.

Role of angiogenesis

Tumor angiogenesis plays a major role in tumor growth and metastasis (45). In order to grow beyond a critical size, tumors must recruit endothelial cells from the surrounding stroma to form their own endogenous microcirculation (46). Tumor progression occurs in two phases: a prevascular, and a vascular phase. The transition between the two phases is termed the 'angiogenic switch' (47). In the first stage, tumor growth is determined by the existing blood supply and it plateaus when the rate of tumor cell proliferation is balanced by the rate of cell death by apoptosis. The vascular phase is characterized by new blood vessel formation, decreased apoptosis (48, 49), exponential growth, tissue invasion and hematogenous spread of tumor cells (50). The angiogenic switch involves either the induction of positive regulators and/or the loss of negative regulators (50–52). Among these, members of the vascular endothelial growth factor (VEGF) and angiopoietin (Ang) families appear to have a predominant role (53). 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 (50–52). 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 (50). There is convincing evidence that inhibition of angiogenesis halts tumor growth and metastatic spread (54, 55).

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 (56, 57). Neovascularization of plaque has been implicated in intra-plaque hemorrhage. Furthermore, recent data indicate 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 (58, 59). In the same animal model, Celeti et al. (60) found that vascular endothelial growth factor (VEGF) promotes plaque neovascularization and growth. The requirement of neovascularization for plaque growth is not so surprising when taken into the context that plaques (particularly those associated with mononuclear infiltrate) are metabolically active. Indeed, macrophages in an inflamed plaque generate sufficient amounts of energy that an increase in temperature over the surrounding tissue can often be detected by thermography (61). Notably, there is a strong correlation between macrophage infiltrate, and the number of plaque vessels, in aortic atheroma of the

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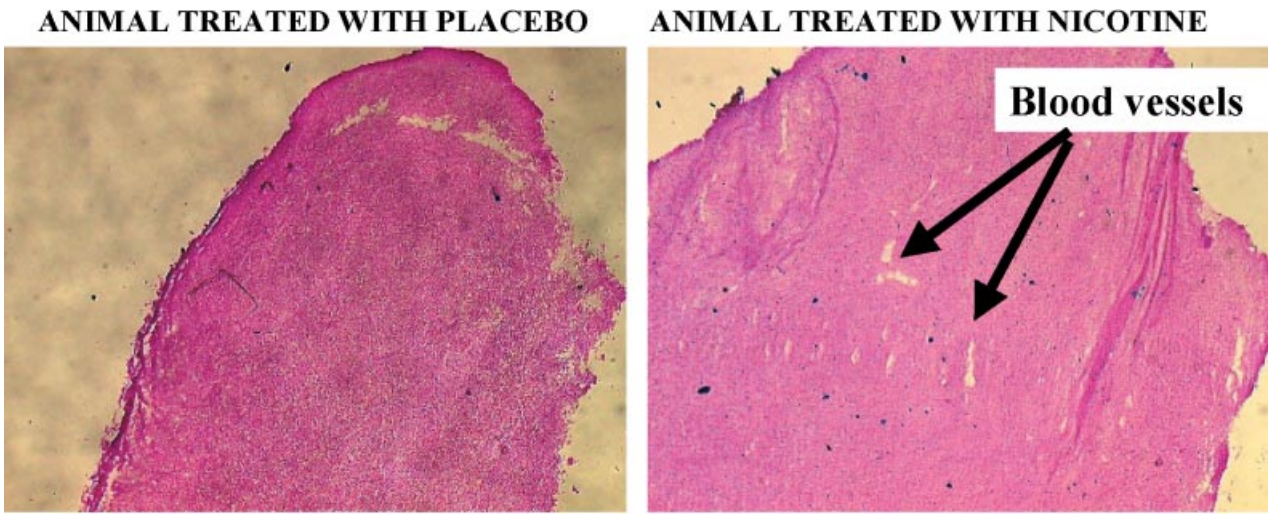

Figure 1. Nicotine stimulates tumor angiogenesis and tumor growth. Low power microphotograph of cross-section of tumor nodules. Lewis lung cancer cells were injected subcutaneously, and mice received vehicle or nicotine in their drinking water, to achieve plasma cotinine levels similar to those observed in moderate smokers. Tumor vascularity was increased by nicotine, and tumor growth was accelerated.

apo E deficient hypercholesterolemic mouse (62). Furthermore, inhibition of plaque angiogenesis reduces macrophage accumulation in the atheroma. These observations are consistent with intravital studies of tissue oxygenation in tumors which demonstrate that significant hypoxia and acidosis occur when the distance from a capillary exceeds 100 μm (63).

To summarize, pathological angiogenesis plays a critical role in the growth and progression of cancer and atherosclerosis. Recent work from our laboratory indicates that there may be a common facilitator of pathological angiogenesis in these two tobacco-related diseases.

A new paradigm for tobacco related diseases

Nicotine is responsible for the psychoactive actions and addictive properties of tobacco (64). These effects are subserved by nicotinic acetylcholine receptors (nAChR) that mediate fast synaptic transmission. Non-neuronal cells including endothelial cells and vascular smooth muscle cells also express nAChR (65, 66). Activation of these receptors in non-neural cells can induce mitosis, differentiation, organization of the cytoskeleton, cell-cell interactions, locomotion, and migration (65–68). Of course, acetycholine (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 (69, 70). Recently, a serendipitous observation in our laboratory revealed an unexpected function of this cholinergic pathway: regulation of angiogenesis (71). This property was unmasked by an investigation in our laboratory which revealed that nicotine is a potent angiogenic factor. In a series of *in vitro* and pre-clinical studies using a variety of models, we found that nicotine is equipotent to basic fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) in its angiogenic effects.

Cultured human endothelial cells exposed to nicotine proliferate more rapidly, are resistant to hypoxia-induced apoptosis, and form capillary like structures in growth factor free collagen gel (71). Notably, these effects were identical to those achieved with VEGF, and were observed at concentrations of nicotine which are attained in the plasma of moderate smokers. In a model of angiogenesis occurring in the setting of inflammation, we found that nicotine augmented fibrovascular growth into an artificial matrix implanted subcutaneously in mice (71).

To determine if nicotine could enhance tumor angiogenesis, we used the *in vivo* Lewis lung cancer model. Sixteen days after 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. This acceleration of tumor growth in the nicotine group corresponded with a 5-fold increase in capillary density within the tumor tissue (Fig 1). The effects of nicotine on tumor growth could be blocked by antagonists of the nAChR, indicating that nicotine was exerting its effects *via* this cholinergic pathway (71).

As mentioned above, in the murine model of atherosclerosis ($ApoE^{-/-}$ mice), growth of advanced plaques depends upon plaque vascularization. Accordingly, we investigated whether nicotine promoted the vascularization and growth of atheroma

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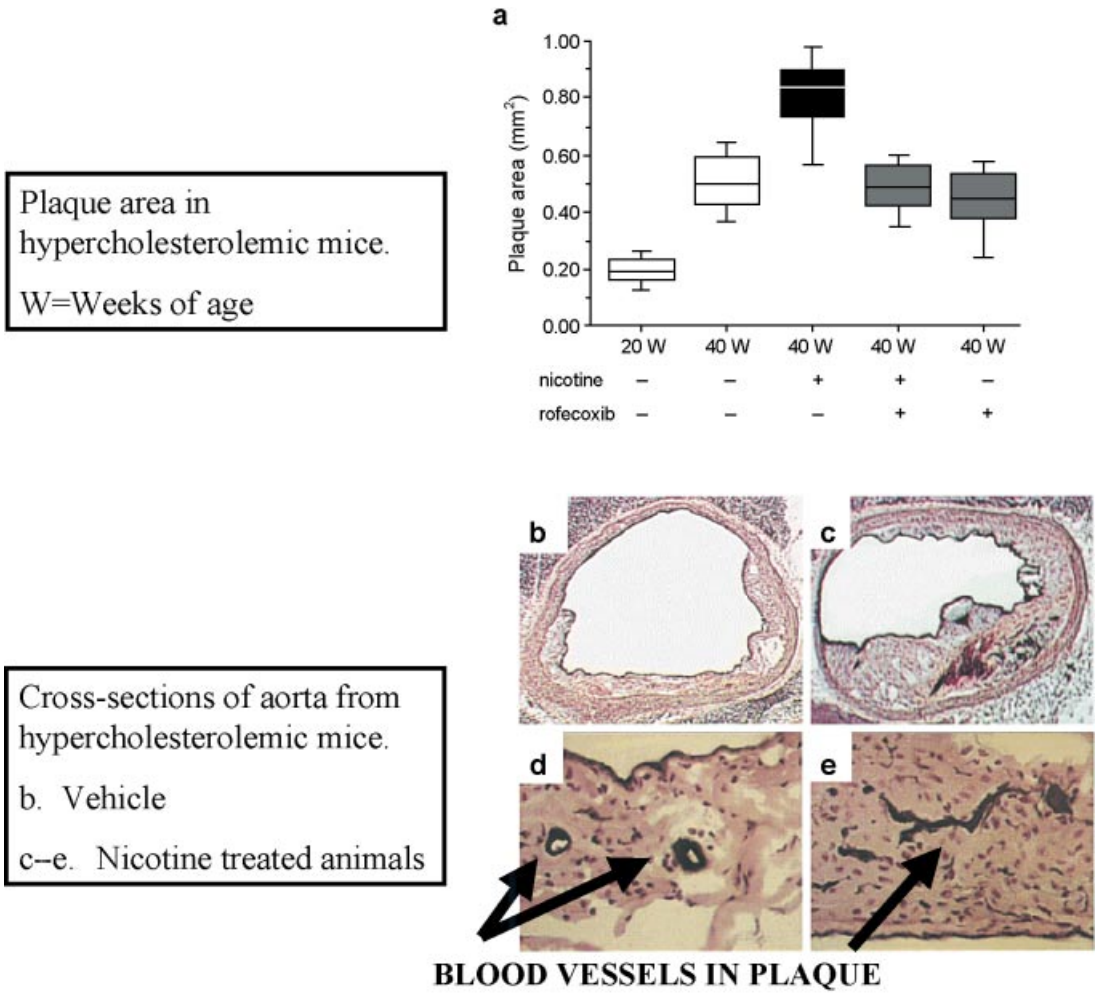

Figure 2. Nicotine accelerates plaque neovascularization and plaque growth. Histogram illustrating growth of atheroma in hypercholesteromic Apo E deficient mice: **a.** Aortic atheroma progresses from 20 to 40 weeks. Nicotine increases plaque growth. The effect of nicotine was abrogated by rofecoxib, an inhibitor of angiogenesis. **b.** Shown are cross sections of the aortic root of mice treated with vehicle (**b** and **d**) or nicotine (**c** and **e**). Intimal vessels were identified by immunohistochemistry for vascular endothelial-cadherin (**d** and **e**). Plaque vascularity is increased in nicotine-treated mice.

(71). Treatment with nicotine for 20 weeks increased by two-fold the number of vascularized plaques, and doubled plaque area in the apo E deficient mice (Fig 2). The effect of nicotine was independent of plasma lipid values, and was blocked by rofecoxib, a known inhibitor of angiogenesis (72).

To determine the functionality of nicotine-induced vasculature, we studied the effect of nicotine in a model of murine hindlimb ischemia. We found that intramuscular injections of nicotine or epibatidine (another agonist of the nAChR) increased capillary density in the ischemic limb. Studies of blood flow by magnetic perfusion imaging revealed that each of these agents were capable of normalizing blood flow in the ischemic limb. These effects were blocked by co-administration of mecamylamine or hexamethonium, antagonists of the nAChR. Similar results were achieved when nicotine was administered *via* the drinking water at a dose that yielded plasma cotinine levels similar to those observed in moderate smokers (71). More recently, we have confirmed the angiogenic effects of nicotine in a rabbit model of hindlimb ischemia, and have learned that arteriogenesis is also enhanced (73). In this model, intra-arterial administration of nicotine increased capillary density in the ischemic limb (angiogenesis), and augmented the number and size of collateral channels (arteriogenesis), as documented by histomorphometry and quantitative angiography. These structural changes were associated with increased limb blood flow as assessed by Doppler flow wire recordings and microsphere studies.

These studies have unmasked a non-neuronal nicotinic cholinergic pathway that regulates blood vessel formation and remodeling. Intriguingly, we have found that there is a specific nAChR expressed on endothelial cells (the α7-nAChR), that mediates the effect of nicotine (or endogenous acetylcholine) on

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angiogenesis (74). In an *in vitro* angiogenesis model, increasing concentrations of the non-selective mecamylamine completely and reversibly inhibited endothelial network formation. Although several nAChR isoforms are expressed on endothelial cells, similar inhibition was only obtained with the selective α7-nAChR antagonist α-bungarotoxin whereas other selective antagonists did not result in significant inhibition of network formation. The α7-nAChR was upregulated during proliferation of subconfluent endothelial cells, or by hypoxia *in vitro*. In a murine model of hindlimb ischemia, we found that the α7-nAChR was upregulated on endothelial cells in vessels of the ischemic limb. Pharmacological inhibition of this nAChR, or genetic disruption of α7-nAChR expression, significantly inhibited angiogenesis in a number of animal models, including angiogenesis in response to inflammation, ischemia, and tumor growth (74). 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).

Our findings are consistent with previous observations that nicotine stimulates endothelial cell proliferation, and promotes the synthesis of growth factors (e.g., FGF) and autocoids (e.g., NO, endothelin, prostacyclin) that may have angiogenic effects (75–79). Nicotine increases the expression of matrix metalloproteinases (75) that facilitate migration of endothelial cells through the extracellular matrix. Moreover, ongoing studies in our laboratory indicate that nicotine potentiates endothelial-monocyte interactions that contribute to arteriogenesis (73). 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.

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 is equally potent as VEGF or FGF, two well characterized angiogenic peptides. These findings raise concerns about prolonged use of nicotine therapies. Nicotine gums and patches are safe and effective adjuncts to tobacco cessation programs when used as directed. The short-term use of transdermal nicotine is not associated with increased ischemic events (80). However, long-term use of nicotine may have unintended consequences. This caveat becomes more important in view of the recent interest in therapies based on chronic stimulation of the nACh receptor. Nicotinergic agonists are being evaluated for treatment of neurological disorders such as Alzheimer's disease and chronic pain syndromes. The potent angiogenic effects of nicotine, and their potential consequences, must now be taken into consideration by investigators who are studying the therapeutic potential of drugs which stimulate nicotinergic acetylcholine receptors.

The unexpected and counterintuitive finding that nicotine is a potent angiogenic agent makes sense when the Janus-like nature of angiogenesis is appreciated. 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 angiogenesis *via* this nicotinergic pathway may have therapeutic utility. Short-term and localized activation of this pathway may ameliorate disorders characterized by inadequate angiogenesis or arteriogenesis. Indeed, the most recent work from our laboratory reveals that therapeutic stimulation of the nicotinergic receptors enhances wound healing in pre-clinical models. Furthermore, to the extent that this pathway plays a role in tumor angiogenesis, nicotinergic antagonists may become a useful adjunct to cancer chemotherapy. Nicotinergic agonists and antagonists may become useful additions to the growing list of candidates for therapeutic modulation of angiogenesis.

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