Cigarette Smoke Components Induce Matrix Metalloproteinase-1 in Aortic Endothelial Cells through Inhibition of mTOR Signaling

Vincent Lemaître, Abdoulaye J. Dabo, and Jeanine D’Armiento

Division of Molecular Medicine, Department of Medicine, Columbia University College of Physicians and Surgeons, New York, New York 10032

Received April 19, 2011; accepted June 27, 2011

Smoking is a major risk factor for heart disease, but the molecular effects of cigarette smoke on vascular cells are poorly understood. In this study, we demonstrate that matrix metalloproteinase-1 (MMP-1), a collagenase expressed in atherosclerosis and aneurysms but not in the normal vessel wall, is induced in the aortic endothelium of rabbits exposed to cigarette smoke. In vitro cigarette smoke extract (CSE) and one of its components, acrolein, inhibit the mammalian target of rapamycin (mTOR)/p70S6K pathway in human endothelial cells, and chemical inhibition of this pathway by rapamycin resulted in elevated MMP-1. Moreover, the tissue inhibitor of metalloproteases-3 (TIMP-3), a major regulator of angiogenesis, is significantly downregulated in aortic endothelial cells treated with CSE, acrolein, or rapamycin. These data indicate that inhibition of mTOR by cigarette smoke components is a key event in the modulation of endothelial MMP-1 and TIMP-3 expression. Our study suggests that circulating smoke components, including acrolein, contribute to vascular diseases through enhanced MMP-1 and decreased TIMP-3 secretion in the endothelium, potentially leading to impaired angiogenesis, matrix disruption, and vessel injury.

Key Words: collagenase; angiogenesis; aortic diseases; endothelium; tobacco; matrix metalloproteinases.

Epidemiological studies demonstrate that smoking is a significant risk factor for heart disease, including atherosclerosis, aneurysm formation and rupture, and stroke (Barnoya and Glantz, 2005; Forsdahl et al., 2009). Even though passive smokers are exposed to much lower doses of toxins, their risk of coronary heart disease is also significantly elevated, indicating that the cardiovascular system is remarkably sensitive to circulating smoke components (Barnoya and Glantz, 2005).

Directly in contact with the bloodstream, the endothelium maintains the integrity of the vessel wall. Endothelial damage and dysfunction lead to an inflammatory response contributing to atherosclerosis and thrombosis (Libby et al., 2006). Studies have demonstrated that cigarette smoke causes oxidative stress and inhibits angiogenesis in endothelial cells in culture (Edirisinghe et al., 2008; Raij et al., 2001) and, in vivo, causes a disruption between carotid endothelial cells and their basement membrane (Mullick et al., 2002).

A key event in the initiation and development of vascular injury is the degradation of the extracellular matrix (ECM) of the vessel wall. Studies on animal models have demonstrated that matrix metalloproteinases (MMPs) are critical for ECM remodeling during atherosclerosis and aneurysm formation (Newby, 2005). In addition to smooth muscle cells and macrophages, MMP-1 (interstitial collagenase-1) is also detected in the endothelium of the atherosclerotic lesion (Galits et al., 1994; Nikkari et al., 1995) and in the aneurysm tissue (Irizarry et al., 1993). MMP-1 is one of the few proteases able to digest the major fibrillar collagens of the vascular wall (types I and III), and it also contributes to inflammation through processing of cytokines and receptors (Schonbeck et al., 1998).

We have previously demonstrated that MMP-1 is induced by cigarette smoke in lung epithelial cells (Mercer et al., 2004), leading to emphysematous changes (D’Armiento et al., 1992; Imai et al., 2001). It is our hypothesis that cigarette smoke components also modulate MMP-1 expression in endothelial cells of major blood vessels, leading to vascular injury. Therefore, we examined the effect of cigarette smoke on MMP-1 expression in aortic endothelial cells in vivo and in culture.

MATERIALS AND METHODS

Animal study. New Zealand white rabbits were purchased from Covance (Princeton, NJ). Female rabbits (1.3–1.5 kg) were exposed to room air (n = 5) or to cigarette smoke (n = 5) 3 h/day, 5 days a week for 10 weeks, in a specially designed chamber (Teague Enterprise, Woodland, CA). The average total particulate matter during smoke exposure was 100 mg/m³. Rabbits were sacrificed by iv injection of pentobarbital (100 mg/kg). All animal experiments were approved by the Institutional Animal Care and Use Committee of Columbia University.

Preparation of cigarette smoke extract. The smoke of one cigarette (10 mg of tar and 0.8 mg of nicotine) was pumped through 25 ml of Dulbecco’s PBS. The smoke extract, mostly containing water-soluble components, was...
adj usted to pH 7.4, filtered, and added immediately to the culture medium, to a final concentration of 0.1–5% vol/vol (Mercer et al., 2004).

**Cells in culture.** Human aortic vascular smooth muscle cells (Lonza, Walkersville, MD) were grown on type I collagen-treated flasks (BD Biosciences, San Diego, CA) using EGM2 media (Lonza). Aortic endothelial cells were used between passages 3 and 10. Cells at a confluence of 80% were treated with cigarette smoke extract (CSE) or acrolein (Sigma-Aldrich, St Louis, MO). Cell viability was assessed using the alamarBlue kit (Invitrogen, Carlsbad, CA), demonstrating the absence of toxicity of CSE at 5% vol/vol.

**Statistical analysis.** Values are presented as mean ± SEM. Statistical significance was assessed using the unpaired two-tailed Student’s t-test for comparison between two given groups and one-way ANOVA with Bonferroni post hoc test for multiple comparisons with p < 0.05 considered significant.

**RESULTS**

**Cigarette Smoke Induces Aortic MMP-1 Expression In Vivo**

Rodents do not have a true homolog for human MMP-1 (Balbin et al., 2001; Vincenti et al., 1998), preventing their use when studying the regulation of MMP-1 in vivo. Rabbits, which possess a gene homologous to human MMP-1 (Fini et al., 1987), were used to study the effects of cigarette smoke on MMP-1 expression in the vascular wall. After 10 weeks of exposure to cigarette smoke, aortic tissues were paraffin embedded for histology and segments of their thoracic aortae were lysed for protein extraction. MMP-1 was induced in the aorta after exposure to smoke (Fig. 1A), colocalizing with the endothelial layer of the vessel (Fig. 1B). MMP-1 was not detected in control tissues from air-exposed rabbits.

**Interstitial Collagenase-1 (MMP-1) Is Induced by CSE in Human Aortic Endothelial Cells in Culture**

The effect of various concentrations of CSE on MMP expression in aortic endothelial cells in culture was examined after 24 h of treatment. Cell viability assays demonstrated absence of toxicity of CSE at concentrations up to 7.5% vol/vol (data not shown). Treatment with CSE, from 0.5 to 5% (vol/vol),
significantly upregulated MMP-1 expression, as determined by Western blotting and densitometry analysis (Figs. 2A and B), and qPCR (Fig. 2C), but did not alter the other collagenases MMP-8 and MMP-14 (data not shown). CSE did not affect the expression of gelatinases (MMP-2 and MMP-9), as assessed by gelatin zymography (Figs. 2A and B). A gene array analysis of aortic endothelial cells treated with CSE (2% vol/vol) for 24 h also showed that MMP-1, but no other MMP, was induced by the CSE (data not shown). Examination of the inhibitors of MMPs (tissue inhibitor of metalloproteases [TIMPs]) by CSE demonstrated that TIMP-1 and TIMP-2 were not regulated by CSE (data not shown), but TIMP-3 expression was significantly downregulated at concentrations of CSE of 2 and 5% (*p < 0.05 and **p < 0.01 vs. nontreated). The expression of TIMP-1 and TIMP-2 was not affected by CSE (data not shown).

CSE Modulates Major MAP Kinases in Aortic Endothelial Cells

A moderate increase in phospho-ERK, p38, and JNK mitogen-activated protein (MAP) kinases was detected in cells treated with CSE. Increased signal was mostly observed after 30 min of treatment (Fig. 3A). ELISA confirmed that CSE caused an increase in phospho-ERK after 20–40 min of treatment and then returning to normal levels after 1 h of treatment (Fig. 3B). The transcription factors c-Jun and ATF-2, two downstream targets of MAP kinases, were also activated after CSE treatment, whereas phosphorylation of NF-κB p65, a marker of NF-κB activation, was not affected after 1 h of treatment with CSE (Fig. 3A). To determine whether MMP-1 upregulation by CSE is controlled by MAP kinases or NF-κB signaling, endothelial cells were treated with specific chemical inhibitors and the expression of MMP-1 was assayed by qPCR. Inhibition of p38 (SB203580), JNK (SP600125), and NF-κB did not prevent MMP-1 induction by CSE (Fig. 3C). On the other hand, baseline MMP-1 expression was repressed by PD184352, but this ERK inhibitor did not abolish MMP-1 upregulation by CSE (Figs. 3C and D).

CSE Inhibits the mammalian target of rapamycin/p70S6K Signaling Pathway

Gene array analysis of smoke-treated endothelial cells revealed an increase in diacylglycerol kinase gamma (DGKG) expression. Induction of DGKG messenger RNA (mRNA) expression by CSE was further confirmed by real-time PCR analysis (relative expression of 5.0 ± 0.9 and 7.1 ± 1.2 after 24 h of treatment with 2 and 5% CSE, respectively, compared with 1.0 ± 0.1 for nontreated cells; p < 0.05). DGKG synthesizes phosphatidic acid through phosphorylation of diacylglycerol. Phosphatidic acid is a critical activator of the mammalian target of rapamycin (mTOR)/p70S6K pathway (Fang et al., 2001), and therefore, we analyzed the activation status of this pathway in endothelial cells treated with cigarette smoke. Surprisingly, treatment with CSE resulted in the loss of phosphorylated p70S6K at residue Thr389, a specific target of the mTOR complex 1 (TORC1) (Dunlop and Tee, 2009; Ma and Blenis,
These data indicate that mTOR/p70S6K pathway is inhibited by CSE in endothelial cells. AMPKα is an inhibitor of TORC1 (Hardie, 2004), but Western blotting analysis showed a moderate decrease in Thr172 phosphorylation, therefore indicating that inhibition of TORC1 after treatment with CSE is not due to AMPKα (Fig. 4A).

**Inhibition of mTOR by Rapamycin and Oligomycin Induces MMP-1 Expression**

To assess the consequences of p70S6K/mTOR inhibition on MMP-1 expression, endothelial cells were treated with rapamycin, a specific inhibitor of TORC1. Rapamycin induced the expression of MMP-1 and downregulated TIMP-3, therefore mimicking the effects of CSE (Figs. 4B and C). Another inhibitor of mTOR, oligomycin, was tested. Oligomycin blocks cellular oxidative phosphorylation by inhibiting the mitochondrial ATPase, leading to activation of AMPKα, which in turn inhibits TORC1 (Gleason et al., 2007). Addition of oligomycin to aortic endothelial cells in culture resulted in increased levels of phospho-AMPKα and decreased phospho-p70S6K (Fig. 4D), together with an induction of MMP-1 expression (Fig. 4E).

**Acrolein, a Component of Cigarette Smoke, Induces MMP-1 Expression and Inhibits the mTOR/p70S6K Pathway in Aortic Endothelial Cells**

Acrolein is an aldehyde component found in high concentrations in cigarette smoke (Fujioka and Shibamoto, 2006). Treatment of aortic endothelial cells with acrolein, at concentrations of 5–10 μM, induced MMP-1 mRNA expression and protein secretion (Figs. 5A and B). Addition of acrolein to the cells was followed by an inhibition of the mTOR/p70S6K pathway (Fig. 5C), with a loss of detectable phosphorylated p70S6K after 4 h of exposure. After 24 h of exposure with acrolein, there were no detectable levels of p70S6K (Fig. 5C), suggesting that acrolein is a key component of cigarette smoke responsible for MMP-1 induction in aortic endothelial cells.
DISCUSSION

In the present study, we show that cigarette smoke induces the expression of MMP-1 in aortic endothelial cells in rabbits and in endothelial cells in culture. MMP-1, an interstitial collagenase that is not present in the normal vessel wall, is significantly elevated in aneurysms and in atherosclerotic lesions, where it is expressed in inflammatory cells, smooth muscle cells, and endothelial cells (Nikkari et al., 1995). The in vivo consequences of elevated MMP-1 in the endothelium of major arteries are not well known, but studies have demonstrated that MMP-1 affects important vascular events, including atherogenesis (Lemaître et al., 2001), angiogenesis (Saunders et al., 2005), and thrombosis (Trivedi et al., 2009).

We previously demonstrated that water-soluble components from cigarette smoke upregulated MMP-1 in small airway epithelial cells (Mercer et al., 2004). This regulation was dependent on the activation of the ERK MAP kinase (Mercer et al., 2004), acting on a specific smoke-responsive region of the MMP-1 promoter (Mercer et al., 2009). In the present study, however, water-soluble cigarette smoke components triggered a short elevation of phospho-ERK in endothelial cells, and chemical inhibition of ERK decreased baseline expression of MMP-1 but did not prevent its induction by CSE. These data indicate that the molecular mechanisms of MMP-1 upregulation by CSE are cell specific. A detailed analysis of various signaling pathways revealed that CSE caused a rapid inhibition of the mTOR/p70S6K signaling pathway in endothelial cells. Activated 70-kDa ribosomal S6 kinase (p70S6K), a major regulator of cell cycle and cellular migration, regulates protein synthesis by phosphorylating the S6 protein of the 40S ribosomal subunit and other translation initiation factors (Ma and Blenis, 2009). Activation of p70S6K occurs through phosphorylation of different residues, with one essential site being Thr389, a target of the mTOR complex 1 (TORC1). TORC1, a complex of mTOR, RAPTOR, and GBL

FIG. 4. Cigarette smoke components inhibit the mTOR/p70S6K signaling pathway in aortic endothelial cells. (A) Inhibition of the mTOR/p70S6K pathway by CSE. Cellular levels of phospho-p70S6K (Thr389), a specific target of mTOR, were decreased after addition of CSE (5% vol/vol). (B) MMP-1 mRNA expression was elevated, and TIMP-3 was downregulated, after 24 h of treatment with the mTOR inhibitor rapamycin, mimicking the effect of CSE (*p < 0.01 vs. nontreated). (C) Western blot analysis of MMP-1 and TIMP-3 protein expression in endothelial cells after treatment with rapamycin for 24 h. MMP-1 secretion was induced, and TIMP-3 levels were decreased by rapamycin (since TIMP-3 is insoluble, the total protein extracts were analyzed on a Western blot, with actin as a loading control). Treatment with CSE (5% vol/vol) also demonstrate decreased TIMP-3 protein in aortic endothelial cells. (D) Oligomycin blocks oxidative phosphorylation, leading to the activation of phospho-AMPKα (Thr172) which subsequently inhibits mTOR, as seen on the Western blot. (E) Western blot analysis showing that inhibition of mTOR by oligomycin results in elevated secretion of MMP-1 after 24 h of treatment.
proteins, is sensitive to rapamycin inhibition and is regulated by various cellular events, including hypoxia, energy stress, and the PI3K-Akt pathway (Ma and Blenis, 2009). Chemical inhibition of mTOR/p70S6K by rapamycin in aortic endothelial cells resulted in MMP-1 upregulation, therefore mimicking the effect of CSE. These data suggest that inhibition of the mTOR/p70S6K pathway by CSE contributes to MMP-1 induction in aortic endothelial cells. Upregulation of MMP-1 by rapamycin has been previously demonstrated in lung fibroblasts, through a JNK/AP-1 pathway (Poulalhon et al., 2006). Further experiments will be required to determine the molecular mechanisms leading to mTOR inhibition by cigarette smoke in the endothelium. Phosphorylation of AMPKα (Dunlop and Tee, 2009), an endogenous inhibitor of mTOR, was not modulated by cigarette smoke, and other possible mechanisms for TORC1 inhibition involve tuberous complexes (TSC1 and 2), the Akt pathway, and molecular changes of the TORC1 complex itself.

In addition to MMP-1 elevation, the expression of the inhibitor of metalloproteinase TIMP-3, but not TIMP-1 and TIMP-2, was repressed by components of CSE, acrolein, and rapamycin. TIMP-3 is a relatively insoluble 24-kDa molecule, which is found exclusively in the ECM (Staskus et al., 1991). A strong inhibitor of most activated MMPs, TIMP-3, has a distinct pattern of expression and regulation compared with other TIMPs (Apte et al., 1995). Clinical and animal studies have implicated TIMP-3 deregulation in various diseases, including Sorsby’s fundus dystrophy (Weber et al., 1994), Alzheimer’s disease (Hoe et al., 2007), and arthritis (Mahmoodi et al., 2005). Importantly, MMP-1 and TIMP-3 are key modulators of angiogenesis, an invasive process that requires MMPs for the degradation of the endothelial basement membrane and cell migration into the ECM (Lamalice et al., 2007). MMP-1 expressed in migrating endothelial cells participates in this invasive process through collagen digestion (Partridge et al., 2000), activation of the endothelial protease activated receptor-1 (Blackburn and Brinckerhoff, 2008), and cleavage of connective tissue growth factor, freeing and activating vascular endothelial growth factor (VEGF) 165 (Hashimoto et al., 2002). MMP-1 is a key enzyme responsible for endothelial cell-mediated collagen degradation leading to capillary tube regression (Saunders et al., 2005), and inhibition of MMP-1 significantly decreases tumor angiogenesis (Blackburn et al., 2007). TIMP-3 blocks the binding of VEGF to its receptor VEGFR-2 on endothelial cells, inhibiting downstream signaling and angiogenesis (Qi et al., 2003). Moreover, TIMP-3 is necessary to induce endothelial tube stabilization (Saunders et al., 2006). Therefore, deregulation of endothelial MMP-1 and TIMP-3 by circulating cigarette smoke components may not only affect the integrity of the surrounding ECM through increased MMP activity but could also impair neovascularization in the diseased vessel wall.

The aldehyde acrolein is a potent irritant found in high concentration in cigarette smoke (Deshmukh et al., 2008), and
acrolein adducts, generated by lipid peroxidation, are detected in macrophages and foam cells of the atherosclerotic lesion (Uchida et al., 1998). In the lungs, acrolein has been shown to increase MMP-9 expression and activity, contributing to excessive mucin production (Deshmukh et al., 2008). The effect of acrolein on MMP-1 expression in endothelial cells was tested. At concentrations found in the bloodstream of smokers (Park et al., 2007), acrolein inhibited the mTOR/p70S6K pathway in aortic endothelial cells. This inhibition was accompanied by an induction of MMP-1 and a decrease in TIMP-3 expression. Our data suggest that, in addition to impairing cholesterol removal in the plaques (Shao et al., 2005), elevated circulating acrolein due to cigarette smoke could also participate in vascular injury through elevation of endothelial MMP-1 and downregulation of TIMP-3.

Studies in our laboratory have demonstrated a critical role for MMP-1 in emphysema (D’Armiento et al., 1992; Imai et al., 2001). An increase in MMP-1 secretion secondary to smoke exposure in aortic endothelial cells leads to the hypothesis that MMP-1 could be a unifying downstream enzyme in smoke-exposure in aortic endothelial cells leading to the hypothesis that MMP-1 inhibition blocks the deleterious effects of cigarette smoke on the endothelium. Future studies using in vivo models will determine if MMP-1 inhibition blocks the deleterious effects of cigarette smoke on the endothelium.

FUNDING

National Institutes of Health (HL086936); American Heart Association (0840108N) to J.D.; American Heart Association (0535405T); Flight Attendant Medical Research Institute, Clinical Innovator Award (062570) to V.L.

REFERENCES


