Smoking-Induced Monocyte Dysfunction Is Reversed by Vitamin C Supplementation In Vivo

Nadina Stadler, Juliane Eggermann, Stefan Vöö, Andrea Kranz, Johannes Waltenberger

Objective—The role of antioxidants in preventing vascular disease remains controversial. Vascular endothelial growth factor (VEGF-A) is important for endothelial and monocyte function. This study investigated the negative effects of smoking on monocyte migratory responsiveness to VEGF-A and the usefulness of vitamin C to prevent smoking-induced monocyte dysfunction.

Methods and Results—The chemotactic response of isolated monocytes from a cohort of 17 non-smokers and 10 smokers toward VEGF-A was assessed. VEGF-A significantly stimulated the migration of monocytes in non-smokers; the monocytes from smokers failed to respond to VEGF-A. Repeated analysis after 2 weeks of vitamin C intake (2g/d) showed a fully restored VEGF-A–induced monocyte migration in smokers. VEGF-A serum levels were not altered by vitamin C. VEGF-A–inducible kinase activity was intact in monocytes from smokers as assessed by in vitro kinase assay. Monocyte dysfunction can be mimicked in vitro by challenging monocytes with a range of reactive oxygen species (ROS).

Conclusions—Stimulation of monocyte migration by VEGF-A was severely attenuated in smokers, and the deficit observed was surmounted by vitamin C supplementation. The negative effects of smoking on monocyte function may translate into adverse impacts on VEGF-A–dependent repair processes such as arteriogenesis. These results propose a causative role of oxidative stress in smoking-induced monocyte dysfunction. (Arterioscler Thromb Vasc Biol. 2007;27:120-126.)

Key Words: smoking ■ monocyte dysfunction ■ free radicals ■ antioxidants ■ growth factors

Smoking is a prominent cardiovascular risk factor known to induce endothelial dysfunction.1,2 The ample mechanisms leading to smoking-related vascular damage are not yet fully understood. Monocytes are important sources of growth factors and their migration into areas of vascular remodeling seems to be a crucial step in the activation of cellular repair processes including wound healing. In a cardiovascular perspective this seems to be true for arteriogenesis describing the growth of preformed collateral arterioles into large arteries.3–5 The positive impact of coronary collateral arteries on the prognosis of patients with coronary artery disease (CAD) has long been established (reviewed by Waltenberger).6

Monocyte migration can be stimulated by several chemotactants, such as vascular endothelial growth factor-A (VEGF-A),6 a peptide growth factor also regulating endothelial cell proliferation and migration.7 VEGF-A plays an important role in maintaining and reestablishing endothelial integrity after injury on the basis of stimulating endothelial proliferation, NO formation,8 and prevention of apoptosis.9 VEGF-A transmits its biological effects via 2 receptor tyrosine kinases, the VEGF-receptor 1 (VEGFR1) and the VEGF-receptor 2 (VEGFR2). Whereas endothelial cells carry both of these receptors,7 monocytes only express VEGFR1,6 which is capable of mediating monocyte migration and tissue factor induction.

In this context 2 important issues have been recognized: (1) VEGF-A–induced migration of monocytes is an important process during inflammation and tissue repair,10 and (2) VEGF-A–induced migration of monocytes is severely impaired under the influence of diabetes mellitus.11 Because of the functional importance of monocytes in the process of arteriogenesis, their impaired migratory response to VEGF-A may explain the reduced ability of diabetic patients to develop coronary collaterals.12,13 Thus it is an issue of high priority to study the influence of other cardiovascular risk factors—such as smoking—on VEGF-A–inducible monocyte function and arteriogenesis. Moreover, the reversibility of risk factor–related monocyte dysfunction has not yet been investigated. Finally, monocyte dysfunction may correlate with endothelial dysfunction, although this aspect remains to be established as yet.
The purpose of the present study was to investigate whether smoking affects the functional response of monocytes to VEGF-A. The cellular damage caused by smoking is largely reflecting enhanced oxidative stress. In this study we are able to demonstrate that the VEGF-A–induced migratory response of monocytes from smokers is severely reduced and that a 2-week period of vitamin C intake can successfully normalize this parameter. Furthermore we found that smoking-related impairment of monocyte function resembles oxidation-induced monocyte dysfunction. This study suggests a preventive role of antioxidant treatment and an overall protective effect of vitamin C on monocyte function.

Materials and Methods
Characterization of Participants and Design of the Study
Healthy male smokers (n=10, median age=29.5 years, 10 to 30 cigarettes/day for >5 years) and healthy male nonsmokers (n=17, median age: 28.7 years) were included in this study. The presence of inflammatory or malignant diseases as well as diabetes mellitus was excluded by history and laboratory tests. Informed consent was obtained according to the requirements of the local ethical committee.

Blood was obtained on day 1 from both smokers and nonsmokers for baseline analysis. Smokers and control subjects received 2 g vitamin C (Hermes Cevitt Brausetabletten) once a day for 14 days, and the smokers did not change their smoking habits during this time period. A second blood sample was obtained from all subjects after 2 weeks of vitamin C intake.

Isolation of Monocytes From Peripheral Venous Blood and Analysis of Monocyte Migration
Monocytes were isolated from 40 mL of heparinized venous blood samples according to the method of Denholm et al with slight modifications. Monocyte chemotaxis was quantified as previously described using a modified 48-well Boyden chamber (Nuclepore) and polycarbonate membranes with a pore diameter of 5 μm (Nuclepore). Cell migration was stimulated with either VEGF-A (rhVEGF-A165, range from 0.1 ng/mL to 10 ng/mL; ReliaTech Braunsweg) or fMLP (formylMetLeuPhe; 10 μmol/L; Sigma) for 90 minutes. The true chemotactic activity of VEGF-A in this assay has previously been documented using checkerboard analysis.

Exposure of Monocytes to Radical and Nonradical Oxidants
Fe(II) or Cu(II) from freshly prepared stock solutions was added to monocytes from healthy nonsmokers to reach the desired final concentrations; for the Fenton reagents, the addition of transition metals was followed after 30 seconds by the addition of H₂O₂. Samples were exposed to transition metals/ROS for 10 minutes at 37°C. The concentrations chosen were rather low and comparable to those used by others when studying ROS-induced cell dysfunction. In addition, these concentrations did not negatively affect the viability of the cells.

To each set of experiments, the chemotactic response of the control sample without ROS was taken as 100% and the migratory viability of the cells.

In addition, these concentrations did not negatively affect the response of the ROS-containing samples were referred to it.

Protection by Radical Scavengers
Shielding agents were added to the monocyte preparations alone or before the addition of oxidants. We tested whether they protect from the negative effects of oxidants (30% solution of H₂O₂ prediluted in PBS, Sigma-Aldrich) or whether they can revert the smoking associated damage. We have used catalase (4550 U/mg, Sigma-Aldrich), DPI (diphenyleuroidonium chloride, 2.5 μmol/L to 10 μmol/L in dimethyl sulfoxide, Sigma-Aldrich) and ebselen (2-phenyl-1,2-benzisoselenazol-3[2H]-one in dimethyl sulfoxide, 2 mol/L, Sigma-Aldrich). All solutions were freshly made in chelex resin-treated buffer just before use. All tests had blanks that contained the agent to study and its effects as potential scavenger were considered only after having established its neutrality in the experimental procedure/non-interference with the migration assay (see above). Isolated monocytes (5×10⁵ cells suspended in 0.5 mL of chelex-treated PBS) were incubated with or without oxidants (10 minutes) in the absence or presence (10 minutes) of catalase, ebselen, and DPI.

Immunoprecipitation and In Vitro Kinase Assay
In vitro phosphorylation of proteins in monocytes via VEGFR1 was performed exactly as described earlier. In brief, cell lysates from monocytes were immunoprecipitated with a phosphotyrosine-specific antibody after stimulation with VEGF-A. The kinase reaction was carried out for 7 minutes at room temperature in the presence of [γ-³²P]-ATP (Amersham). Samples were separated by SDS-PAGE, fixed in 2.5% glutaraldehyde, incubated with 1 mol/L KOH to remove serine-bound phosphate and gels were dried. Radioactive bands were quantified on a Phospholmager (Fuji).

Immunoradiometric Assay
The serum-level of VEGF-A was analyzed using blood from nonsmokers as well as from smokers on days 1 and 14. Samples were stored at –20°C until analysis. An immunoradiometric assay (IRMA) with 2 monoclonal antibodies specific for VEGF-A, generously supplied by Genentech Inc (South San Francisco, Calif), was performed as described previously. The monoclonal antibody B2.6.2 recognizing VEGF-A₁₆₅ and VEGF-A₁₈₉, and the monoclonal antibody A4.6.1 recognizing VEGF-A₁₆₅, VEGF-A₁₈₉, and VEGF-A₁₆₅ were used. Plates were coated with B2.6.2 (5 μg/mL) for 16 hours, blocked with PBS/BSA 0.5%Tween 80 0.03%, and washed before addition of either serum samples or VEGF-A₁₆₅ control. A4.6.1 was [¹²⁵I]-labeled using the Chloramin-T method and was added to all wells. After 2 hours of incubation, radioactivity was counted using an automated gamma counter (LKB Wallac 1277 Gammamaster, LKB-Pharmacia). The limit of detection in our assay was 20 pg/mL.

Cell Viability
Cell viability following ROS treatment was determined by propidium iodide (PI) staining. Samples containing 1×10⁵ cells were analyzed by fluorescence-activated-cell sorter (FACS) using the CellQuest software (Becton Dickinson). The percentage of viable (PI-negative) cells per sample was calculated.

Statistical Analysis
Results of the migration assay as well as the VEGF-A serum levels in the smoking group after 2 weeks of high-dose vitamin C were compared with the baseline results using a two-sided exact Wilcoxon test for paired samples. Data are described as median and [25%; 75th percentiles] and shown as mean±standard deviation (SD; in the figure). The Student t test was used to assess significance of results in Figure 3. A value of P<0.05 was considered significant.

Results
Smoking Is Associated With the Inability of VEGF-A to Induce Monocyte Migration
Migration of monocytes from healthy male nonsmokers (n=17) was significantly stimulated with VEGF-A to 143.9 (130.4; 166.5)% as compared with the 100% baseline control value (P<0.001). This level of stimulation was reached at 1.0 ng/mL VEGF-A (Figure 1). VEGF-A at a concentration of 0.3 ng/mL resulted in a similar effect (148.9% above unstimulated baseline). In sharp contrast, migration of monocytes from smokers (n=10) on day 1 could not be stimulated...
with VEGF-A in this assay, which was true for all different VEGF-A concentrations tested (0.1 ng/mL to 10 ng/mL): monocyte migration measured 101.8 [100; 117.9]% of the unstimulated baseline value, when stimulated with VEGF-A at 1 ng/mL (Figure 1). With the use of VEGF-A at 0.1 ng/mL, 0.3 ng/mL, and 10 ng/mL, migration measured 97.8 [96.0; 100.7]%, 100.1 [96.6; 105.9]% and 94.6 [88.5; 100.0]%, respectively. All these values were significantly below the results obtained for nonsmokers ($P<0.001$).

To find out whether the impairment of VEGF-A–induced monocyte chemotaxis is a general phenomenon or based on a specific defect within the VEGF-A/VEGFR1 signaling pathway, we stimulated monocytes from both groups with the potent tyrosine kinase-receptor independent chemotactic tripeptide fMLP. Unlike the effect seen after stimulation with VEGF-A, the chemotactic response of monocytes to fMLP ($10^{-8}$ mol/L) was very similar in smokers on day 1 as compared with nonsmokers (225.4 [199.3; 259.1]% and 173.1 [150; 250.1]%, respectively; data not shown). Given the lack of difference between the chemotactic behavior of monocytes from the 2 groups, we concluded that the migratory pathway activated by fMLP is not affected by smoking and we did not pursue this matter further.

**The Smoking-Related Impairment of Monocyte Chemotaxis Is Reversible by High Doses of Vitamin C**

In the smoking group chemotaxis analysis was repeated after 2 weeks of vitamin C intake (2 g/d). Interestingly, a complete restitution of VEGF-A–inducible monocyte migration was observed. The migratory response to VEGF-A (1 ng/mL) measured 146.0 [118.3; 159.3]% when referred to the unstimulated baseline (Figure 1). This increase in migratory response was statistically significant ($P<0.001$). In contrast, in nonsmokers, a 2-week supplementation with vitamin C was without effect. The migratory response in this case measured 138.7 [121.8; 156.2]%.

The VEGF-A–inducible kinase activity of VEGFR1 was intact in monocytes from smokers as assessed by in vitro kinase assay (Figure 2). There was no reduction in the tyrosine phosphorylation levels in monocytes isolated from smokers, which indicated a proper tyrosine-kinase function of VEGFR1. Among others, VEGF-A stimulation results in the activation of p46, p69, and p120, likely representing p46Shc, p69Shp, p69Src, and p120GAP as previously identified by our group.

**ROS Abolish the Capacity of Monocytes to Migrate Toward VEGF-A**

A short-time (10 minutes) exposure of monocytes to transition metals and ROS was sufficient to abolish the ability of...
monocytes to migrate toward VEGF-A (Figure 3). The migratory response of monocytes was practically annulled when using transition metals at concentrations of 10 μmol/L, and Fenton reagents at concentrations as low as 10 μmol/L Fe(II)/Cu(II)+100 μmol/L H₂O₂. The ROS-induced modifications of the migratory responses shown in Figure 3 depict noxious effects by the reagents (P<0.05 in all cases) that translated in an impaired monocyte function, ie, in the incapacity of monocytes to migrate toward VEGF-A. The cell viability, measured by FACS analysis, was not affected by exposure to ROS (<5% PI positive cells, results not shown).

In an attempt to identify the nature of ROS that leads to the impairment of monocyte migration in smokers we have studied 3 different antioxidants, namely, catalase to check for a role of H₂O₂, DPI to block the NADPH oxidase, and ebselen, a seleno-organic compound known to show glutathione peroxidase-like activity. Preincubation with either agent had no significant influence on VEGF-A–induced monocyte migration in smokers (Figure 4), hence excluding the sole impact of H₂O₂ in the effects observed. In fact, monocytes from smokers were sensitive to the addition of DPI in vitro; a short time preincubation with added catalase completely reversed the noxious effects of H₂O₂ (Figure 4). Like catalase, the addition of DPI in vitro resulted in a nonsignificant modulation of VEGF-A–induced migration to a median of 82.7%, thereby excluding NADPH oxidase activation as a sole mechanism to explain smoking induced attenuation of VEGF-A–induced monocyte migration (data not shown). Ebselen could not be reasonably used in our migration assay as it impaired VEGF-A–induced migration of monocytes both in smokers and in nonsmokers, even at rather low concentrations such as 2 μmol/L (data not shown). Taken together, the likelihood that neither NADPH oxidase nor H₂O₂ alone could act as main culprits in the negative functional effects observed in monocytes from smokers gives way to the assumption that other reactive oxygen species, eg, hydroxyl radicals, products of Fenton reagents, peroxynitrite or others might play a yet more important role in substantiating the cellular dysfunction observed.

**Discussion**

In the present study we demonstrate a marked impairment of VEGF-A–induced monocyte migration in young and apparently healthy smokers. This functional cellular deficit was completely reversible after intake of vitamin C (2 g/d) over a period of 2 weeks. Even with a small number of individuals tested, the observed differences were highly significant. The molecular basis of this cellular impairment is likely to be a signal transduction defect in monocytes downstream of VEGFR1, because the apparent kinase activity of VEGFR1 seemed intact as seen by in vitro phosphorylation assay. We have previously described a similar pattern of intact VEGFR1 kinase activity associated with reduced VEGF-A–induced monocyte migration in monocytes from patients with diabetes mellitus. This finding correlates well with the reduced ability of diabetic CAD patients to develop coronary collaterals (arteriogenesis). Recruitment and activation of monocytes plays a major role during various repair processes such as wound healing, vascular remodelling, and arteriogenesis. Vascular growth and repair is largely achieved by attraction, adhesion, invasion, and activation of circulating cells, mostly monocytes. Hence monocyte dysfunction could represent a very early sign of cardiovascular pathology detectable in individuals at risk. Growth factors such as VEGF-A or MCP-1 released by invading cells produce an environment that facilitates arte-
riolar growth. Insufficient recruitment of monocytes may translate into defective repair processes. Thus, mice lacking the receptor for the chemokine MCP-1 show delayed dermal wound healing.17 Furthermore, other animal studies have shown that treatment with MCP-1 can result in accumulation of monocytes/macrophages around preformed arterioles and in an increased growth of collateral vessels.18 Because an impaired response of monocytes to VEGF-A correlates well with reduced arteriogenesis11 and because monocyte function is crucial for wound repair,17 our data lead us to speculate that collateralization may be impaired in smokers with CAD. Such a correlation between smoking and impaired collateral formation appears to be likely, but remains to be demonstrated in clinical studies.

Little is known about function or specific dysfunction of monocytes in smokers. Our study was able to show that smoking inhibits VEGF-A–induced monocyte migration, and this defect can be prevented by vitamin C intake. Several clinical studies have shown healing defects of bone fractures and gastric ulcers in smokers.19–22 Although more data are needed, our data could represent a preamble in support of the idea that smoking-related defects in wound healing may at least in part be attributable to monocyte dysfunction.

The results of our study on smoking-related monocyte dysfunction may reflect what has previously been observed for endothelial dysfunction.23 There is clear evidence for endothelial dysfunction in apparently healthy smokers,24,25 As endothelial cells from patients are difficult to obtain, only in vivo assessment of endothelial function such as acetylcholine-induced changes in forearm blood-flow25 are feasible. Because both, endothelial as well as monocyte function, are critical parameters for vascular repair, the test system used in the present study might be of clinical relevance, because it is likely to have a predictive value for VEGF-A–dependent repair processes.

The functional basis of smoking-induced monocyte dysfunction is not well understood. The fact that VEGF-A–induced migration of monocytes was more severely affected than fMLP-induced migration suggests that processes depending on tyrosine phosphorylation are more susceptible to smoking-induced oxidative damage than G protein–coupled serine-threonine phosphorylation events. There is evidence for a causative role of oxidative stress and damage by free radicals.1,26 One study demonstrated that tobacco smoke rapidly induces complex oxidant-mediated stress responses (upregulation of heat shock protein 70 and heme oxygenase-1 expression, loss of mitochondrial membrane potential) in both human vascular endothelial cells and circulating monocytes.27 Another study was able to show that smoking a single cigarette severely reduced concentrations of antioxidants and combined concentrations of nitrate and nitrite in plasma, an effect that was rapidly reversible by intake of the oral antioxidant and precursor of the cellular tripeptide glutathione, GSH, N-acetyl cysteine.28 This finding is in accordance with our data that show complete reversibility of the functional defect in monocytes after intake of yet another water-soluble antioxidant, vitamin C.

Cigarette smoke is well known to contain a multitude of different potential sources of oxidants, among them metal ions such as iron and copper which can catalyze cellular damage via formation of the highly reactive hydroxyl radicals. We hypothesized that ROS/reactive oxygen species could directly play a role in the dysfunction detected in this study. We tested this hypothesis by choosing a battery of species able to trigger oxidative damage and testing their effect on the VEGF-A–induced migratory response of monocytes from healthy males; a short-time (10 minutes) exposure to these species was sufficient to suppress the capacity of monocytes to migrate toward VEGF-A (Figure 3). Likewise, the positive effect of various antioxidant reagents could possibly be explained by their ability to scavenge ROS. Catalase alone was not able to even partially restore the capacity of monocytes to migrate toward VEGF-A (Figure 4). DPI, a NADPH oxidase inhibitor, showed no protective effect either. Ebselen, thought to be an effective mimic of glutathione peroxidase, the enzyme known to catalyze the reduction of hydroperoxides at the expense of thiol reducing equivalents, exerted a direct detrimental effect on the current cellular system. These protection experiments indicate that H2O2 alone cannot be made responsible for the monocyte dysfunction observed, and other reactive oxygen species might act as potential effectors. Such a possibility is not unlikely as free radical scavenging occurs through complex metabolic pathways. So far though, we are unable to assign a well-defined mechanism of action to clearly substantiate our positive findings. Among others, vitamin C is able to react with various reactive oxygen and nitrogen species (eg, hydroxyl radicals, –OH, superoxide anions, O2–, and peroxynitrite, respectively).

Numerous epidemiologic studies have reported on the beneficial effects of vitamin C or vitamin C-rich foods to prevent cancer, heart disease, and even asthma. Evidence is accumulating that direct supplementation with vitamin C has many beneficial effects, either by increasing the GSH concentration in the blood or by limiting oxidative degradation of cellular components.29 However, a number of studies such as those recently reviewed by Duvall et al30 suggest that the majority of evidence to date fails to support beneficial effects of vitamin C supplementation. The topic remains controversial.

A recent study by Moller et al31 showed that vitamin C supplementation decreases oxidative DNA damage in mononuclear blood cells of smokers. This paper supports the notion that the effect of vitamin C is short-lived and that a slow release formulation of the antioxidant is able to grant a protective effect after long-term supplementation.

To compensate for the low rate constants of reaction between superoxide and antioxidant vitamins such as ascorbic acid (>104 M–1S–1 at pH 7.4),34 relatively high doses of vitamin C (2 g/d) have been given to our subjects. Still, in the absence of definitive evidence, it is possible that the beneficial effect of vitamin C in our study is based on yet another mechanism, different from that described above. Another argument for using a high dose of vitamin C was that smokers have an increased requirement of both α-tocopherol and ascorbic acid.42

Whereas there is scant information available on the efficiency of antioxidant vitamins to reverse monocyte dysfuc-
tion, many studies describe the use of the antioxidant vitamin C in smokers to reduce parameters of endothelial dysfunction. Whereas Scott et al.\textsuperscript{23} report that vitamin C supplementation did not affect the tobacco-induced vascular activation, earlier studies\textsuperscript{24} showed that oral vitamin C was able to improve smoking-induced arterial wall stiffness by reducing endothelial dysfunction. Several other studies showed a clear benefit of vitamin C particularly regarding short-term effects after either intraarterial application\textsuperscript{23,25,37} or intravenous application.\textsuperscript{39} Others were able to demonstrate an antioxidant effect of vitamin C on either monocytes or platelets using the same oral dose as we did in our study (2 g/d).\textsuperscript{38,40,41} It is also important to note that high doses of vitamin C had no effect on the improvement of endothelial function or other read-outs in healthy individuals, although they showed great benefit in smokers.\textsuperscript{23} Finally, it is important to mention that the oral administration of vitamin C (2 g/d) as used in our study resulted in a significant increase in vitamin C plasma levels to 8.29±11.8 μmol/L.\textsuperscript{38}

In our study, complete reversibility of the impaired VEGF-A–induced monocyte migration in apparently healthy smokers was found after as little as 2 weeks of vitamin C intake. VEGF-A serum levels were normal at baseline and remained unchanged. This indicates that there are no compensatory mechanisms activated within the VEGF-system, and that the smoking-related defect is localized within the target cell, ie, the monocyte. This is partly in contrast to the situation in diabetic patients, who show a significantly elevated serum level of VEGF-A.\textsuperscript{11}

Our study design does not allow any conclusions about long-term effects of vitamin C as yet. Based on our findings, further testing of similar doses of oral ascorbic acid in the long-term prevention of vascular disease in smokers would seem warranted.

In conclusion, we report a severely reduced chemotactic response of monocytes from smokers on stimulation with VEGF-A, and this finding was fully reversible after intake of high doses of the antioxidant vitamin C for as little as 2 weeks. Considering the prominent role of appropriate monocyte function for vascular and nonvascular repair processes, these findings underscore the importance of smoking as a cardiovascular risk factor. Based on our data, supplementary vitamin C intake could be a safe and effective way of vitamin C intake could be a safe and effective way of reducing smoking-induced vascular damage in smoking-cessation refractory individuals.

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### Disclosures

None.

### References


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