Vascular Endothelial Growth Factor and the Risk of Smoking-Related COPD*

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Study objectives: Vascular endothelial growth factor (VEGF) signaling may be required for maintenance of the alveolar structures, and alveolar septal cell apoptosis could contribute to the pathogenesis of COPD presenting emphysematous changes; however, the common mutation at position 936 in the 3′ untranslated region of the VEGF gene, a C to T substitution (the C allele was denoted as 1, and the T allele as 2), VEGF936*2, has been reported to be associated with significantly lower VEGF plasma levels. Based on these concepts, we hypothesized that VEGF936*1/2 polymorphism may be linked to the development of COPD.

Design: The differences in VEGF936*1/2 allele frequency were examined in 113 patients with smoking-related COPD and two control groups (101 smoker/ex-smoker control subjects and 102 population control subjects) using the polymerase chain reaction-restriction fragment length polymorphism technique.

Results: VEGF936*1/2 allele frequencies did not differ among the groups: 0.792/0.208 in COPD patients, 0.822/0.178 in smoker/ex-smoker control subjects, and 0.842/0.152 in population control subjects.

Conclusion: The 936 C/T polymorphism of the VEGF gene (including both homozygous and heterozygous) was not associated with the development of COPD (odds ratio, 1.23; 95% confidence interval, 0.760 to 1.995).

Key words: emphysema; gene polymorphism; vascular endothelial growth factor

Abbreviations: AP-4 = activator protein-4; bp = base pair; PCR = polymerase chain reaction; 3′-UTR = 3′ untranslated region; VEGF = vascular endothelial growth factor

The chronic airflow limitation characteristic of COPD is caused by a mixture of small airway disease (obstructive bronchiolitis) and parenchymal destruction (emphysema).1 It is generally accepted that cigarette smoking is by far the most important risk factor for COPD. Nevertheless, not all smokers acquire clinically significant COPD, which suggests that genetic factors must modify each individual’s risk. It is believed that many genetic factors increase the risk of acquiring COPD. A study2 demonstrated an increased risk of COPD within families with COPD probands. Some of this risk may be due to shared environmental factors, but several studies in diverse populations also suggest a shared genetic risk.3

Alveolar septal cell apoptosis may contribute to the pathogenesis of COPD. Apoptosis occurs in vascular endothelial and/or alveolar epithelial cells in patients with COPD presenting emphysematous changes of the lung, and is associated with reduced expression of vascular endothelial growth factor (VEGF) in the lung.4 VEGF receptor signaling is required for maintenance of the alveolar structures, and withdrawal of VEGF leads to endothelial cell apoptosis in vitro and in vivo.5 The loss of endothelial cells may be caused both by loss of VEGF or a faulty VEGF signaling.5

Based on these concepts, we hypothesized that a reduced ability of vascular endothelial and alveolar epithelial cells to produce VEGF may be linked to...
the development of COPD and emphysematous changes in the lungs. Cigarette smoking may act to decrease the expression of VEGF and its receptor 2 (KDR/Flk-1). A reduced ability to produce VEGF and/or a decreased expression of VEGF receptor 2 may lead to apoptosis of these cells triggered by direct exposure to inhaled insults, such as tobacco smoking, potentially contributing to the destruction of lung tissue observed in these patients.

It was reported that there were three common mutations in the 3’ untranslated region (3’-UTR) of the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in the VEGF gene; one of them, a cytosine (C) to thymine (T) substitution in

Materials and Methods

Subjects

The patients group consisted of 113 patients with smoking-related COPD and two control groups (a smoker control group and a population control group) using the polymerase chain reaction (PCR)-restriction fragment length polymorphism technique. The purpose of the present study was to determine whether VEGF936*1/2 polymorphism was associated with the presence of smoking-related COPD.

Detection of VEGF Polymorphism

The base composition at 936 of the VEGF gene was determined with the PCR-restriction fragment length polymorphism technique. Genomic DNA was obtained from blood lymphocytes using the Qiaamp DNA Blood Mini Kit (Qiagen; Valencia, CA). The 3’ untranslated region of the VEGF gene was amplified, and the PCR conditions were similar to those already described: the 5’ primer was 5’-AAGGAAGAGAGACTCTGGGAGG and the 3’-primer was 5’-TTATGTATGATGCGGTCGCTC-CAACGC. PCR conditions were as follows: genomic DNA was amplified using 0.2 μmol/L concentrations of the primers, 100 μmol/L of each deoxynucleotide triphosphate, 10 mmol/L Tris, 1.5 mmol/L MgCl2, 50 mmol/L KCl, and 0.1% Triton X-100 (Sigma-Aldrich Japan; Tokyo, Japan). Cycling was as follows: 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min for 30 cycles, followed by 60°C for 1 min and 72°C for 5 min. The PCR product was ethanol precipitated and digested with NlaIII (New England Biolab; Beverly, MA) and analyzed on a 3% NuSieve agarose gel (FMC BioProducts; Rockland, ME). DNA products were visualized by ethidium bromide staining. The VEGF936*1 allele would not be digested (208 base pair [bp]), while the VEGF936*2 allele would be digested into two fragments (122 bp and 86 bp, respectively).

Statistical Analysis

The difference in allele distribution and allele frequency among the groups was examined for statistical significance by χ² test for independence, and with the Fisher exact test when appropriate. Age, smoking index expressed as pack-years, and pulmonary function parameters were compared using the Mann-Whitney U test; p < 0.05 was considered statistically significant.

Results

Age, smoking history, and pulmonary function data of COPD patients and smoker control subjects are summarized in Table 1. No significant differences were observed in age or smoking history between the two groups.

The results of the comparison of the base composition at position 936 of the VEGF gene are summarized in Table 2. The distribution of the genotype was not statistically different between patients and smoker control groups, nor between patients and population control groups. χ² analysis showed that the patients group had no higher VEGF936*2 frequency than either the smoker control or population control group. Presence of the VEGF936*2 allele (including both homozygous and heterozygous sub-

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Table 1—Age, Smoking, and Pulmonary Function in Patients With COPD and Smoker Control Subjects*

<table>
<thead>
<tr>
<th>Variables</th>
<th>Age, yr</th>
<th>Smoking, pack-yr</th>
<th>FEV₁, % pred</th>
<th>FEV₁/FVC, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>COPD patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n = 69)</td>
<td>62.5 ± 0.7</td>
<td>51.9 ± 2.3</td>
<td>48.2 ± 1.0</td>
<td>48.7 ± 1.0</td>
</tr>
<tr>
<td>Female (n = 44)</td>
<td>63.3 ± 0.6</td>
<td>54.6 ± 1.9</td>
<td>46.1 ± 1.5</td>
<td>48.0 ± 1.1</td>
</tr>
<tr>
<td>Smoker control subjects</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n = 59)</td>
<td>64.4 ± 0.8</td>
<td>48.2 ± 2.4</td>
<td>91.0 ± 1.1</td>
<td>91.6 ± 1.7</td>
</tr>
<tr>
<td>Female (n = 42)</td>
<td>65.2 ± 0.7</td>
<td>51.0 ± 2.0</td>
<td>91.1 ± 1.5</td>
<td>90.6 ± 1.6</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SEM.

Table 2—Genotype and Allele Frequencies in Patients With COPD, Smoker Control Subjects, and Population Control Subjects*

<table>
<thead>
<tr>
<th>Groups</th>
<th>Genotype Frequency†</th>
<th>Allele Frequency‡</th>
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<tbody>
<tr>
<td></td>
<td>VEGF936*1/1</td>
<td>VEGF936*1/2</td>
</tr>
<tr>
<td>COPD patients (n = 113)</td>
<td>69 (61.0)</td>
<td>41 (36.3)</td>
</tr>
<tr>
<td>Smoker control subjects (n = 101)</td>
<td>66 (65.3)</td>
<td>34 (33.7)</td>
</tr>
<tr>
<td>Population control subjects (n = 102)</td>
<td>70 (68.6)</td>
<td>32 (31.4)</td>
</tr>
</tbody>
</table>

*Data are presented as No. (%) or %.
† Genotype frequency: patients vs smoker controls, p = 0.52; patients vs population controls, p = 0.25.
‡ Allele frequency: patients vs smoker controls, p = 0.44; patients vs population controls, p = 0.17.

Dysregulated VEGF expression in marked VEGF-induced angiogenesis is linked to tumor growth and metastases, rheumatoid arthritis, and diabetic retinopathy. The increase in active VEGF protein from cells exposed to hypoxia is partly because of an increased transcription rate, mediated by binding of the transcription factor to a hypoxia responsive element in the VEGF gene. The precise mechanisms by which the VEGF plasma levels are lower in subjects expressing the VEGF 936 T allele are currently unknown. Analysis of potential transcription factor binding sites showed that the 936 C to T mutation led to the loss of a potential binding site for activator protein-4 (AP-4). AP-4 is a helix-loop-helix transcription factor that enhances the expression of several genes by binding to specific enhancer sites. The potential AP-4 binding site abolished by the 936 CT mutation may be an explanation for the association between this mutation and lower VEGF plasma levels. Another possible explanation of the association between this mutation and lower VEGF plasma levels could be a linkage disequilibrium between this mutation and another yet unknown functional mutation elsewhere in the VEGF gene sequence. Future studies using reporter gene constructs are necessary to determine if the alleles of the VEGF936*2 polymorphism have a direct effect at the gene transcription level and ultimately at the protein production level, in the alveolar epithelial cell lines.
Disappearance of lung tissue in COPD may lead to the progressive loss of capillary endothelial and epithelial cells associated with the loss of the extracellular matrix. The loss of these cells might occur through the process of programmed cell death (apoptosis). COPD is a heterogeneous disease, and several predisposing genetic risk factors may be involved in its pathogenesis. Among them, VEGF polymorphism could be associated with the development of smoking-related COPD. In patients with COPD, there might be the reduced VEGF expression or the impairment of the signal transduction via VEGF receptor 2, either because of a reduction in the number of endothelial cell receptors or due to impairment of the VEGF receptor/tyrosine kinase activity. VEGF signaling may be required for the maintenance of adult lung alveolar structures. Cigarette smoking may act by decreasing the expression of VEGF and its receptor 2, thus resulting in lung septal cell death. Kasahara and coworkers reported increased septal cell death in human emphysematous lungs, which was associated with reduced lung expression of VEGF and VEGF receptor 2. In addition, Grazia et al reported that there appears to exist a subgroup of patients with emphysema and significantly decreased or undetectable plasma levels of VEGF, and very low or undetectable plasma VEGF may characterize patients with end-stage emphysema.

In this study, we hypothesized that the reduced ability of patients with VEGF 936*2 allele to produce VEGF, when exposed to tobacco smoking or hypoxia, was involved in the apoptotic changes of vascular endothelial and alveolar epithelial cells. Patients with COPD tend to be hypoxemic, and hypoxia is a strong inducer of the VEGF gene. Theoretically, in patients with COPD, a decreased VEGF production might contribute to an increase of alveolar septal cells apoptosis. We found no association between VEGF936*1/2 alleles and the occurrence of COPD. This result, however, may not deny the important role of VEGF in the development of emphysematous changes in patients with COPD. It was reported that VEGF induced the expression of antiapoptotic proteins and acted as a survival factor for endothelial cells. If the amount of VEGF is reduced below threshold levels, alveolar endothelial cells could indeed die as a consequence of a failing VEGF-dependent endothelial cell maintenance program. Therefore a reduction of VEGF in the lungs of patients with emphysema could be one of the several factors facilitating alveolar septal cell apoptosis. In the future, the correlation of VEGF production, initiated by any stimulus, such as tobacco smoking, with a specific genotype may allow the identification of individuals predisposed to acquire VEGF-mediated COPD. Detailed disease association studies may reveal that other yet unknown functional mutations elsewhere in the VEGF gene sequence are linked to a particular type of patients with COPD.

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