Long-term Nicotine Exposure Increases Aortic Endothelial Cell Death and Enhances Transendothelial Macromolecular Transport in Rats

Shing-Jong Lin, Chuang-Ye Hong, Mau-Song Chang, Benjamin N. Chiang, and Shu Chien

Long-term nicotine exposure has been suggested as an initiating factor in atherogenesis. Dying or dead endothelial cells have been shown to make significant contributions to the local enhancement of transendothelial macromolecular transport. Since cigarette smoking is one of the major risk factors for atherosclerosis, we examined the hypothesis that smoking accelerates atherogenesis by increasing the frequency of endothelial cell death and hence transendothelial macromolecular transport. Sixteen male Sprague-Dawley rats were given nicotine at a weight-adjusted dose of 5 mg/kg body wt per day in their drinking water over a period of 6 weeks. A group of 16 age-matched male Sprague-Dawley rats not exposed to nicotine and maintained over the same time period served as the control group. In en face preparations of thoracic aorta, immunoglobulin G-containing dying or dead endothelial cells were identified by the indirect immunoperoxidase method, and endothelial leakage to Evans blue–albumin (EBA) complexes (5 minutes after intravenous injection) was visualized by fluorescence microscopy. The results showed that in nicotine-treated rats, 51% of dead endothelial cells were associated with EBA leakage, which was responsible for 57% of total EBA leaky foci. Both the frequency of endothelial cell death (0.94±0.11% versus 0.40±0.04%, p<0.0001 by two-tailed, unpaired Student’s t test) and the number density of EBA leaky foci (6.45±1.23/mm² versus 3.30±0.49/mm², p<0.05 by two-tailed, unpaired t test) were significantly greater in nicotine-treated rats than in control rats. It is concluded that long-term nicotine exposure, as experienced with cigarette smoking, may increase the frequency of endothelial cell death, resulting in an enhanced transendothelial leakage to macromolecules, such as low density lipoproteins, and leading to the acceleration of atherogenesis.

Key Words • atherosclerosis • cell death • cell mitosis • endothelium • Evans blue–albumin • nicotine • permeability • transport

Cigarette smoking is generally accepted to be one of the major risk factors for coronary heart disease.1-6 The potential for developing coronary heart disease in male cigarette smokers is approximately two times greater than in male nonsmokers.7 Large epidemiological surveys have shown that the incidence of morbidity and mortality from coronary heart disease increases progressively with the number of cigarettes smoked.4,8 Smoking cessation has been demonstrated to be associated with a decline in cardiovascular death.9 Autopsy studies have also revealed the increased occurrence of advanced atherosclerotic lesions in the coronary arteries from cigarette smokers.10-12 Despite these facts, however, the pathogenic mechanisms by which cigarette smoking accelerates the development of atherosclerosis are still not clearly understood.

Smoking or long-term nicotine administration has been shown to cause endothelial injury, as indicated by the increase in the number of circulating endothelial cells13-16 and the increase in endothelial permeability,17,18 as well as the morphological changes of the endothelium.19-23 Repeated endothelial damage has been suggested as an initiating factor in the pathogenesis of atherosclerosis.24,25 Our recent studies have demonstrated that immunoglobulin G (IgG)–containing dying or dead endothelial cells may make a significant contribution to the local enhancement in transendothelial macromolecular transport.26,27 Since cigarette smoking has a strong epidemiological association with atherosclerosis and coronary heart disease, in the present investigations we examined the hypothesis that smoking, through long-term nicotine consumption, accelerates atherogenesis by increasing the frequency of endothelial cell death and hence of transendothelial macromolecular transport.

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Methods

Animals and Treatments

Thirty-two 2-month-old male Sprague-Dawley rats were randomly assigned to two groups. The test group of 16 rats weighing 298±10 g was given nicotine at a weight-adjusted dose of 5 mg/kg body wt per day in their drinking water over a 6-week period. It was estimated that each rat consumed about as much nicotine per day as a person who smoked 50–100 cigarettes per day. The nicotine, in the form of its hydrogen tartrate salt (Sigma Chemical Co., St. Louis, Mo.), was added to the drinking water. Drinking bottles were covered with aluminum foil to prevent any light-induced decomposition of the nicotine. The 16 control rats weighing 294±9 g received no nicotine in their drinking water and were maintained over the same time period. Both groups of rats were allowed free access to drinking water and standard rat chow. The body weights and water consumptions of all rats were recorded throughout the whole experiment.

To establish the plasma nicotine levels and to evaluate the physiological effects of nicotine after long-term oral consumption of nicotine, a group of 15 nicotine-treated rats was studied. Systolic blood pressures, heart rates, and body temperatures were measured before and 1, 2, 3, 4, and 5 weeks after they started drinking the nicotine-containing water. Systolic blood pressures were measured without anesthesia by the tail plethysmographic method by using a Narco PE-300 electrophyg

Endothelial Cells

Methods for Detecting Dying or Dead Endothelial Cells

The indirect IgG immunocytochemistry method developed by Hansson and Schwartz was used to identify and quantify IgG-containing dying or dead endothelial cells in the aortic endothelium.

Reagents. The IgG antibodies used in the present study were goat IgG, goat anti-rat IgG, and horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG, all of which were purchased from Sigma. The 1-mg vial of goat anti-rat IgG was reconstituted to a 1-mL solution with distilled water. These antibodies were used at optimal dilutions of 1:1,500. Dilutions were in phosphate-buffered saline (PBS: NaCl 120 mmol/l and KCl 2.7 mmol/l in phosphate buffer 10 mmol/l, pH 7.4, at 25°C; obtained from Sigma) containing 1% ovalbumin (Sigma).

Preparation of aortic tissue for indirect immunocytochemistry. After 1 hour of immersion in 10% formalin solution, the adventitial tissue was carefully removed with fine forceps under a dissecting microscope. The thoracic aorta was excised and cut open longitudinally along the ventral surface. The aortic specimen was dissected into six pieces, each about 5 mm long. To make en face preparations, each aortic piece was pinned with the endothelial side up onto a dental wax plate and rinsed several times for 10 minutes each with PBS. Then all six aortic pieces from the thoracic aorta were incubated for 90 minutes with HRP-conjugated rabbit anti-goat IgG at a dilution of 1:1,500 in PBS containing 1% ovalbumin for 90 minutes. One aortic piece from the ascending aorta was used as a control and was incubated in PBS containing 1% ovalbumin with goat IgG at a dilution of 1:1,500 for 90 minutes. After the first incubation, the aortic pieces were rinsed three times for 10 minutes each with PBS. Then all six aortic pieces from the thoracic aorta and the control piece from the ascending aorta were incubated for 90 minutes with HRP-conjugated rabbit anti-goat IgG at a dilution of 1:1,500 in PBS containing 1% ovalbumin. After the second incubation, the aortic pieces were rinsed three times for 10 minutes each with PBS. HRP was visualized by a modification of the method described by Graham and Karnovsky. The aortic specimens treated with HRP-conjugated rabbit anti-goat IgG were first incubated for 15 minutes in 0.5 mg/ml diaminobenzidine in 50 mM tris(hydroxymethyl)aminomethane buffer, pH 7.6, and then for 3 minutes in the same solution containing 0.02% H2O2.

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overnight before hematoxylin staining and viewing under a fluorescence microscope.

Quantification of IgG-containing dying or dead endothelial cells. After overnight immersion in a 10% formalin solution, the aortic specimens were rinsed with PBS and stained with Harris' hematoxylin for 30 seconds. The IgG-containing endothelial cells and mitotic endothelial cells were identified and counted on en face preparations under a fluorescence microscope equipped with a diascopic phase-contrast attachment. The distributions of IgG-containing endothelial cells and mitotic endothelial cells were correlated with the distribution of macromolecular tracer (EBA) leaky foci.

Criteria for Identifying Mitotic Figures and IgG-Containing Dead Endothelial Cells

With hematoxylin staining, normal endothelial cells display their characteristics of large, ellipsoidal or ovoid, lightly stained, purple-colored nuclei and specific alignment and were easily recognized in en face preparations of the aorta. Different stages of endothelial cell division in the mitotic phase of the cell cycle were identified according to the following morphological criteria. Cells with visible, condensed chromosomes and an intact nuclear envelope were defined as being in prophase. Cells with their chromosomes aligned along the metaphase plate halfway between the poles but without a nuclear envelope were defined as being in metaphase. Cells with separated chromatids that were being pulled toward the poles were defined as being in anaphase. Cells with chromatids clustered at each pole, a contractile ring creating a cleavage furrow, and a re-formed nuclear envelope were defined as being in telophase. Cells with a brown-stained cytoplasm and normally shaped or pyknotic nuclei were defined as HRP-stained IgG-containing dying or dead endothelial cells.

Fluorescence Microscopy

A Nikon epifluorescence microscope (Microphot-FX) equipped with a diascopic phase-contrast attachment was used for detecting and quantifying EBA leaky foci in en face preparations of the thoracic aorta. The EBA fluorescence was studied with a combination of an excitation filter at 450–490 nm, a dichroic mirror at 510 nm, and a barrier filter at 520 nm or alternatively, with an excitation filter at 510–516 nm, a dichroic mirror at 580 nm, and a barrier filter at 590 nm. Color slides for photomicrographs were made with Kodak Ektachrome P800/1600 professional color-reversal film 5020.

Statistical Analysis

In the present studies, the differences in age, body weight, frequencies of endothelial cell death and mitosis, and number density of EBA leaky foci between control rats and nicotine-treated rats were analyzed by two-tailed, unpaired Student's t test. The changes in systolic blood pressure, heart rate, and body temperature before and at different times during the nicotine drinking period in treated rats were analyzed by a one-way analysis of variance (ANOVA).  

<table>
<thead>
<tr>
<th>Parameters</th>
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<tr>
<td>Final</td>
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<td>3.5</td>
<td>NS</td>
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<tr>
<td>Body weight (g)</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>294±9</td>
<td>298±10</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Final</td>
<td>418±13</td>
<td>397±11</td>
<td>NS</td>
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<tr>
<td>Gain</td>
<td>124±12</td>
<td>99±12</td>
<td>NS</td>
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<td>Frequency of EC death (%)</td>
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<td>0.94±0.11</td>
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</tr>
<tr>
<td>Frequency of EC mitosis (%)</td>
<td>0.017±0.005</td>
<td>0.007±0.002</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>No. density of EBA leaky foci (per mm²)</td>
<td>3.30±0.48</td>
<td>6.45±1.19</td>
<td>&lt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

EC, endothelial cell; EBA, Evans blue–albumin; NS, not significant. Values are mean±SEM.

The t test used was two-tailed, unpaired Student's t test.

Results

No statistically significant differences in body weights were found between the nicotine-treated group and the control group at any stage of the experiment, as shown in Table 1. There was also no significant difference in mean daily fluid consumption between the two groups. The entire endothelial surface of each aortic segment was systematically scanned under a fluorescence microscope. The number of endothelial cells scanned was determined by dividing the total endothelial surface area of each aortic segment by the average surface area of a single endothelial cell, which was determined by AgNO₃ staining of the cell boundary followed by computerized image analysis, and was found to be 520 μm². 2,26,27,32,37,38 The number of IgG-containing dying or dead endothelial cells, the number of mitotic endothelial cells, and the number of EBA leaky foci were counted in six segments of the thoracic aorta from each rat. Data from the six segments of each rat were summed. The frequency of endothelial cell death or mitosis was the number of IgG-containing or mitotic endothelial cells, respectively, expressed as a percentage of the total number of endothelial cells scanned. The number density of EBA leaky foci was calculated by dividing the total number of EBA leaky foci by the total endothelial surface area scanned in square millimeters. As shown in Table 1, the frequency of endothelial cell death was significantly higher (0.94±0.11% versus 0.40±0.04%, p<0.0001, as shown in Figure 1), but the frequency of endothelial cell mitosis was significantly lower (0.007±0.002% versus 0.017±0.005%, p<0.05, as shown in Figure 2) in nicotine-treated rats than in control rats. The number density of EBA leaky foci per square millimeter of endothelial surface area was also significantly higher (6.45±1.19/mm² versus 3.30±0.48/mm², p<0.05, as shown in Figure 3) in nicotine-treated rats than in control rats, suggesting a greater permeability in the nicotine-affected endothelium.

Under the fluorescence microscope, EBA leakage in the aorta was visualized as discrete spots or larger foci.
in en face preparations of the thoracic aorta, as previously described.26,32 Figure 4 shows an EBA leaky focus (orange area) located close to an aortic branch (dark area) in a fluorescence color photomicrograph of an en face preparation of a rat thoracic aorta stained with hematoxylin. At higher magnifications, a cluster of HRP-stained IgG-containing endothelial cells was found to be collocated with this EBA leaky focus, as shown in Figures 5 and 6. Some of the rounded cells with large, darkly stained nuclei that were also seen in this EBA leaky focus could be mononuclear cells. Table 2 summarizes the relation between EBA leakage and endothelial cell death or mitosis in both groups of rats, as observed in the present study. A high degree of correlation was found between EBA leakage and endothelial cell death or mitosis. In nicotine-treated rats, an average of 51.4±4.6% of IgG-containing dead endothelial cells was found to be associated with EBA leakage, which contributed to 57.4±3.6% of total EBA leaky foci; also, 80.6±3.5% of mitotic endothelial cells were associated with EBA leakage, but these mitotic endothelial cells accounted for only 2.1±0.4% of the total EBA leaky foci. Similarly in control rats, 42.5±3.3% of dead endothelial cells and 72.6±2.3% of mitotic endothelial cells were located within EBA leaky foci, which were responsible for 46.0±2.6% and 7.4±1.8%, respectively, of the total EBA leaky foci. Since the frequency of endothelial cell death is much higher than the frequency of endothelial cell mitosis in both groups of rats, it can be concluded that dead endothelial cells contributed to the majority of the EBA leakage in rat aortas.

The changes in systolic blood pressure, heart rate, and body temperature before and at different times...
after the 15 rats started drinking the nicotine-containing water are shown in Table 3. Systolic blood pressures were significantly increased (ANOVA) with time in these nicotine-treated rats, without significant alterations in heart rates or body temperatures. Plasma nicotine levels in these 15 rats determined after 6 weeks of drinking the nicotine-containing water ranged from 650.6 ng/ml to 1,489.0 ng/ml with a mean±SEM of 1,011.1 ±56.5 ng/ml, as shown in Table 4.

**Discussion**

There exists a close connection between cigarette smoking and the development of arterial diseases, especially coronary heart disease.1-6 Epidemiological studies have also shown a graded relation between the intensity of smoking and the risk for coronary heart disease.6'8 The intensity and duration of the effects of cigarette smoke and injected nicotine on heart rate, blood pressure, and tracheal pressure have been shown to correspond to the blood levels of nicotine.30 The long-term administration of nicotine to rabbits was also demonstrated to cause rapid and extensive changes in the aortic endothelium, such as numerous focal areas of “ruffled” endothelium,19 which were similar to those seen during the early stages of spontaneous atherosclerosis in pigeons.39 Therefore, it has been suggested that nicotine may be one of the major components in cigarette smoke responsible for the effects of smoking on the cardiovascular system.

The long-term treatment of animals with nicotine in their drinking water has been shown to induce a gradual increase in the systolic blood pressure.40 In the present studies, the systolic blood pressures of rats began to rise significantly after the initiation of drinking nicotine-containing water, from 122±2 mm Hg, and reached a peak pressure of 133 ±2 mm Hg at the end of the third week of treatment; afterward, blood pressure was maintained at high levels throughout the rest of the nicotine treatment period. The heart rate of these rats showed an initial fall and then a rise during the nicotine treatment period, although the changes were not statistically significant. It is, therefore, indicated that long-term drinking of nicotine-containing water has caused some physiological effects on the cardiovascular system and that these effects might partly contribute to the in vivo mechanisms of endothelial damage induced by nicotine.41 Plasma nicotine concentration in human smokers after smoking two cigarettes (1.5 mg of nicotine per cigarette) has been shown to be ~30 ng/ml.42 After

### Table 2. Relation Between Evans Blue–Albumin Leakage and Endothelial Cell Mitosis or Death in Thoracic Aortas of Control and Nicotine-Treated Rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Nicotine treated</th>
<th>Total</th>
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</thead>
<tbody>
<tr>
<td>No. of rats</td>
<td>16</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>Mitotic ECs with EBA leakage (%)</td>
<td>72.6±2.3</td>
<td>80.6±3.5</td>
<td>76.6±2.2</td>
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<tr>
<td>Dead ECs with EBA leakage (%)</td>
<td>42.5±3.3</td>
<td>51.4±4.6</td>
<td>46.9±2.9</td>
</tr>
<tr>
<td>EBA leaky foci with mitotic ECs (%)</td>
<td>7.4±1.8</td>
<td>2.1±0.4</td>
<td>4.7±1.0</td>
</tr>
<tr>
<td>EBA leaky foci with dead ECs (%)</td>
<td>46.0±2.6</td>
<td>57.4±3.6</td>
<td>51.7±2.4</td>
</tr>
</tbody>
</table>

ECs, endothelial cells; EBA, Evans blue–albumin. Values are expressed as mean±SEM.
6 weeks of drinking nicotine-containing water at a dose of 5 mg/kg body wt per day, the plasma levels of nicotine in our nicotine-treated rats ranged from 650.6 ng/ml to 1,489.0 ng/ml with a mean of 1,011.1 ng/ml. In comparison with the plasma nicotine levels found in human smokers, these levels in nicotine-treated rats were approximately equivalent to those of a human who had smoked 50–100 cigarettes, although plasma nicotine levels in humans after smoking the same number of cigarettes have been variable. The variation in plasma nicotine levels among nicotine-treated rats may explain the variability of the data observed in the present studies.

A number of studies have demonstrated that exposure to cigarette smoke or nicotine produces morphological alterations in the vascular endothelium that are indicative of endothelial injury. These changes include endothelial cell swelling, greater cytoplasmic vacuolation, subendothelial edema, increased formation of microvilli and numerous surface projections, opening of the endothelial junctions, etc. Smoking or nicotine consumption has also been shown to have a desquamating effect on endothelial cells, as indicated by a significant increase in the number of macromolecular EBA leaky foci per square millimeter of endothelial surface area was significantly higher than that in the control endothelium (6.45±1.19/mm²) than in control endothelium (3.30±0.48/mm²). These findings indicated that the nicotine-treated aortic endothelium exhibited quantitatively greater permeability to macromolecules. The finding that 51% of IgG-containing dying or dead endothelial cells were associated with EBA leakage and increased with the number of cigarettes smoked. All these findings provide a pathogenic basis that may underlie the relation between cardiovascular problems and cigarette smoking.

Although short- or long-term exposure to nicotine has been shown by numerous studies to have direct damaging effects on the vascular endothelium, none of these studies has directly quantified the frequency of cell death or mitosis in the nicotine-affected aortic endothelium nor the association of such specific events as cell turnover with endothelial permeability. In an autoradiographic study, after exposure of mice to 5 weeks of nicotine, the thymidine labeling index was significantly higher in the nicotine-affected endothelium than in control. However, the thymidine labeling index per se is not an adequate parameter to represent the actual rate of cell loss or cell mitosis in the aortic endothelium. Similarly, although increased circulating endothelial cell counts after nicotine or cigarette smoke exposure could reflect the increased rate of endothelial cell loss, it does not provide quantitative information on the frequency of cell death in the aortic endothelium. Indirect IgG immunocytochemistry has been regarded as a reliable method for quantifying IgG-containing dying or dead endothelial cells in arteries. By using this technique, we were able to quantify the frequency of cell death in nicotine-affected rat aortic endothelium at 0.94±0.11%, which is significantly greater (p<0.0001) than that in control rats (0.40±0.04%). These findings provide direct quantitative evidence that long-term nicotine exposure increases the rate of cell death or cell loss in the rat aortic endothelium, although this might be an indirect effect of nicotine.

The intact endothelium but not the deendothelialized intima has been shown to be the dominant mass transfer resistance for 125I-albumin transport across the aortic wall. The consequence of endothelial injury could be an increase in endothelial permeability to macromolecules. The work done by Lees and coworkers suggested that low density lipoproteins were selectively accumulated in regions where the endothelium was actively regenerating after injury. Extracts of cigarette smoke have been shown to cause dose-dependent increases in albumin flux across the pulmonary endothelium in vitro through their effects on cytoskeletal elements. By a semiquantitative analysis with light microscopy, cigarette smoke exposure has been demonstrated to produce increased endothelial permeability to peroxidase in the rat thoracic aorta, which was proportional to the length of the smoking period. In the present investigation, we found that the number of macromolecular EBA leaky foci per square millimeter of endothelial surface area was significantly higher (p<0.05) in nicotine-exposed aortic endothelium (6.45±1.19/mm²) than in control endothelium (3.30±0.48/mm²). These findings indicated that the nicotine-treated aortic endothelium exhibited quantitatively greater permeability to macromolecules. The finding that 51% of IgG-containing dying or dead endothelial cells were associated with EBA leakage and that these cells accounted for 57% of total EBA leaky foci in this study is consistent with our previous reports that dying or dead endothelial cells make significant contributions to macromolecular leakage into the arterial wall. It is thus suggested that by increasing the rate of endothelial cell death, long-term nicotine consumption could enhance transendothelial macromolecular transport.

In the present study, it is noteworthy that in contrast to a higher rate of endothelial cell death, the frequency of cell mitosis in the nicotine-affected rat aortic endothelium (0.007±0.002%) was significantly lower (p<0.05) than that in the control endothelium (0.017±0.005%). These observations suggest that long-term nicotine expo-
sure not only increases endothelial cell death but also inhibits endothelial cell mitosis. Similar to the findings in our previous reports, these events were associated with EBA leakage, but these mitotic endothelial cells were responsible for only a small proportion of total EBA leaky foci, i.e., 2% in nicotine-treated rats. Therefore, although long-term nicotine exposure decreased the rate of endothelial cell mitosis, this decrease did not significantly reduce the enhanced endothelial permeability resulting from the marked increase in the frequency of endothelial cell death. In other words, it is the increase in the rate of endothelial cell death that accounts for the majority of the nicotine-induced enhancement in aortic endothelial permeability.

In a study in which cigarette smoke was shown to have a progressive and long-term effect on thoracic aorta endothelial permeability to peroxidase, some intercellular clefts were strongly labeled by the tracer, which suggested an increased junctional passage of macromolecular tracer in the nicotine-affected aorta. In addition, the intimal ultrastructure of human umbilical arteries from newborns of smoking mothers revealed degenerative changes of the endothelium, with focal loss of closed intercellular junctions, suggesting the opening of the endothelial junctions with subsequent formation of subendothelial clefts induced by tobacco smoking. A quantitative ultrastructural study has also demonstrated that the intercellular cleft morphology was significantly (p<0.005) less complex and the subendothelial edema was greater in the chronically nicotine-exposed aortic endothelium than in the control endothelium of mice. The authors have suggested that the nicotine-exposed endothelium is more permeable than the unexposed endothelium. Together, all these lines of ultrastructural evidence indicate that open or leaky endothelial junctions might be an important pathway responsible for the greater permeability in cigarette smoke- or nicotine-affected endothelium.

Leaky endothelial junctions associated with cell death or mitosis have been suggested as an important pathway through which macromolecules leak into the intima. Since long-term nicotine exposure markedly increases the rate of endothelial cell death, it is reasonable to postulate that the increase in endothelial cell death induced by long-term nicotine administration would lead to the enhancement of transendothelial macromolecular transport through leaky junctions around dead endothelial cells. However, the additional leakage of macromolecules directly through the dead endothelial cells per se cannot be excluded.

In conclusion, the present study has provided experimental evidence in support of the hypothesis that long-term nicotine exposure, as experienced by cigarette smokers, may markedly increase the frequency of endothelial cell death, resulting in an enhancement of transendothelial leakage to macromolecules such as low density lipoprotein and the initiation of a cascade of complex processes. These eventually lead to the acceleration of atherogenesis and the greater occurrence of coronary heart disease in smokers. The results of the present investigation thus make contributions to our understanding of the pathogenic relation between cigarette smoking and the development of atherosclerosis.

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