Dysfunctional Lipoproteins from Young Smokers Exacerbate Cellular Senescence and Atherogenesis with Smaller Particle Size and Severe Oxidation and Glycation

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Until now, there has been limited information on the effects of smoking on atherogenesis and senescence in the context of lipoprotein parameters, particularly in young smokers who have smoked fewer than 10 cigarettes per day for 3 years. In this study, lipoprotein profiles and functions were compared between smoker (n = 21) and control groups (n = 20). In the smoking group, ferric ion reduction abilities of serum and high-density lipoprotein (HDL) fractions were significantly reduced, and low-density lipoprotein (LDL) was severely oxidized. All lipoprotein particles from the smoker group showed higher advanced glycation end products with more triglyceride (TG) content compared with the control group. Lipoproteins from smokers showed faster agarose gel electrophoresis as well as greater smear band intensity in SDS-PAGE due to oxidation and glycation. LDL from smokers was more sensitive to oxidation and promoted foam cell formation in macrophages. Gel filtration column chromatography revealed that the protein and cholesterol peaks of VLDL and LDL were elevated in the smoker group, whereas those of HDL were reduced. Human dermal fibroblast cells from the smoker group showed severe senescence following treatment with HDL2 and HDL3. Although HDL from young smokers showed impaired antioxidant ability, smaller particle size, and increased TG content, cholesteryl ester transfer protein activities were greatly enhanced in the serum and HDL fractions of the smoker group. In conclusion, smoking can cause production of dysfunctional lipoproteins having a smaller particle size that exacerbate senescence and atherogenic progress due to oxidation and glycation.

Key words: smoking; lipoprotein; glycation; oxidation; atherosclerosis; senescence.

Cigarette smoking is a major and independent risk factor of cardiovascular disease (CVD) (Howard et al., 1998) that is mediated through multiple interrelated mechanisms, including increased oxidative stress, endothelial injury and dysfunction, altered blood coagulation, and derangements of lipid composition and metabolism (Grundy, 1995). It has been well known that smoking impairs endothelial function and creates a vascular environment that contributes to the development of atherosclerosis (Ceremajer et al., 1993; Widlansky et al., 2003). Although there has been controversy about changes in serum cholesterol levels in response to smoking (Slagter et al., 2013), it is generally accepted that smokers have highly elevated levels of serum triglyceride (TG) and low-density lipoprotein cholesterol (LDL-C) (Gossett et al., 2009). Furthermore, smokers showed lower levels of cholesterol in high-density lipoprotein cholesterol (HDL-C) compared with non-smokers (Campbell et al., 2008; Craig et al., 1989). Generally, smoking is associated with increased atherogenic factors in the serum, including elevated levels of serum TG and reduced levels of HDL-C (Nakamura et al., 2009). Smoking frequency and intensity are also associated with increased atherogenic lipoprotein contents, as increased serum TG levels contribute to generate small dense LDL (Griffin et al., 1994) and dysfunctional HDL (Park et al., 2010b, Park and Cho, 2011a). HDL exerts many beneficial effects on health due to its antioxidant, anti-inflammatory, and anti-thrombotic effects (Cho, 2009a). In addition to alteration of the serum lipid profile, smoking causes elevation of inflammatory parameters such as C-reactive protein and serum amyloid A, as previously described (Tracy et al., 1997).

Although there have been several reports on the increased risk of myocardial infarction (MI) and adverse blood lipid profiles in smokers, there is limited information on the changes in lipoprotein properties brought about by smoking. Furthermore, there is no report investigating the function and composition of individual lipoproteins in young smokers.

Accordingly, this study was designed to compare lipid and lipoprotein properties in young smokers who have been smoking fewer than 10 cigarettes per day based on interviews and self-statements. We compared four classes of lipoproteins, very low-density lipoprotein (VLDL), LDL, HDL2, and HDL3, which were individually separated from the subjects based on structural and functional modifications, in order to assess their contributions to the atherogenic process.
MATERIALS AND METHODS

Subjects. Young male smokers (n = 21, 24 ± 1 years old) and control subjects (n = 20, 23 ± 2 years old) with similar age were voluntarily recruited from students who were enrolled at Yeungnam University (Gyeongsan, Korea). We included subjects in the smoking group who had been smoking about 10 cigarettes per day for 3 years without a history of an endocrinological disorder. We excluded heavy alcohol consumers (more than 30 g of EtOH consumed per day) as defined in a previous report. We excluded heavy alcohol consumers (30 g EtOH/day) as defined in previous report (Pöschl and Seitz, 2004) in order to eliminate cancer risk and those who had consumed any prescribed drugs to treat hyperlipidemia or diabetes mellitus. Informed consent was obtained from all individuals prior to enrollment in the study, and the Institutional Review Board at the Medical Center of Yeungnam University (Daegu, Korea) approved this protocol.

Plasma analysis. Blood was drawn from overnight fasting young male smokers and controls. Blood was collected in Vacutainers (BD Sciences, Franklin Lakes, NJ) containing EDTA (final concentration, 1 mM). Plasma was isolated by low-speed centrifugation and stored at −80°C until analysis. Blood parameters including total cholesterol (TC), TG, glucose, and hepatic enzyme concentrations were determined using an automatic blood analyzer (AU4500 Chemistry Analyzer; Olympus, Tokyo, Japan). Serum uric acid concentrations were determined by a standard method described previously (Caraway, 1955).

Isolation and characterization of lipoproteins. VLDL (d < 1.019 g/ml), LDL (1.019 < d < 1.063), HDL-2 (1.063 < d < 1.125), and HDL-3 (1.125 < d < 1.225) were isolated from individual patient and control sera via sequential ultracentrifugation (Havel et al., 1955) with slight modification (Cho, 2012). Samples were centrifuged at 100,000 × g for 24 h at 10°C using a Himac CP-90α centrifuge (Hitachi, Tokyo, Japan). The protein concentrations of the lipoproteins were determined via the Lowry protein assay, as modified by Markwell et al. (1978) with bovine serum albumin as the standard. Phospholipid (PL) in lipoprotein was determined by previously described method (Chen et al., 1956). The concentration of oxidized species in LDL was determined by the thiobarbituric acid reactive substances (TBARS) method using malondialdehyde (MDA) as the standard (Blois, 1958). To compare the extent of glycation between the groups, the content of advanced glycation end products (AGEs) in the individual lipoproteins was determined by reading the fluorometric intensities at 370 nm (excitation) and 440 nm (emission), as described by our group (Park et al., 2010a). All lipoproteins were subjected to the electrophoresis on 0.5% agarose gels, to compare extent of glycation and oxidation (Noble, 1968); electromobility of each lipoprotein is known to depend on its intact charge and size. The gels were then dried and the bands stained with 0.125% Coomassie Brilliant Blue.

Electron microscopy. Transmission electron microscopy (TEM) was performed with a Hitachi electron microscope (model H-7600; Ibaraki, Japan) operating at 80 kV. HDL was negatively stained with 1% sodium phosphotungstate (PTA; pH 7.4) with a final apolipoprotein concentration of 0.3 mg/ml in tris buffered saline. Five microliter of the HDL suspension was blotted with filter paper and immediately replaced with a 5 µl droplet of 1% PTA. After a few seconds, the stained HDL fraction was blotted onto a Formvar carbon-coated 300 mesh copper grid and then air-dried. The shape and size of HDL were determined by TEM photography at a magnification of ×40,000 according to our previous report (Park et al., 2010b).

Functional assays. As antioxidant assays, the ferric-reducing ability (FRA) was determined using the method described by Benzie and Strain (1996). For cholesteryl ester transfer assays, reconstituted HDL (rHDL)-containing apoA-I and cholesteryl (CE) olate was synthesized using trace amounts of [3H]-cholesteryl olate (TRK886, 3.5 µCi/mg of apoA-I; GE Healthcare, Piscataway, NJ) as the CE donor. The CE-transfer reaction occurred in 300 µl reaction mixtures that contained each of the serum samples (20 µl) as a cholesteryl ester transfer protein (CETP) source, rHDL-agarose (50 µg, 0.25 mg/ml), and human LDL (50 µg, 0.25 mg/ml) as CE-donor and CE-acceptor, respectively (Cho, 2009b).

Acetylation of LDL. The acetylation of LDL (acLDL) was performed using saturated sodium acetate and acetic anhydride according to a previously described method (Fraenkel-Conrat, 1957). After acetylation and subsequent dialysis, acLDL protein content was determined and acLDL particles filtered through a 0.22-µm filter (Millex; Millipore, Bedford, MA) prior to use.

Uptake of LDL and acLDL into macrophages. The extent of LDL or acLDL uptake into macrophages was assessed as reported previously to compare anti-atherosclerotic activity using a cellular model. Differentiated, adherent macrophages were then rinsed with warm PBS and incubated with 400 µl of fresh RPMI-1640 medium containing 1% FBS, 50 µl of acLDL (50 µg of protein in PBS), and 50 µl of each HDL (50 µl, 1 mg/ml) for 48 h at 37°C in a humidified incubator. After incubation, the cells were stained with Oil red O solution (0.67%) to visualize the lipid species in the cell. Culture medium (0.2 ml) was then analyzed by the TBARS assay to evaluate changes in levels of oxidized species with a MDA standard. Quantification of Oil red O-stained area in the cell was carried out via computer-assisted morphometry using the Image Proplus software (version 4.5.1.22; Media Cybernetics, Bethesda, MD).

Fast protein column chromatography. To compare the elution profile of plasma between the smokers and control, pooled...
plasma from each group was applied to a Superose 6 10/300GL column (GE Healthcare, Uppsala, Sweden), which was pre-equilibrated with a solution of 20 mM Tris buffer at pH 8.0 and 150 mM NaCl, with a flow rate of 0.25 ml/min using the AKTA purifier system (GE Healthcare). Absorbance of the eluate at 280 nm was monitored and collected in a fraction collector (0.3 ml/tube). An aliquot of each fraction was analyzed for cholesterol.

Anti-senescence assay. Primary human dermal fibroblast (HDF) cells were cultured and cellular senescence-associated-β-gal activity was compared with the degree of senescence, as described previously (Dimri et al., 1995; Park and Cho, 2011a). For induction of senescence, cells at passage 9 (~40% confluence) were exposed to each HDL fraction (0.1 mg/ml of protein). Cells were fixed for 5 min in 3% paraformaldehyde in PBS, washed three times in PBS, and incubated in SA-β-gal staining solution (40 mM citric acid/phosphate [pH 6.0], 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl2, and 1 mg/ml of 5-bromo-4-chloro-3-indolyl-X-galactosidase) for 16 h at 37°C. The percentage of blue cells was calculated under light microscopic observation.

Zebrafish and embryos. Wild-type zebrafish and embryos were maintained according to standard protocols (Nusslein-Volhard and Dahm, 2002). Zebrafish maintenance and experimental procedures were approved by the Committee of Animal Care and Use of Yeungnam University (Gyeongsan, Korea). Zebrafish and embryos were maintained in a system cage (3L volume, acrylic tank) and 6-well plates, respectively, at 28°C during treatment under a 14:10 h light:dark cycle.

Microinjection of zebrafish embryos. In order to compare antioxidant and anti-inflammatory activity of HDL between smokers and control, HDL3 from the each group was injected into zebrafish embryo as our previous report (Park and Cho, 2011b). Embryos at 1 day post-fertilization were individually injected by microinjection using a pneumatic micropipette (PV820; World Precision Instruments, Sarasota, FL) equipped with a magnetic manipulator (MM33; Kantec, Bensenville, IL) with a pulled micropipillary pipette-using device (PC-10; Narishigen, Tokyo, Japan). To minimize bias, injections were performed at the same position on the yolk. Filter-sterilized solution of each HDL (1 mg/ml, 50 nL) was injected into flasks of embryos. Following injection, live embryos were observed under a stereomicroscope (Motic SMZ 168; Hong Kong) and photographed using a Motic cam 2300 CCD camera.

Imaging of reactive oxygen species. After injection with rHDL, changes in the levels of reactive oxygen species (ROS) in the larvae were imaged by dihydroethidium (DHE; cat no. 37291, BioChemika) staining as previously described (Owusu-Ansah et al., 2008). Images were obtained by fluorescence microscopy (Ex = 588 nm and Em = 605 nm) on a Nikon Eclipse TE2000 instrument (Tokyo, Japan). In order to avoid bias, red fluorescence was measured in the trunk area away from the injected site.

Data analysis. All data are expressed as mean ± standard deviation from at least three independent experiments with duplicate samples. Data comparisons were assessed by Student’s t-test using the SPSS version 14.0 program (SPSS, Inc., Chicago, IL).

RESULTS

Serum Profiles

Although the serum lipid profiles of all subjects were within a normal range, the smoker group showed 1.6-fold higher TG levels than the control group (Table 1). Further, the smoker group showed no significant difference in HDL-C levels or LDL-C levels compared with the control. The smoker group showed a significantly lower percentage of HDL-C/TC (~23%) compared with the control group (~29%). Interestingly, the smoker group showed significantly higher serum TG/HDL-C levels compared with the control group, although there was no difference in the TC/HDL ratio or LDL/HDL ratio. The hepatic inflammatory profile, based on aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities, was significantly higher in the smoker group compared with the control group. However, serum glucose and uric acid levels were not significantly different between the groups.

Plasma obtained from smokers showed much less ferric ion reducing ability compared with control plasma (Supplementary fig. 1A). After 30 min of incubation, plasma (10 μL) from smokers showed a 2.8-fold increase in A593 from initial level, whereas the control showed a 4.7-fold increase (p < 0.005 vs. smoker) from initial level. This result indicates an up to 40% decrease in antioxidant ability in the smoker group compared with the control based on ferric ion reduction ability.

Lipoprotein Properties

As shown in Table 2, the smoker group showed ~1.5–2.0-fold higher cholesterol and TG contents in VLDL and LDL fractions than the control group. In particular, the smoker group showed almost twofold higher TG contents in the VLDL and LDL fractions compared with the control. The VLDL fraction from smokers had threefold higher PL content than that of the control group. The HDL2 fraction from the smoker group also showed almost twofold higher TG contents in the VLDL and LDL fractions compared with the control. The HDL2 fraction from the smoker group also showed twofold higher TG content as well as 1.8-fold lower PL content compared with the control group, although both HDL2 fractions showed similar cholesterol contents. Regarding TC content, the HDL3 fraction from the smoker group showed 24% lower TC content compared with the control, whereas the VLDL and LDL fractions from smokers showed higher TC levels. Lastly, the smoker group showed impaired HDL3-associated...
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**TABLE 1**

<table>
<thead>
<tr>
<th>Plasma Profiles of Smoker and Non-Smoker</th>
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<tbody>
<tr>
<td>Smoker (n = 21)</td>
</tr>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
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<tr>
<td>TG (mg/dL)</td>
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<tr>
<td>HDL-cholesterol (mg/dL)</td>
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<tr>
<td>LDL-cholesterol (mg/dL)</td>
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<tr>
<td>TG/HDL ratio</td>
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<tr>
<td>LDL/HDL ratio</td>
</tr>
<tr>
<td>AST (IU/L)</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
</tr>
<tr>
<td>Serum glucose (mg/dL)</td>
</tr>
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<td>Uric acid (mg/dL)</td>
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</tbody>
</table>

C, cholesterol; CETP, cholesteryl ester transfer protein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TC, total cholesterol; TG, triglyceride; VLDL, very low-density lipoprotein. *p < 0.05 versus control; **p < 0.01 versus control.

FRA, with 33% lower FRA than the control HDL3 fraction (p < 0.01 vs. smoker), as shown in Supplementary figure 1B.

**Lipoproteins from Smokers are More Oxidized**

As shown in Figure 1A, LDL from the young smoker (YS) group (YS-LDL) was more susceptible to cupric ion-mediated oxidation. After 60 min of exposure to Cu²⁺, YS-LDL showed a 2.6-fold increase in conjugated diene level (A₂₃₄), whereas the control showed a 2.4-fold increase in A₂₃₄ (p < 0.05). Similarly, YS-LDL was more susceptible to oxidation after 30 min without Cu²⁺ exposure, as shown in Figure 1A (black triangles).

Quantification of oxidized species (MDA) in each LDL fraction by TBARS showed that YS-LDL and HDL3 from smokers had 2.1-fold and 1.4-fold higher MDA content compared with the control, respectively (Fig. 1B), although the smoker group showed higher MDA levels in all lipoprotein fractions. These results indicate that lipoproteins from the smoker group contained more oxidized species and were more sensitive to oxidation than those of the control group.

**Glycated Species and Modification of Electrophoretic Properties**

All lipoprotein fractions in plasma from the smoker group showed significantly higher AGE contents (Fig. 1C). VLDL and LDL in the smoker group showed 25% (p < 0.001 vs. control) and 12% (p < 0.01 vs. control) greater glycation compared with the control group, respectively, per an equal amount of protein. The smoker group showed 57% (p < 0.01 vs. control) and 12% higher AGE contents in the HDL2 and HDL3 fractions, respectively, compared with the control, indicating that HDL2 was more sensitive to glycation due to smoking. Interestingly, the highest TG content in HDL2 from smokers (Table 2) was directly associated with increased AGE content in the LDL and HDL3 fractions. Electron microscopy revealed that HDL from smokers had a smaller particle size with more distorted shape and unclear contours compared with that of the control, as shown in photo of Figure 1C.

Agarose gel electrophoresis found that VLDL and LDL from smokers showed stronger and thicker band intensities with faster electromobility compared with the same amount of control proteins, suggesting that apo-B containing lipoprotein was well developed in response to smoking. As shown in Figure 2A, LDL and HDL from smokers showed faster agarose gel electromobility than those of the controls, indicating greater oxidation in the LDL and HDL fractions. Especially in the HDL3 fraction, the smoker group showed greater smear band intensity, more migration to the bottom of the gel, and larger particles, as indicated by the arrowhead. SDS-PAGE revealed weaker apoA-I band (28 kDa) intensities with a slightly upward shift in HDL2 and HDL3 fractions from the smoker group as well as a multimerized band (indicated by the arrow head in YS-HDL3 of Fig. 2B), which is similar to HDL from the elderly in our previous report (Park et al., 2010b; Park and Cho, 2011a). Further, the apoA-I band in HDL2 from smokers disappeared and shifted upward.

The apoC band in HDL2 from smokers was stronger, as indicated by the arrowhead in Figure 2B. Western blot analysis using apoA-I antibody also revealed that the apoA-I band in the HDL2 fraction from smokers showed diminished intensity and shifted upward, indicating modification of apoA-I induced by smoking (Fig. 2B and C).

**CETP Activity**

As shown in Figure 3, plasma (20 μl) from smokers showed 1.3-fold higher CE-transfer activity (32.6 ± 3.7% of CE-transfer) compared with the control (25.9 ± 3.0% of CE-
Lipid and Protein Compositions in Lipoproteins

<table>
<thead>
<tr>
<th>Young smoker (YS)</th>
<th>Young non-smoker (YN)</th>
</tr>
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<tbody>
<tr>
<td>TC (mg/ml)</td>
<td>TC (mg/ml)</td>
</tr>
<tr>
<td>VLDL</td>
<td>TC (mg/ml)</td>
</tr>
<tr>
<td>0.5 ± 0.1**</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Total amount (mg)</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>1.8 ± 0.7**</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>LDL (mg/ml)</td>
<td>1.9 ± 0.8**</td>
</tr>
<tr>
<td>1.2 ± 0.1**</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>Total amount (mg)</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>4.8 ± 1.4**</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>HDL2 (mg/ml)</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>1.9 ± 0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>Total amount (mg)</td>
<td>2.0 ± 0.4</td>
</tr>
<tr>
<td>7.7 ± 0.6**</td>
<td>2.9 ± 0.6</td>
</tr>
<tr>
<td>HDL3 (mg/ml)</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>0.5 ± 0.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Total amount (mg)</td>
<td>0.6 ± 0.21*</td>
</tr>
<tr>
<td>1.8 ± 0.1</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td>Total amount (mg)</td>
<td>0.5 ± 0.1*</td>
</tr>
<tr>
<td>1.5 ± 0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>Total amount (mg)</td>
<td>0.4 ± 0.1*</td>
</tr>
<tr>
<td>5.7 ± 1.7</td>
<td>3.7 ± 1.3</td>
</tr>
</tbody>
</table>

YS, Young-Smoker; YN, Young-Non smoker; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; TC, total cholesterol; TG, triglyceride; TP, total protein.

* p < 0.05 versus control; ** p < 0.01 versus non-smoker.

FIG. 1. Comparison of oxidation of lipoproteins between the smoker and control groups. The data are expressed as mean ±SD from three independent experiments with duplicate samples. YS, young smoker; YN, young non-smoker.

Cellular Uptake of LDL Into Macrophages

THP-1 cells were stained with Oil red O to evaluate the extent of LDL uptake in the smoking and control groups after 48 h of incubation. As shown in Figure 4A, individual smoker LDL-treated cells showed stronger staining intensity than control LDL (YN-LDL)-treated cells, indicating that young smoker LDL (YS-LDL) was more easily taken up into macrophages by putative phagocytosis under the same protein amount of LDL.

In the presence of acLDL, YS-HDL2-treated cells showed stronger Oil red O staining than YN-HDL2-treated cells, indicating that smoker HDL had less inhibitory effects against cellular uptake of acLDL. TBARS analysis with cell media showed that cells treated with YS-LDL and YN-LDL contained 49 and 44 nmoles of MDA, respectively. Treatment with acLDL resulted in the highest production of MDA, whereas co-treatment with YN-HDL2 and YS-HDL2 reduced MDA production to 29 and 40 nmoles, respectively. These results indicate that YS-LDL treatment caused maximal oxidation of the product, whereas YS-HDL2 resulted in loss of anti-atherosclerotic activity. HDL from the young and normal groups (YN-HDL) prevented phagocytosis, whereas HDL from young smokers (YS-HDL) did not prevent acLDL uptake into macrophages.

As shown in Supplementary figure 2A, smoker LDL was more easily taken up into macrophages compared with normal LDL under the same protein amount of LDL, as visualized Oil red O staining. It is well known that modified LDL is taken...
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FIG. 2. Expression of apolipoproteins in HDL (panel C). HDL2 and HDL3 purified via ultracentrifugation were equally diluted, after which the same amount of protein (5 μg) was loaded in each lane and immunodetected using apoA-I antibody raised against full-length apoA-I (ab7613; Abcam). Numbers below the bands indicate relative band intensity (BI), which was analyzed using Gel Doc XR (Bio-Rad). The data are expressed as mean ± SD from three independent experiments with duplicate samples.

FIG. 3. Comparison of CETP activity in lipoproteins between the smoker and control groups. Same amount of protein in the individual fraction (20 mg of total protein) was utilized as a source of CETP. Human LDL (0.25 mg/ml of protein) and reconstituted high-density lipoprotein (rHDL) containing [3H]-cholesterol were used as a CE-acceptor and CE-donor, respectively. Error bars indicate the standard deviation from three independent experiments with duplicate samples. YS, young smoker; YN, young non-smoker. *p < 0.05; **p < 0.01.

FIG. 4. Cellular uptake of purified LDL into macrophages. PMA-differentiated THP-1 cells were incubated with 50 μl of purified LDL (1 mg of protein/ml) or NBD-LDL (1 mg of protein/ml) from the representative smoker or control groups in the presence of 450 μl of RPMI-1640 media. Extent of cellular uptake of lipoproteins into macrophages was compared by Oil red O staining (A), and green fluorescence was quantified (B). Cells were photographed using a Nikon Eclipse TE2000 microscope at ×600 magnification.

Lipoprotein Profile from Column Chromatography

As shown in Figure 5, peaks of the VLDL and LDL fractions were remarkably elevated in plasma from smokers, whereas the size of the HDL peak was reduced. Smoker VLDL and LDL fractions had higher cholesterol contents than the control, whereas smoker HDL had a lower cholesterol content. However, peak size and elution time of the albumin peak were very similar between the groups (Fig. 5A). Cholesterol contents of the VLDL and LDL fractions were remarkably elevated in smokers, whereas cholesterol content in HDL was reduced (Fig. 5B). These results make a good agreement with lipoprotein...
FIG. 5. Elution profiles of pooled sera from each group using size exclusion column chromatography with the AKTA purifier system equipped with a Sepharose 6 10/300GL column. Elution profile was monitored by absorbance at 280 nm (A280) in panel A and determination of cholesterol content in panel B.

FIG. 6. Acceleration of cellular senescence in HDFs treated with HDL from smokers. Representative image of SA-β-gal positive HDFs (blue staining). Cells were treated with HDL (112 μg of protein/5 ml of media) from passages 11–15 and measured as described in the Materials and Methods section. Cell images were captured using a Nikon Eclipse TE2000 microscope (Tokyo, Japan) at ×400 magnification.

Composition analysis as shown in Table 1, suggesting that smokers had elevated VLDL and LDL fractions with enriched cholesterol contents compared with those of the control, whereas the HDL fraction was reduced.

Induction of Cellular Senescence

Cellular senescence was accelerated by treatment with smoker HDL2 and HDL3, as evidenced by SA-β-gal staining in Figure 6. HDL from smokers caused much stronger blue intensity in HDF cells compared with control cells at the same passage. By passage 14, the percentage of cells stained positive for SA-β-gal had increased up to 30% by HDL3 treatment in the smoker group, whereas HDL3 from non-smokers resulted in 6% SA-β-gal-positive cell staining (Supplementary figure 3).

In the control group, the percentage of SA-β-gal-positive cells had diminished upon HDL3 treatment compared with HDL2 treatment at the same dosage (0.022 mg of protein/ml of media). However, HDL3 from smokers exacerbated senescence more than HDL2. Usually, in a normal state, HDL3 has more potent antioxidant and anti-aging activities than HDL2. However, the current result suggests that even moderate smoking could impair the anti-senescence activity of HDL.

HDL from Smokers Causes Increased Oxidative Stress in Zebrafish Embryos

The survivability of zebrafish embryos was reduced to 61% upon injection of smoker HDL, whereas non-smoker HDL induced 80% survival, as shown in Supplementary Figure 4A. In the presence of oxLDL, injection of HDL from smokers resulted in the lowest survivability (46%), whereas control HDL injection resulted in 68% survivability. Developmental speed of embryos was more attenuated by smoker HDL in both the absence and presence of oxLDL, as shown in Supplementary figure 4B. DHE staining revealed that smoker HDL-injected embryos showed increased ROS production based on higher red intensity. Co-injection of oxLDL and smoker HDL3 resulted in the slowest developmental speeds at 52 h post-fertilization as well as the highest ROS production. This result shows that normal HDL could protect embryos from oxLDL toxicity, whereas HDL from smokers could not.

DISCUSSION

Smoking is an independent risk factor of CVD as well as a number of cancers due to increased oxidative stress and inflammation, which have been linked to the pathogenesis of chronic diseases in smokers. Although many reports have demonstrated changes in serum lipid profiles due to smoking, none have characterized purified lipoproteins particularly in younger smokers in their early 20s.

Elevation of the hepatic inflammatory profile in smokers, as indicated by AST and ALT activities, was in good agreement with a previous report (Jang et al., 2012). Smoker VLDL particles were well developed and showed enrichment of cholesterol, TG, and protein compared with control, as shown in Table 2 and Supplementary figure 5.

In the current study, young male smokers showed higher TG contents in serum as well as all lipoprotein fractions (Tables 1 and 2) with reduced antioxidant activity, which was in good agreement with previous reports (James et al., 2000; Morrow et al., 1995). Lipoproteins from smokers were more oxidized (Fig. 1), similar to a previous report (Frei et al., 1991). Furthermore, lipoproteins from smokers showed different electrophoretic properties and increased apoA-I multimerization (Fig. 2).

Smokers also showed elevated CETP activities in serum and lipoproteins, especially in HDL fractions (Fig. 3), which was similar to previous reports (Dullaart et al., 1994; Freeman et al., 1998). In support of these results, cigarette-smoking men show higher plasma lipid transfer protein levels with reduced content of cholesteryl ester in HDL fraction (Dullaart et al., 1994).
Smoker LDL was more easily taken up into macrophages, and YS-HDL could not prevent uptake of acetylated LDL into macrophages (Fig. 4). This suggests that lipoprotein was modified to produce advanced glycated end products, as suggested previously (Cerami et al., 1997). LDL from smokers was more easily taken up into macrophages, resulting in acceleration of foam cell production (Supplementary fig. 2A) as well as increased cholesterol influx (Supplementary fig. 2B) compared with LDL from non-smokers. These results provide mechanistic evidence that cigarette smoking is directly related to progression of atherosclerosis (Burns, 2003; Howard et al., 1998). In the same context, size exclusion chromatography found that protein and cholesterol levels were higher in VLDL and LDL fractions from smokers, whereas protein and cholesterol contents in HDL were reduced (Fig. 5). These differences in particle content were in good agreement with the lipoprotein component results, as shown in Table 2.

Recently, Okada et al. (2013) investigated facial changes caused by smoking in twins and found that aging was more associated with the smoking twin than the non-smoking twin. Although the reason for facial aging in smokers is unknown, our current results can be used to provide a molecular mechanism for the effects of tobacco on dermal fibroblasts. Smoker HDL$_2$ and HDL$_3$ fractions induced remarkable cellular senescence in HDF cells compared with normal HDL (Fig. 6 and Supplementary fig. 3). Smoker HDL$_2$ also increased inflammatory death in zebrafish embryos with attenuated developmental speed (Supplementary fig. 4). These differences in lipoprotein activities between the two groups indicate that smokers had a greater atherogenic lipoprotein profile along with pro-inflammatory properties.

Although the smoker and control groups were of a similar body mass index and age, smokers showed significantly higher serum glucose and TG levels compared with the control group. Lipoproteins from smokers were strikingly enriched with TG (Table 2). It is well established that serum TG level is an independent risk factor of inflammatory disease. Our result of increased TG content in lipoproteins from smokers is in good agreement with previous studies, which found that TG levels are an important and independent predictor of coronary artery disease (CAD) and stroke in the Asia-Pacific region (Patel et al., 2004). Further, VLDL, LDL, and HDL$_2$ fractions from the smoker group were more oxidized and glycated, and they exhibited greater uptake into macrophages with increased production of oxidized species than those of the control group. These results are in good agreement with previous reports that TG-rich lipoproteins are pro-atherogenic (Ginsberg, 2002). TG-enriched LDL is easily transformed into small dense LDL (sdLDL), which is associated with an increased risk of diabetes and CVD (Hirayama and Miida, 2012). The sdLDL can easily be oxidized and glycated via interactions with ROS and carbohydrates in the blood (Griffin et al., 1994). Therefore, serum levels of sdLDL are strongly correlated with progression of aging, diabetes mellitus, and CAD (Gardner et al., 1996). More YS-LDL was taken up into macrophages than LDL from the control group (Fig. 4), which is in agreement with a previous report that sdLDL enhances foam cell formation in atheroma (Tani et al., 2011).

CETP is an atherogenic protein and thus could affect HDL composition and functionality. As such, several CETP inhibitors have been developed in clinical trials. Cigarette smoking is associated with significantly increased risk of early onset MI only among carriers of the Taq1B1 allele; current smokers carrying the B1B1 and B1B2 genotypes display 9.4 ($p < 0.001$) and 8.4 ($p < 0.001$) year reductions, respectively, at the age of first onset of MI compared with non-smokers (Goldenberg et al., 2007). CETP activity is inversely correlated with HDL-C and apoA-I levels in acute phase (Kim et al., 2010). Further, young athletes show much lower CETP activity and expression, whereas HDL-C and apoA-I levels in HDL are remarkably elevated (Lee et al., 2009). These results correlate well with a previous report that found CETP activity is enhanced in smoking non-obese men and smoking diabetic men (Dullaart et al., 1991).

Similar to oxidative stress, glycation of lipoproteins is another modification that exacerbates atherosclerosis and diabetic complications. In the current study, VLDL and LDL from smokers were highly glycated (Figs. 1 and 2), indicating that LDL was smaller and denser than native LDL, as sdLDL can be preferentially glycated. Highly glycated LDL increases CC chemokine receptor-2 expression in macrophages and monocyte chemoattractant protein-1-mediated chemotaxis (Isoda et al., 2008).

Because no reports have compared the extent of oxidation, inflammation, and aging among individual lipoproteins, we characterized lipoprotein properties in young male smokers in this study. Characteristics of smoker HDL are very similar with those of elderly HDL (Park and Cho, 2011a,b); they share smaller HDL particle size, oxidation, and glycation (Park et al., 2010b), induction of severe cellular senescence in human dermal cells (Park and Cho, 2011a), and pro-inflammatory death of zebrafish embryos (Park and Cho, 2011b). Similar to our current results, Stein’s group reported that smoking cessation at ~1 year causes elevation of HDL-C and large HDL particle concentrations (Gepner et al., 2011).

In conclusion, we showed that the lipoprotein properties of young male smokers are more oxidized and glycated compared with those of controls. LDL from smokers promoted increased foam cell formation via accelerated phagocytosis of macrophages. LDL$_3$ from the smoker group showed increased glycation with reduced antioxidant ability compared with that of the non-smoker group. Smoker HDL exacerbated cellular senescence and embryonic cell death due to elevation of ROS production. These modifications, including oxidation and glycation of lipoproteins, are related to pro-senescence parameters in the blood of young smokers. It should be understood that lipoproteins modified by smoking could cause rapid progress of atherosclerosis, diabetes, and senescence, even in young, moderate smokers.
SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci. oxfordjournals.org/.

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REFERENCES


Nakamura, K., et al. (2009). Does cigarette smoking exacerbate the effect of total cholesterol and high-density lipoprotein cholesterol on the risk of cardiovascular diseases? Heart. 95, 909–916.


