Lipoprotein-Associated Phospholipase A2 Regulates Macrophage Apoptosis via the Akt and Caspase-7 Pathways

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Aim: Mutations in lipoprotein-associated phospholipase A2 (Lp-PLA2) are related to atherosclerosis. However, the molecular effects of Lp-PLA2 on atherosclerosis have not been fully investigated. Therefore, this study attempted to elucidate this issue.

Methods: Monocytes were isolated from randomly selected healthy male volunteers according to each Lp-PLA2 genotype (wild-type Lp-PLA2 [Lp-PLA2 (V/V)], the heterozygous V279F mutation [Lp-PLA2 (V/F)] and the homozygous V279F mutation [Lp-PLA2 (F/F)]) and differentiated into macrophages. The level of apoptosis in the macrophages following incubation without serum was measured using the annexin V/propidium iodide double staining method, and the underlying mechanisms were further examined using a culture cell line.

Results: The average plasma Lp-PLA2 concentration [Lp-PLA2 (V/V): 129.4 ng/mL, Lp-PLA2 (V/F): 70.7 ng/mL, Lp-PLA2 (F/F): 0.4 ng/mL] and activity [Lp-PLA2 (V/V): 164.3 nmol/min/mL, Lp-PLA2 (V/F): 100.9 nmol/min/mL, Lp-PLA2 (F/F): 11.6 nmol/min/mL] were significantly different between each genotype, although the basic clinical characteristics were similar. The percentage of apoptotic cells was significantly higher among the Lp-PLA2 (F/F) macrophages compared with that observed in the Lp-PLA2 (V/V) macrophages. This induction of apoptosis was independent of the actions of acetylated low-density lipoproteins. In addition, the transfection of the expression plasmid of V279F mutant Lp-PLA2 into Cos-7 cells or monocyte/macrophage-like U937 cells promoted apoptosis. The knockdown of Lp-PLA2 also increased the number of apoptotic cells. Among the cells expressing mutant Lp-PLA2, the caspase-7 activity was increased, while the activated Akt level was decreased.

Conclusions: The V279F mutation of Lp-PLA2 positively regulates the induction of apoptosis in macrophages and Cos-7 cells. An increase in the caspase-7 activity and a reduction in the activated Akt level are likely to be involved in this phenomenon.


Key words: Apoptosis, Atherosclerosis, Macrophage, Phospholipase

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Introduction

Lipoprotein-associated phospholipase A2 (Lp-PLA2) is a member of the PLA2 superfamily and consists of 441 amino acids in humans¹,². This protein was first identified as platelet-activating factor acetylhydrolase (PAF-AH) due to its ability to hydrolyze and inactivate PAF in vitro³,⁴. It was later found that this protein is produced in monocytes, macrophages and mast cells, as well as platelets⁵,⁷, and circulates in association with low-density lipoprotein (LDL; 80-85%) via an interaction with apolipoprotein B in addition to a minor portion of high-density lipopro-
tein (15-20%) and very-low-density lipoprotein. Similar to its effects on PAF-AH, this protein cleaves the oxidized phospholipids in the sn-2 chain to generate lysophosphatidylcholine and oxidize non-esterified fatty acids. Based on its characteristics, this protein was additionally named Lp-PLA2.

The gene name of Lp-PLA2 is PLA2G7. This gene is formed by 12 exons located in chromosome 6 in humans. The PLA2G7 gene includes several single nucleotide polymorphisms, one of which is the transversion of 994G-T in exon 9 of the gene, resulting in the replacement of Val at amino acid 279 to Phe (V279F). The homozygous V279F mutation of Lp-PLA2 [Lp-PLA2 (F/F)] induces not only the complete loss of enzymatic activity, but also a deficiency of the enzyme in the serum due to the blockade of its secretion from cells. Lp-PLA2 (F/F) was initially discovered in the Japanese population, with an incidence of approximately 4%.

Approximately 30% of the Japanese population has the heterozygous V279F mutation [Lp-PLA2 (V/F)], and its enzymatic activity is half that of wild-type Lp-PLA2 [Lp-PLA2 (V/V)]. In addition to its detection in the Japanese population, the V279F mutation of Lp-PLA2 has been found in other Asian populations, such as Korean, Taiwanese and Chinese groups, although it has not been identified in Caucasian populations.

There are reports that the deficiency of plasma Lp-PLA2 in Japanese due to its homozygous mutation elevates the risk of cardiovascular diseases, including stroke, coronary artery disease and abdominal aortic aneurysm. In contrast, the largest study conducted to date demonstrated that carriage of the V279F null allele is protective for cardiovascular diseases among South Korean men. In addition, several groups have shown that a high plasma Lp-PLA2 level is associated with an increased risk of atherosclerosis and cardiovascular diseases. Therefore, some controversy exists over whether Lp-PLA2 has protective or adverse effects on diseases related to atherosclerosis. The discrepancies observed between clinical studies likely depend on the possible bidirectional effects of Lp-PLA2 on atherosclerogenic factors; for example, it reduces the level of the pro-inflammatory factor PAF, while it also produces lysophosphatidylcholine and oxidized non-esterified fatty acids from oxidized LDLs.

**Aim**

In an attempt to shed some light on the above discrepancies, we investigated the molecular consequences of having the Lp-PLA2 (F/F) phenotype. It has been reported that macrophage death is correlated with the vulnerability of plaque in atherosclerotic lesions. Therefore, in this study, we examined the effects of Lp-PLA2 mutations on macrophage apoptosis and its underlying mechanisms by mainly focusing on the caspase pathway.

**Methods**

**Study Participants**

Among men 40 to 79 years of age living in Kusatsu city, Shiga, Japan, 2,381 were randomly selected based on age strata and sent an invitation to participate in the baseline survey of the Shiga Epidemiological Study of Subclinical Atherosclerosis (SESSA). Of these individuals, 1,094 agreed to participate and underwent examinations, 1,050 of whom also agreed to undergo analyses of their personal genetic information, including Lp-PLA2 genotypes, with written informed consent. Genomic DNA was extracted from peripheral blood leukocytes. The SNP rs16874954 (V279F) of the PLA2G7 gene was genotyped via allelic-specific oligonucleotide hybridization, with the following results: 693 (66.0%) for Lp-PLA2 (V/V), 329 (31.3%) for Lp-PLA2 (V/F) and 28 (2.7%) for Lp-PLA2 (F/F). This genotype distribution is consistent with that predicted if the population is in Hardy-Weinberg equilibrium. Simultaneously, plasma aliquots prepared from fasting blood samples were collected and used to measure the Lp-PLA2 activity according to a colorimetric activity method assay (diaDexus Inc., South San Francisco, CA, USA) in which 1-myristoyl-2-(4-nitrophenoxy)phosphatidylcholine was used as a substrate. The activity (nmol/min/mL) was calculated from the slope of the kinetic absorption curve (405 nm) using the slope of the standard phenol curve. The Lp-PLA2 concentration was measured according to an enzyme-linked immunoassay (PLAC test; diaDexus Inc.). The participants were randomly selected from each Lp-PLA2 genotype group by the subteam at the Department of Health Science, and, after obtaining further written informed consent, blood samples were again drawn in order to assess the level of apoptosis using the assay described below. All clinical information for the selected participants, including the Lp-PLA2 genotype, was completely closed to the subteam at the Department of Biochemistry and Molecular Biology, who carried out the in vitro experiments, until the end of the study. The protocol of this study was reviewed and approved by the institutional review board of Shiga University of Medical Science (No. 17-83, 17-84-1, 19-61).
Isolation and Culture of Human Monocyte-Derived Macrophages

The buffy coat layer was collected from the blood sample (50 mL) of each participant, and human monocytes were isolated via density gradient centrifugation (OptiPrep, Sigma-Aldrich, St. Louis, MO, USA), as previously described. The isolated monocytes (4.5 × 10⁶ cells/well in a 6-well plate) were incubated in 2 mL of RPMI1640 complete medium (Nacalai Tesque, Kyoto, Japan), which contains 2 mM of glutamine, 5% human serum (Sigma-Aldrich), antibiotic solution (100 unit/mL of penicillin, 0.1 mg/mL of streptomycin and 0.25 μg/mL of amphotericin B; Wako Pure Chemical Industries, Osaka, Japan) and 5 mM of ethylenediaminetetraacetic acid (EDTA) and replated on a 96-well plate with a clear bottom and black wall (Corning Inc., Corning, NY, USA), as previously described. The isolated monocytes were cultured for more than 24 hours in RPMI1640 complete medium. The cells were subsequently detached using phosphate-buffered saline (PBS) containing 8 mg/mL of lidocaine (Sigma-Aldrich) and 5 mM of ethylenediaminetetraacetic acid (EDTA) and replated on a 96-well plate with a clear bottom and black wall (Corning Inc., Corning, NY, USA) at a density of 8 × 10⁴ cells/well. The cells were further cultured in RPMI1640 complete medium for five days, changing the culture medium every other day, and then used in the apoptosis assay, as described below.

Apoptosis Assay

In order to induce apoptosis, we treated monocyte-derived macrophages, U937 cells or Cos-7 cells for 16 hours in serum-free medium. Under some conditions, 10 μg/mL of acetylated LDL (AcLDL; Invitrogen, Carlsbad, CA, USA), 10 μg/mL of acyl-coenzyme A: cholesterol acyltransferase (ACAT) inhibitor (kindly gifted from GlaxoSmithKline, Middlesex, UK), caspase-7 inhibitor (Merck, Darmstadt, Germany) or Akt inhibitor (Merck) was added to the serum-free medium. After 16 hours of incubation, the cells were stained using the Vybrant Apoptosis Assay kit (Invitrogen), which includes Alexa Fluor 488-labeled annexin V and propidium iodide, according to the manufacturer’s instructions. Cell images of monocyte-derived macrophages were obtained in over six non-overlapping microscopic fields of each well under each treatment condition using an Olympus IX-71 microscope (Olympus, Tokyo, Japan) equipped with a cooled CCD camera CoolSNAP HQ (Nippon Roper, Tokyo, Japan). The overlay images were produced using the Meta Morph software program (Molecular Devices, Sunnyvale, CA, USA). The cells stained with Alexa Fluor 488-labeled annexin V alone, not propidium iodide, were counted as apoptotic cells. The cell count in each microscopic field was obtained and averaged independently by two researchers with no knowledge of the genotype of the monocytes/macrophages. The proportion of apoptotic cells is shown as the percentage of the total cell number.

Expression Vector Construction for Lp-PLA2 and its Mutant

Human Lp-PLA2 cDNA was generated via reverse transcription PCR using mRNA extracted from human cultured cells and forward and reverse primers tagged with NotI and SalI sites: 5’-GATCCTGCGCGCCCGCAAGATGGTGCCACCACAATTG-3’ (forward; F1) and 5’-GATCTGGATCATTGTATTTCTCATTTCGGAAG-3’ (reverse; R1), respectively, where the restriction sites are indicated by underlined text. The amplified fragment was inserted into the multiple cloning site of the pFLAG-CMV5 vector (Sigma-Aldrich) to express C-terminally FLAG-tagged Lp-PLA2. The expression vector for mutant Lp-PLA2, in which Val at amino acid 279 is replaced by Phe (V279F), was prepared according to a two-step site-directed mutagenesis strategy using the pFLAG-CMV5-Lp-PLA2 vector as a template. Briefly, two PCR fragments were obtained as the first step: one was amplified using a pair consisting of the F1 primer shown above and the reverse 5’-GCAACGTTTATTCAGACTCTAGTGTCGCCTGC-3’ primer, and the other was amplified using a pair consisting of the forward 5’-CTGAATAAGATATTGTCGCCTGC-3’ primer and R1 primer shown above. The mutated codon is indicated by underlined text. PCR was subsequently performed using these two PCR products and the set of F1 and R1 primers in order to obtain the mutant Lp-PLA2 (V279F) fragment. This fragment was then inserted into the multiple cloning site of the pFLAG-CMV5 vector. The DNA sequence of the fragments inserted into the pFLAG-CMV5 vector was verified using the DNA sequencer PRISM 3130xl (Applied Biosystems, Foster City, CA, USA) at the Central Research Laboratory, Shiga University of Medical Science.

Cell Culture and Transfection

Cos-7 and U937 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Nacalai Tesque) and RPMI1640 medium, respectively, supplemented with 10% (v/v) heat inactivated fetal bovine serum and antibiotic solution (100 unit/mL of penicillin, 0.1 mg/mL of streptomycin and 0.25 μg/mL of ampho-
cells were counted.

**Knockdown of Lp-PLA2**

In order to knockdown the expression of Lp-PLA2 in the U937 cells, the small interference RNA (siRNA) method was applied, as previously described\(^3^2\). Stealth RNAi duplexes against human Lp-PLA2 and a Stealth RNAi negative control duplex were purchased from Invitrogen. The sequences of the RNAi duplexes against human Lp-PLA2 were as follows: 5’-UCAUGGGUUUAUAGUUGCUGCUGUA-3’ (#1), 5’-GAAACAAGAGGAGGAGACACAUAUA-3’ (#2), 5’-CAGACUUAATGUUUGAUCACACUAA-3’ (#3). siRNA transfection was performed using the Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

**Statistical Analysis**

The statistical significance of the data was determined using two-tailed Student’s t-test. A p value of <0.05 was considered to be statistically significant.

**Results**

**Plasma Lp-PLA2 Concentration and Activity of Each Genotype**

The plasma Lp-PLA2 concentration and activity were measured in all samples of each genotype. As shown in Table 1, the concentration and activity were significantly different between each genotype. In the Lp-PLA2 (V/F) group, the concentration and activity were roughly half of those observed in the Lp-PLA2 (V/V) group. In the Lp-PLA2 (F/F) group, these values were remarkably low; in particular, the concentration was almost zero. These results are consistent with the findings of a previous report \(^1^3\). Despite the significant plasma Lp-PLA2 concentration and activity, the baseline clinical characteristics related to atherosclerogenic factors were not significantly different between the three groups (Table 2).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of samples</th>
<th>Concentration (ng/mL)</th>
<th>Activity (nmol/min/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V/V</td>
<td>10</td>
<td>129.4 ± 32.8</td>
<td>164.3 ± 31.1</td>
</tr>
<tr>
<td>V/F</td>
<td>6</td>
<td>70.7 ± 15.6*</td>
<td>100.9 ± 23.7*</td>
</tr>
<tr>
<td>F/F</td>
<td>7</td>
<td>0.4 ± 1.1*†</td>
<td>11.6 ± 15.7*†</td>
</tr>
</tbody>
</table>

The data are presented as the mean ± S.D. *p < 0.01 vs V/V, †p < 0.01 vs V/F.
Correlation between the Lp-PLA2 Genotype and Macrophage Apoptosis

We investigated whether the Lp-PLA2 genotype is correlated with the occurrence of apoptosis using macrophages differentiated from isolated monocytes. The percentage of apoptotic macrophages was lowest in the Lp-PLA2 (V/V) group and highest in the Lp-PLA2 (F/F) group, with statistical significance (Fig. 1), thus indicating that plasma Lp-PLA2 plays a protective role in macrophage apoptosis. Furthermore, cellular cholesterol accumulation is reportedly involved in macrophage apoptosis. In the present study, when macrophages were incubated in the presence of AcLDL, no additional changes in the percentage of apoptotic macrophages were observed in any group (Fig. 2A), compared with the results observed in the absence of AcLDL (Fig. 1). Similar results were obtained in the presence of the ACAT inhibitor (Fig. 2B), although there are opposing reports that ACAT inhibitors suppress oxidized LDL-induced macrophage apoptosis\(^{33}\) and, in contrast, accelerate macrophage apoptosis\(^{34}\). Moreover, incubation of macrophages with both AcLDL and ACAT inhibitor has been shown to induce apoptosis via the cellular accumulation of free cholesterol in an endoplasmic reticulum (ER) stress-dependent manner\(^{35}\). However, we found that the administration of neither AcLDL nor ACAT inhibitor enhanced macrophage apoptosis, although a slight trend toward a reduction in apoptosis was noted in the Lp-PLA2 (F/F) group (Fig. 2C). These data suggest that macrophage apoptosis occurred independently of LDL overload and free cholesterol accumulation inside the cells in this study.

Molecular Characteristics of Lp-PLA2 and its Mutant in the Induction of Apoptosis

We next assessed the molecular mechanism accounting for the markedly high level of macrophage apoptosis observed in the Lp-PLA2 (F/F) group. For this purpose, we used monocyte/macrophage-like U937 cells and transfected the empty vector (mock) or the FLAG-tagged expression vector of the wild-type (WT) or V279F mutant Lp-PLA2 into the cells. The expression levels of FLAG-Lp-PLA2-WT and FLAG-Lp-PLA2 (V279F) were similar (Fig. 3A). The induction of apoptosis in the U937 cells expressing FLAG-Lp-PLA2 (V279F) tended to be higher than that observed in the cells expressing FLAG-Lp-PLA2-WT and was significantly increased comparing with that noted in the mock-transfected cells (Fig. 3C and E). The same experiments were performed in Cos-7 cells, showing similar results (Fig. 3B, D and F), suggesting that the effect of the V279F mutation of Lp-PLA2 on apoptosis is independent of the cell line.

In order to further confirm the protective effects of Lp-PLA2 on apoptosis, a knockdown experiment was performed in U937 cells. The expression of Lp-PLA2 was most effectively reduced by siRNA #1 against Lp-PLA2 (Fig. 4A). Therefore, this siRNA was applied in the subsequent experiments. The number of apoptotic cells was higher among the Lp-PLA2-knockdown U937 cells than the control cells (Fig. 4B).

### Table 2. Baseline clinical characteristics of each group

<table>
<thead>
<tr>
<th>Genotype</th>
<th>V/V</th>
<th>V/F</th>
<th>F/F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples</td>
<td>10</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Age (years)</td>
<td>59.6 ± 7.0</td>
<td>62.4 ± 4.9</td>
<td>63.2 ± 7.5</td>
</tr>
<tr>
<td>BMI</td>
<td>24.9 ± 2.9</td>
<td>24.6 ± 3.6</td>
<td>25.0 ± 2.9</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>128 ± 15</td>
<td>131 ± 16</td>
<td>133 ± 11</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>77 ± 10</td>
<td>78 ± 8</td>
<td>78 ± 8</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>210 ± 38</td>
<td>191 ± 42</td>
<td>213 ± 32</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>133 ± 38</td>
<td>121 ± 42</td>
<td>124 ± 27</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>50 ± 14</td>
<td>47 ± 15</td>
<td>58 ± 19</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>138 ± 82</td>
<td>115 ± 46</td>
<td>158 ± 75</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.4 ± 0.4</td>
<td>5.7 ± 0.5</td>
<td>5.9 ± 0.7</td>
</tr>
<tr>
<td>Alcohol intake (g/day)</td>
<td>32 ± 26</td>
<td>43 ± 16</td>
<td>46 ± 51</td>
</tr>
<tr>
<td>Brinkman index</td>
<td>934 ± 656</td>
<td>489 ± 444</td>
<td>867 ± 724</td>
</tr>
</tbody>
</table>

The data are presented as the mean ± S.D. There were no statistically significant differences in any of the parameters between the groups.

BMI: body mass index, BP: blood pressure, TC: total cholesterol, LDL-C: low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol, TG: triglyceride.
sis38), were significantly increased in the FLAG-Lp-PLA2-WT-expressing U937 and Cos-7 cells after starvation; this increase was subsequently suppressed by the expression of FLAG-Lp-PLA2 (V279F) (Fig.5C).

In order to confirm the involvement of caspase-7 and Akt in Lp-PLA2-related apoptosis, the caspase-7 or Akt inhibitor was used in FLAG-Lp-PLA2-WT- or FLAG-Lp-PLA2 (V279F)-expressing cells. When cells expressing FLAG-Lp-PLA2 (V279F) were treated with the caspase-7 inhibitor, the number of apoptotic cells decreased (Fig.6A and B). In contrast, when cells expressing FLAG-Lp-PLA2-WT were treated with the Akt inhibitor, the number of apoptotic cells increased (Fig.6C and D). Collectively, these results suggest that Akt and caspase-7 may at least partly play a role in the apoptosis induced by mutations at V279F of Lp-PLA2.

**Discussion**

We, for the first time, found that the V279F mutation of Lp-PLA2 facilitates the induction of apoptosis.

Since it is well known that cleaved caspases, which constitute the activated form of caspases, play important roles in the induction of apoptosis36), we examined the levels of cleaved caspases in both U937 and Cos-7 cells. We found that, in both U937 and Cos-7 cells, the level of cleaved caspase-7 was increased by the expression of FLAG-Lp-PLA2 (V279F) rather than that of FLAG-Lp-PLA2-WT, although the levels of cleaved caspase-3 and cleaved caspase-9 were similar in association with the expression of FLAG-Lp-PLA2-WT and FLAG-Lp-PLA2 (V279F) in both the U937 and Cos-7 cells (Fig.5A). PARP has been reported to contribute to DNA repair and stabilization, and the cleavage of PARP is often observed in the process of apoptosis37). In the present study, the amount of cleaved PARP product was also similar between the FLAG-Lp-PLA2-WT- and FLAG-Lp-PLA2 (V279F)-expressing U937 and Cos-7 cells (Fig.5B). On the other hand, the levels of phosphorylated and thus activated Akt, which inhibits the progression of apoptosis38), were significantly increased in the FLAG-Lp-PLA2-WT-expressing U937 and Cos-7 cells after starvation; this increase was subsequently suppressed by the expression of FLAG-Lp-PLA2 (V279F) (Fig.5C).

Thus indicating the regulatory role of Lp-PLA2 in apoptosis.

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**Discussion**

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Macrophage Apoptosis Induced by Lp-PLA2

express endogenous Lp-PLA2; thus, ectopically expressed mutated Lp-PLA2 may be unable to overcome the protective effects of endogenous Lp-PLA2 on apoptosis in these cells. Finally, the cultured cells themselves may be resistant to apoptosis-inducing stimuli, including serum starvation. Although further experiments are needed, this study suggests that the V279F mutation of Lp-PLA2 promotes apoptosis, even in cultured cells other than macrophages.

The theory that an increase in apoptosis in macrophages results in the suppression of the atheroscle-

 apoptosis in human macrophages. In the present study, in cultured U937 and Cos-7 cells expressing mutated Lp-PLA2, the induction of apoptosis increased, although the difference was not remarkable, as observed in the macrophages with the genotype of Lp-PLA2 (F/F). There are several potential reasons why ectopically expressed mutated Lp-PLA2 exerted a limited effect on apoptosis in these cultured cells. First, the transfection level of Lp-PLA2 in the cultured cells may not have been adequate to sufficiently induce apoptosis. Second, the cultured cells may

Fig. 2. No effect of acetylated LDL or ACAT inhibitor was observed on the induction of macrophage apoptosis.

The percentage of apoptotic macrophages in each Lp-PLA2 genotype in the presence of AcLDL (A), ACAT inhibitor (B) or both AcLDL and ACAT inhibitor (C) was calculated after the apoptosis assay. The data are presented as the mean + S.D.
Fig. 3. Induction of apoptosis by the ectopic expression of mutant Lp-PLA2 in the U937 and Cos-7 cells.

(A and B) The expression levels of wild-type (WT) and V279F mutant Lp-PLA2 ectopically expressed in U937 cells (A) and Cos-7 cells (B) were evaluated using Western blotting. An empty vector (Mock) was transfected as a negative control. The band density of FLAG-Lp-PLA2 was adjusted according to that of GAPDH, which was used as the internal control. A.U.: arbitrary units. (C and D) Representative FACS images of U937 cells (C) and Cos-7 cells (D) transfected with an empty vector or the plasmid of FLAG-tagged wild-type or V279F mutant Lp-PLA2. The cells were stained with Alexa Fluor 488-labeled annexin V and propidium iodide after the apoptosis assay. Cells present in the lower right compartment were counted as apoptotic cells, the percentage of which is indicated on each image. (E and F) The results of the statistical analyses of the percentage of apoptotic cells are indicated. The data are presented as the mean ± S.D.
Macrophage Apoptosis Induced by Lp-PLA2

Lp-PLA2 removes oxidatively truncated phospholipids, which have been demonstrated to target the mitochondrial compartment, where they induce mitochondrial damage and intrinsic caspase cascade activation, thus resulting in the promotion of apoptosis. In the present study, we did not obtain any obvious results showing that the overexpression of Lp-PLA2-WT in cultured cells reduces apoptosis compared with that observed in the mock-transfected cells (Fig. 3). Although it is not clear why there is such a discrepancy in results between previous studies and the current study, the differences may depend on differences in the experimental models used in each study. For example, in our experiments, apoptosis was induced via serum starvation, not phospholipid overload.

There is a recent article explaining the anti-apoptotic effects of Lp-PLA2. Lp-PLA2 removes oxidatively truncated phospholipids, which have been demonstrated to target the mitochondrial compartment, where they induce mitochondrial damage and intrinsic caspase cascade activation, thus resulting in the promotion of apoptosis. In the present study, we did not obtain any obvious results showing that the overexpression of Lp-PLA2-WT in cultured cells reduces apoptosis compared with that observed in the mock-transfected cells (Fig. 3). Although it is not clear why there is such a discrepancy in results between previous studies and the current study, the differences may depend on differences in the experimental models used in each study. For example, in our experiments, apoptosis was induced via serum starvation, not phospholipid overload.

AcLDL and ACAT inhibitor are known to be moderators of the atherosclerotic process. Some reports have shown that apoptosis in macrophages and/or differentiated foam cells is an atherogenic factor, as various kinds of cytokines produced and stored in these cells are robustly released following apoptosis, with subsequent stimulation of surrounding cells, such as vascular smooth muscle cells, thus promoting atherosclerosis. In contrast, it has been reported that the occurrence of macrophage apoptosis in the early phase of the atherosclerotic process negatively regulates the progression of atherosclerotic lesions by preventing the differentiation of macrophages to foam cells and reducing the cytokine production. It is currently unclear whether the increased apoptosis observed in macrophages obtained from the participants with the Lp-PLA2 genotype positively or negatively contributes to the progression of atherosclerosis. Therefore, further clinical studies are required to elucidate this issue.

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Fig. 5. Involvement of cleaved caspase-7 and activated Akt in V279F mutant Lp-PLA2-induced apoptosis.

(A and B) Cleavage of caspases and PARP in U937 and Cos-7 cells expressing wild-type or V279F mutant Lp-PLA2. The numbers below the blot represent the findings of the comparison of the relative density of the cleaved caspases and PARP between the wild-type and V279F mutant Lp-PLA2-expressing cells. The value of wild-type Lp-PLA2-expressing cells was set at 1. (C) The level of activated Akt was evaluated according to the amount of phosphorylated Akt on Western blotting. The graph represents the band density of phosphorylated Akt in the wild-type and V279F mutant Lp-PLA2-expressing U937 and Cos-7 cells before and after serum starvation. The density of each cell type was adjusted according to that of total Akt. A.U.: arbitrary units. The data are presented as the mean ± S.D.
Fig. 6. Effects of caspase-7 inhibitor or Akt inhibitor on V279F mutant Lp-PLA2-induced apoptosis.

(A and B) Representative FACS images of U937 cells (A) and Cos-7 cells (B) expressing V279F mutant Lp-PLA2 in the presence or absence of caspase-7 inhibitor. The cells were stained with Alexa Fluor 488-labeled annexin V and propidium iodide after the apoptosis assay. Cells present in the lower right compartment were counted as apoptotic cells, the percentage of which is indicated on each image. (C and D) Representative FACS images of U937 cells (C) and Cos-7 cells (D) expressing Lp-PLA2-WT in the presence or absence of Akt inhibitor. The same procedures were conducted as described above. The results of the statistical analyses of the percentage of apoptotic cells are indicated on the right side of each representative FACS image. The data are presented as the mean ± S.D.
Tabas's research group to increase the accumulation of intracellular free cholesterol, which induces apoptosis via endoplasmic reticulum stress pathways. However, these reagents did not enhance the apoptosis induced by serum starvation in any of the Lp-PLA2 genotypes of macrophages evaluated in this study. In the study conducted by Tabas's research group, macrophage apoptosis was induced by the administration of AcLDL and ACAT inhibitor only. In contrast, in our study, the macrophages were treated with AcLDL and ACAT inhibitor in addition to the apoptosis-inducing stimulation of serum starvation. Moreover, macrophages obtained from different species may affect the tendency of apoptosis induction. For example, in this study, we used human monocyte-derived macrophages, whereas Tabas's research group used murine macrophages. Unlike the effect of ACAT inhibitor on the accumulation of free cholesterol in rodent macrophages, ACAT inhibition has been shown in several studies to reduce the uptake of AcLDL and facilitate free cholesterol efflux in human macrophages. The different experimental procedures used in this study may therefore have resulted in no significant effects of AcLDL and ACAT inhibitor on macrophage apoptosis.

One of the important processes of apoptosis is the activation of the caspase cascade, in which one caspase is cleaved by another. For example, initiator caspase-9 cleaves and activates executioner caspases, caspase-7 and caspase-3. The functions of caspase-3 and caspase-7 in the process of apoptosis appear to be redundant; however, there are reports that a non-redundant function of caspase-3 and caspase-7 exists, especially in inflammation. Moreover, in some cases, the mode of cleavage of caspase-7 differs from that of caspase-3; caspase-7, but not caspase-3, can be cleaved by caspase-1. Since the results of this study indicate that, among the caspases tested, only cleaved caspase-7 is increased by the expression of mutated Lp-PLA2, this caspase, not caspase-3, is likely to be specifically cleaved and become involved in the progression of apoptosis induced by mutated Lp-PLA2. However, why mutated Lp-PLA2-dependent apoptosis is predominantly mediated by caspase-7 remains to be elucidated.

It has been shown that cleaved caspase-7 further cleaves PARP, which translocates apoptosis-inducing factor (AIF) to the nucleus, where AIF induces DNA fragmentation and chromatin condensation, leading to apoptosis. In the present study, the levels of cleaved PARP were similar between the WT and mutated Lp-PLA2-expressing cells. Therefore, the activated caspase-7 induced by mutated Lp-PLA2 appears to mediate apoptosis via pathways independent of PARP. The precise mechanisms by which caspase-7 activates the PARP-independent pathway and the molecules that participate in this pathway are currently unidentified.

Akt is a protein kinase and signaling molecule that is activated by phosphoinositide-dependent kinase downstream of phosphatidylinositol 3 (PI3)-kinase. In this study, the activation of Akt was suppressed in mutated Lp-PLA2-expressing cells and contrarily increased in WT Lp-PLA2-expressing cells after the apoptosis assay. However, the molecular mechanism by which Lp-PLA2 induces the activation of Akt is completely unclear. Since Lp-PLA2 is present in the circulating blood flow, this protein is thought to act via cell surface membrane molecules to activate Akt, which subsequently localizes inside the cell. Given that PI3-kinase is activated by several growth factor receptors, including platelet-derived growth factor receptors, circulating Lp-PLA2 may directly or indirectly interact with such receptors in order to activate the PI3-kinase-Akt axis, thereby preventing apoptosis. In addition, the relationship between Akt and caspase-7 has not been elucidated, although it is known that Akt inhibits the activity of caspase-9. Further studies are thus needed to address these issues.

In an animal experimental model, darapladib, a novel selective Lp-PLA2 inhibitor, has been shown to reduce the development of advanced coronary atherosclerosis and affect the coronary artery gene expression. However, the precise molecular mechanisms by which darapladib exerts beneficial effects on coronary atherosclerosis have not been clearly demonstrated as of yet. Based on the results of our present study, there is a possibility that darapladib increases macrophage apoptosis in the early phase of atherosclerosis. Furthermore, the safe administration of darapladib with a consequent reduction in the plasma Lp-PLA2 activity was recently shown in one clinical study. Therefore, darapladib may be of clinical use in the future to prevent atherosclerosis-related cardiovascular diseases.

**Conclusion**

The V279F mutation of Lp-PLA2 positively regulates the induction of apoptosis in macrophages and cultured cells. In this study, although several other caspases were not activated in Lp-PLA2 (V279F)-expressing cells, an increase in the caspase-7 activity and reduction in the activated Akt level appear to constitute at least one underlying mechanism promoting apoptosis.
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Conflicts of Interest

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