Novel Therapeutic Strategy for Atherosclerosis

Ribozyme Oligonucleotides Against Apolipoprotein(a) Selectively Inhibit Apolipoprotein(a) But Not Plasminogen Gene Expression

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**Background**—Because mechanisms of atherosclerosis by lipoprotein(a) [Lp(a)] have been postulated in the decrease in active transforming growth factor-β conversion by decreased plasmin, selective decrease in apolipoprotein(a) [apo(a)] independent of plasminogen may have therapeutic values. Although antisense can decrease apo(a), its application may be difficult because of very high homology of apo(a) gene to plasminogen. Thus we used ribozyme strategy that actively cleaves targeted genes to selectively inhibit apo(a) expression.

**Methods and Results**—We constructed ribozyme oligonucleotides containing phosphorothioate DNA- and RNA-targeted kringle 4 of the apo(a) gene that showed 80% homology to plasminogen. Transfection of human apo(a) gene produced Lp(a) in medium of HepG2 cells, whereas Lp(a) could not be detected in control cells. Cotransfection of ribozyme and apo(a) gene resulted in the decrease in mRNA of apo(a) but not plasminogen. Moreover, marked decrease in Lp(a) was also observed in the medium transfected with ribozyme and apo(a) gene compared with apo(a) gene alone (P<0.01), whereas there was no significant change in plasminogen level between ribozyme-transfected and control cells. Incubation of human vascular smooth muscle cells (VSMC) with conditioned medium from apo(a)–transfected HepG2 cells resulted in a significant increase in VSMC number, whereas addition of conditioned medium from cells cotransfected with ribozyme oligonucleotides and apo(a) gene resulted in no VSMC growth (P<0.01). DNA-based control oligonucleotides and mismatched ribozyme oligonucleotides did not have an inhibitory effect on Lp(a) production.

**Conclusions**—Overall, our data revealed that transfection of ribozyme against the apo(a) gene resulted in the selective inhibition of the apo(a) but not the plasminogen gene, providing novel therapeutic strategy for treatment of high Lp(a), a risk factor for atherosclerosis. 

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**Key Words:** atherosclerosis ■ lipoproteins ■ vasculature ■ genes ■ muscle, smooth

A high serum concentration of lipoprotein(a) [Lp(a)] is a risk factor for atherosclerosis, restenosis after angioplasty, cardiac disease, and stroke.\(^1\)–\(^4\) Lp(a) consists of LDL with an additional protein component, apolipoprotein(a) [apo(a)], a homolog of plasminogen.\(^7\) Lp(a) and apo(a) enhance proliferation of human vascular smooth muscle cells (VSMC) in culture by inhibiting activation of plasminogen to plasmin, thus blocking the proteolytic activation of transforming growth factor (TGF)-β.\(^5\)–\(^10\) On the other hand, Lp(a) has been postulated to bind to endothelial and macrophage cells and extracellular components such as fibrin and to inhibit cell-associated plasminogen activation.\(^11\) In addition to the atherogenic action of Lp(a), apo(a) has been reported to be related to vascular diseases, as transgenic mice harboring the human apo(a) gene showed the development of atherosclerosis.\(^11\)–\(^15\) Therefore, prevention of atherosclerosis induced by high Lp(a) requires a decrease in apo(a) without any effects on plasminogen production. With this aim, researchers have speculated that the removal of Lp(a) from plasma may reduce coronary events. Indeed, prevention of restenosis has been reported by reducing Lp(a) level by LDL apheresis.\(^16\)–\(^18\) Although apheresis is an effective method for reducing both plasma Lp(a) and LDL cholesterol levels, to perform apheresis in all patients with high Lp(a) levels is not practical. Therefore, we focused on molecular approaches to reduce high level of Lp(a).

Initially, we thought that antisense oligonucleotides (ON) directed against apo(a) RNA might be effective to reduce the Lp(a) level. One strategy for combating disease processes has been to target to the transcriptional process. Two approaches have been used to accomplish this. One is the use of antisense ON that are complementary to the mRNA of interest. The
second approach is the use of ribozymes, a unique class of RNA molecules that not only store information but also process catalytic activity. Ribozymes are known to catalytically cleave specific target RNA leading to degradation, whereas antisense ON inhibit translation by binding to mRNA sequences on a stoichiometric basis. Theoretically, ribozymes are more effective for inhibiting target gene expression. Moreover, it appears to be very difficult to use the antisense strategy to decrease apo(a) separate from plasminogen because the structure of the apo(a) gene has a very high degree of homology to the plasminogen gene. Therefore, we used a novel therapeutic strategy, ribozyme technology, to selectively inhibit apo(a) gene expression apart from plasminogen.

Methods

Synthesis of Ribozyme ON and Selection of Sequence Targets

The sequences of ribozyme ON were as follows:

- **RZ 120:** 5′-AGCAUUCUGUAGUUGUGAGGGAGC-AACCAUAAGGC-3′
- **RZ 151:** 5′-GUGCUGAAGUGAGUGUGAGGC-AACUCUGUA-3′
- **RZ 164:** 5′-AGUGGUGAGUGAGUGUGAGGAC-AACGUGCCUCG-3′
- **mRZ 151:** 5′-GUGCUGCGGUGAGUGUGAGGAC-AACGUGCCUCG-3′
- **DNA-RZ151:** 5′-GTGCCCTCGATCTGATGAGTCCGTGAGGA-9
- **mRZ 151:** 5′-AGUGGUGAGCUGUGAGUGAGGAC-AACGUGCCUCG-3′
- **DNA-RZ151:** 5′-GTGCCCTCGATCTGATGAGTCCGTGAGGA-9

Ribozyme ON were synthesized and purified by chromatography. Synthetic ON were washed with 70% ethanol, dried, and dissolved in sterile Tris-EDTA buffer (10 mmol/L Tris, 1 mmol/L EDTA). The supernatant was purified over a NAP 10 column (Pharmacia) and quantified by spectrophotometry.

Mismatches ribozyme 151 (mRZ 151) and DNA-based ON (DNA-RZ 151) with no catalytic activity were used as negative controls.

Construction of Plasmids

A recombinant apo(a) [r-apo(a)] molecule containing 17 kringle 4–like domains, as well as the kringle 5–like and protease-like domains, was assembled from apo(a) cDNA clones. An apo(a) expression vector containing the 5-untranslated region, the signal sequence, the first 5 kringle 4–like repeats, and 291 bp of the size kringle repeat of apo(a) was driven by the cytomegalovirus promoter.

Koschinsky et al reported that the engineered protein (predicted mass of 250 kDa) contains 17 copies of the apo(a) domain, which resembles kringle 4 of plasminogen, followed by the plasminogen-like kringle 5 and protease-like domain of apo(a). Atherogenesis in transgenic mice expressing this human mini–apo(a) gene has been reported.

Preparation of HVJ Liposomes

We used the hemagglutinating virus of Japan (HVJ)-coated liposomes that have been reported to be highly efficient for transfection of cells in culture. Briefly, phosphatidylserine, phosphatidylcholine, and cholesterol were mixed in a weight ratio (1:4:8:2) to create a lipid mixture. Purified HVJ (Z strain) was inactivated by UV irradiation before use. The liposome suspension was mixed with HVJ, and free HVJ was removed by sucrose density gradient centrifugation. The final concentration of encapsulated ON was calculated as previous reported. An r-apo(a) molecule containing 17 kringle 4–like domains, as well as the kringle 5–like and protease-like domains, was assembled from apo(a) cDNA clones reported previously.

HepG2 cells (1 × 10⁶) were seeded onto plates and grown to confluence. Cells were washed 3 times with balanced saline solution containing 2 mmol/L CaCl₂ and then incubated with 1 mL HVJ-liposome complex (10 μg encapsulated DNA) at 4°C for 5 minutes followed by 37°C for 30 minutes. The cells were then washed and fed fresh medium containing 10% calf serum and placed in a CO₂ incubator.

Cell Culture

Human aortic VSMC (passage 3) were obtained from Clonetics Corp and cultured in modified MCD8131 medium supplemented with 5% fetal calf serum, 100 U/mL penicillin, 100 mg/mL streptomycin, 10 ng/mL epidermal growth factor, 2 ng/mL basic fibroblast growth factor, and 1 μmol/L dexamethasone in the standard fashion.

Cells were incubated at 37°C in a humidified atmosphere of 95% air/5% CO₂ with medium changes every 2 days. These cells showed the specific characteristics of VSMC by immunohistochemical examination and morphological observation. Briefly, human aortic VSMC also tested positive for α-actin and negative for expression of factor VIII antigen. All the cells were used within passages 3 to 6.

HepG2 cells were obtained from American Tissue Culture Collection.

Transfection of Ribozyme ON Into Cultured Cells

To document the successful transfection of the cells, we examined the production of Lp(a). HepG2 cells were seeded onto 6-well plates (Corning, NY) at a density of 5 × 10⁴ cells/cm² and cultured for 24 hours. HepG2 cells were seeded onto 96-well plates and grown to confluence. Cells were washed 3 times with BSS containing 2 mmol/L CaCl₂ and then incubated with 1 mL HVJ-lipoprotein–DNA complex (2.5 mg lipid, 10 μg encapsulated DNA, and 1 μmol/L ribozyme or control ON) at 4°C for 5 minutes followed by 37°C for 30 minutes (total 35 minutes). The cells were then washed and fed fresh medium containing 10% calf serum and placed in a CO₂ incubator. Twenty-four hours after transfection, the medium was changed and the cells were incubated for an additional 48 hours.

To study the release of apo(a) and Lp(a), transfected cells (48 hours after transfection) were washed and fed with 1 mL defined serum-free (DSF) medium containing medium supplemented with insulin (5 × 10⁻⁷ M), transferrin (5 mg/mL), and ascorbate (0.2 mmol/L).

Fifty-four hours later, conditioned medium was collected, centrifuged at 600g for 10 minutes, and stored at −20°C. The concentration of Lp(a) in the medium was determined by enzyme-immunoassay with anti–Lp(a) antibody, as described below.

To study the effects of ribozyme ON on Lp(a) production, ribozyme ON was also transfected into HepG2 cells at 72 hours after transfection of the apo(a) gene. Immediately after transfection, the medium was changed to fresh DSF, and the cells were incubated for an additional 48 hours to study the release of apo(a) and Lp(a). Forty-eight hours later, conditioned medium was collected, centrifuged at 600g for 10 minutes, and stored at −20°C.

Measurement of Lp(a) and Plasminogen Concentrations in Cultured Cells

Anti–Lp(a) polyclonal antibody (15 μg/mL) was coated on 96-well plates (Corning) at 37°C for 2 hours. Medium supernatants were diluted 3-fold with 10 mmol/L Tris-HCl (pH 8.0) containing 0.85% sodium chloride and 1% BSA. After blocking with 1% BSA in PBS, conditioned medium was added to each well and the preparation was incubated for 2 hours at room temperature. Wells were washed 3 times with PBS containing 0.025% Tween 20 (PBS-Tween), conjugated anti–Lp(a) polyclonal antibody (diluted 500– to 600-fold) was added, and the preparation was incubated for 2 hours at room temperature. After washing with PBS-Tween, wells were incubated with color reagent (3,3,5,5-tetramethylbenzidine) in 24 mmol/L citric acid buffer, pH 5.0, containing 0.03% hydrogen peroxide. The enzyme reaction was halted by adding 0.5 mol/L H₂SO₄, and absorbance at 450 nm was measured. Electrophoresis and immunoblotting were also performed. Briefly, agarose gel (1%) electrophoresis was performed with the Titan GEL Lipoprotein Electrophoresis kit (Helena Laboratory). After transfer onto a nitrocellulose membrane (Bio-Rad) with a nova blot electrophoretic transfer kit (Pharmacia-LKB), membranes were soaked overnight in PBS con...
containing 1% BSA at 4°C to effect blocking. These nitrocellulose membranes were washed and soaked in PBS containing 3% BSA with 500-fold diluted, peroxidase-labeled anti-mouse IgG antibody (Dako Co). After washing, membranes were again soaked in PBS containing 0.025% 3,3-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxidase at room temperature for 20 minutes for color development.

Western blotting and enzyme immunoassay of plasminogen were also performed with anti-human plasminogen antibody.22 Conditioned medium was subjected to 15% SDS-PAGE at 36 mA for 3 hours. Blots without the primary antibody were performed as a negative control. The proteins were transferred to a filter overnight at 125 mA. The blots were incubated overnight with antiplasminogen antibody (at 10 μg/mL) and then incubated with conjugated secondary antibodies and substrates (Promega) in conjunction with the ECL chemiluminescence system (Amersham) for detection of bound primary antibody.

RNA Analysis
Levels of apo(a) and plasminogen mRNA were measured by reverse transcription polymerase chain reaction (RT-PCR). RNA was extracted with the use of RNAzol (Tel-Test Inc) from HepG2 cells transfected with apo(a) or control vector at 3 days after transfection.21 We used primers that can make 2 different bands corresponding to apo(a) and plasminogen mRNA, respectively. GAPDH primers were purchased from Clontech. Extreme care was taken to avoid contamination of tissue samples with trace amounts of experimental RNA. Aliquots of RNA (0.5 μg) were amplified simultaneously by PCR (35 cycles) with the same reagents by individuals who were blinded to the identity of the samples and compared with a negative control (primers without RNA). Preliminary results demonstrated that amplification products at these cycles are within the linear range. Amplification products were electrophoresed through 2% agarose gels and stained with ethidium bromide. To ensure that the RT-PCR–amplified product reflected transcribed target RNA without significant DNA contamination, RNA samples treated with RNase A or amplified without RT were amplified simultaneously as negative controls. These samples did not result in a visual band. At least 3 aliquots of each DNA and RNA sample were subjected to separate PCR amplifications in all experiments.

Cell Counting Assay
In this study, we measured cell number by using a WST cell-counting kit, which is similar to the MTT assay (Wako).23 Tetrabromofluorescein salt has been used to develop a quantitative colorimetric assay for cell growth. The assay detects living but not dead cells. In this study, we used an alternative to MTT, that is, sulfofibratinetetrazolium salt, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1), because this compound produces a highly water-soluble formazan dye, which makes the assay procedure easier to perform. Briefly, 16.3 mg WST-1 and 0.2 mmol/L 1-methoxy-5-methylphenazinium methyl sulfate were dissolved in 20 mmol/L HEPES buffer (pH 7.4). Then, 10 μL of the reaction solution was immediately added to 100 μL of culture medium per well, and the cells were incubated for an additional 15 minutes. The plates were read on a Bio-Rad model 3550 microplate reader, with a test wavelength of 450 nm and a reference wavelength of 650 nm. We confirmed that serum-stimulated increase in cell number is associated with increased absorbance at 450 nm (data not shown). Briefly, 50,000 cells per well reflects an absorbance of 1 under the manufacturer’s recommended conditions. The sensitivity of the WST assay is double that of the MTT assay. In our experimental conditions, an increase in absorbance of 0.2 reflects an increase in cell number to 20,000 cells per well.

Effect of Cotransfection of Ribozyme ON on Growth of VSMC
The ability of conditioned medium to increase cell growth in a paracrine manner was also examined. HepG2 cells for transfection were seeded onto 6-well plates (Corning) at a density of 5×10³ cells/cm², cultured for 24 hours, and grown to confluence. Cells were then transfected with apo(a) or control vector. After transfection, the medium was changed and the cells were incubated for an additional 48 hours. After 48 hours, ribozyme ON or DNA-based control ON were transfected into HepG2 cells transfected with apo(a) or control vector. To study the biological effect of locally produced Lp(a) on growth of VSMC, cotransfected HepG2 cells (48 hours after transfection) were washed and fed with 1 mL of DSF. Forty-eight hours later, conditioned medium was collected, centrifuged at 600 g for 10 minutes, and stored at −20°C.29 The test VSMC were seeded onto 24-well tissue culture plates. Quiescent VSMC (placed in DSF with 0.05% FCS for 48 hours after 80% confluence to make quiescent) were treated with the conditioned media collected from HepG2 cells transfected with either the apo(a) expression or control vector, diluted 1:1 with fresh medium. After 48 hours, the medium was changed to fresh DSF diluted 1:1 with the conditioned medium. Four days later, cell growth assay was performed as described above.

In Vitro Transfection of FITC-Labeled Ribozyme ON
FITC-labeled ribozyme ON were kindly provided by Hitachi Co Ltd (Tsukuba, Japan). FITC was labeled on the 5’ ends of the ON. HepG2 cells were grown in 8 chamber slides to subconfluence. Transfection of ON was performed under the following protocols: (1) 500 μL of HVJ-liposome complex with FITC-labeled phosphorothioate ON (3 μmol/L) was incubated for 5 minutes at 4°C and for 30 minutes at 37°C. (2) HVJ complex without ON was incubated for 5 minutes at 4°C and for 30 minutes at 37°C. After changing to fresh medium with 5% calf serum, the cells were incubated in a CO₂ incubator. Cells were fixed with 3% paraformaldehyde at 24 and 72 hours after transfection. After mounting, cells were examined by fluorescent microscopy.24

Materials
Human r-apo(a) was purified from the culture medium of Chinese hamster ovary cells and transfected with expression plasmid containing human apo(a) cDNA.22,23 r-Apo(a) then was mixed with human apo B, allowing the formation of recombinant Lp(a) [r-Lp(a)]. r-Lp(a) was used as a positive control for Western blotting.

Statistical Analysis
All values are expressed as mean±SEM. All experiments were repeated at least 3 times. ANOVA with subsequent Bonferroni test was used to determine differences in multiple comparisons. A value of P<0.05 was considered statistically significant.

Results
We constructed “hammerhead” ribozyme ON containing phosphorothioate DNA and RNA targeted to kringle 4 of the apo(a) gene, which showed 80% homology.20 Hammerhead ribozymes are known to cleave the specific sequences GUH (H=C, U, or A), but not other sequences.19 In kringle 4, 3 possible sites could be detected as target sequences for ribozyme catalysis. As shown in Figure 1A, ribozyme 151 (RZ 151) can cleave the mRNA of apo(a) at +151, considered on the basis of the sequence reported by McLean et al,20 whereas the plasminogen gene theoretically cannot be cleaved by RZ 151. The other 2 ribozyme ONs directed against +120 (RZ 120) and +164 (RZ 164) also can cleave apo(a) mRNA. However, RZ 120 may also cleave plasminogen, because the plasminogen message also contains the sequence GUC. RZ 164 may also be very effective to cleave only apo(a) mRNA but not plasminogen mRNA as GUA, which is the target site in apo(a) mRNA for RZ 164 is different from AUC in plasminogen mRNA. However, RZ 164 may have an antisense effect against plasminogen be-
cause of the high complementary of sequences. Therefore, we chose RZ 151 as ribozyme ON against apo(a). Indeed, comparison of the inhibitory effect of 3 different kinds of ribozyme ON showed that RZ 151 is most effective in the inhibition of Lp(a) production (data not shown).

To increase the resistance to degradation by nucleases, ribozyme ONs were constructed by chemical modification of the phosphoribose backbone of RNA to phosphorothioate. \(^{35-37}\) This modification does not influence catalytic activity. Furthermore, the synthesis of DNA-RNA hybrid hammerheads (chimeric ribozyme) used in this study, in which some ribonucleotides outside the catalytic core are replaced with 2'-deoxyribonucleotides, results in increased resistance to nucleases and a 6-fold enhancement of catalytic activity. \(^{37}\) To further enhance the efficiency and stability, we used a Sendai-virus (HVJ)-liposome delivery system, which is reported to bypass endocytosis, thereby avoiding degradation in lysosomes. \(^{21,24-26}\) Fluorescence could be observed in the cellular components at 1 day after transfection, consistent with the previous report. \(^{34}\) Fluorescence from FITC-labeled ribozyme ON was sustained up to 3 days after transfection (Figure 1B). In contrast, no fluorescence was observed in cells transfected with HVJ-liposome complex alone (Figure 1B) and untreated cells (data not shown).

Because the apo(a) gene is absent in rodents and nearly all subprimate species except the groundhog and hedgehog, there are limitations to available animal models. Therefore we used genetically modified HepG2 cells that were transfected with human apo(a) gene. As shown in Figures 2 and 3, transfusion of human apo(a) gene into HepG2 cells resulted in a significant increase in Lp(a) production assessed by Western

\(\text{Figure 1. A, Scheme of ribozyme strategy. GUU is cleavage site for ribozyme ON against apo(a). In contrast, GUU is GGU at this location in plasminogen RNA, resulting in no cleavage by ribozyme ON (RZ 151). Underlined sequences are phosphorothioate DNA; other sequences are phosphoribose RNA. Italic sequences in plasminogen gene are mismatched compared with apo(a) gene. B, Representative fluorescence microscopy of FITC-labeled ribozyme ON in vitro cultured HepG2 cells at 3 days after transfection. Control indicates transfected cells with HVJ-liposome complex alone; ribozyme, cells transfected with HVJ-liposome complex containing FITC-labeled ribozyme ON. This experiment was repeated 3 times.} \)

\(\text{Figure 2. A, Effect of ribozyme ON of apo(a) on Lp(a) production at 2 days after transfection assessed by Western blotting. CV indicates HepG2 cells transfected with control vector; apo(a), HepG2 cells transfected with apo(a) vector and DNA-based control ON (DNA-RZ 151); ribozyme, HepG2 cells transfected with apo(a) vector and ribozyme ON (RZ 151); Lp(a), purified Lp(a) from plasma. B, Inhibitory effect of ribozyme ON of apo(a) on Lp(a) production at 2 days after transfection assessed by EIA. apo(a) indicates HepG2 cells transfected with apo(a) vector and DNA-based control ON (DNA-RZ 151); ribozyme, HepG2 cells transfected with apo(a) vector and ribozyme ON (RZ 151); and control, untransfected HepG2 cells. n=6 per group. P<0.01 versus apo(a).} \)
blotting and EIA with anti–Lp(a) antibody. Cotransfection of apo(a) ribozyme ON but not DNA-based control ON (DNA-RZ 151) significantly decreased Lp(a) assessed by Western blotting and EIA. In contrast, mismatched ribozyme ON (mRZ 151) did not show any inhibitory effect on Lp(a) production [RZ151, 0.032±0.005; mRZ151, 0.175±0.033; apo(a) vector alone, 0.189±0.012; absorbance at OD 450, not significant]. Similarly, antisense ON directed against the same sequences of RZ 151 also did not inhibit Lp(a) production (data not shown). In the present study, we used DNA-based control ON (DNA-RZ 151) and mismatched ribozyme ON (mRZ 151) as negative control because both control ONs have no catalytic activity. The specificity of ribozyme ON to cleave apo(a) was also confirmed by EIA against plasminogen (Figure 3A). There was no significant difference in plasminogen concentration among the conditioned medium from transfected HepG2 cells with control vector, HepG2 cells cotransfected with apo(a) vector, and DNA-based control ON (DNA-RZ 151) and HepG2 cells cotransfected with apo(a) vector and ribozyme ON (RZ 151). Moreover, apo(a) mRNA was also decreased by ribozyme ON treatment compared with control ON treatment, whereas plasminogen mRNA was not decreased in HepG2 cells transfected with ribozyme ON (Figure 3B). These in vitro studies suggest that a reduction of apo(a) but not plasminogen expression could be achieved by RZ 151 ON. Finally, we examined the effects of ribozyme ON on Lp(a)–stimulated VSMC growth. Consistent with the previous findings that Lp(a) has a mitogenic action on VSMC, treatment with the conditioned medium from HepG2 cells transfected with apo(a) vector resulted in a significant increase in number of VSMC compared with that from cells transfected with control vector (Figure 3C). Of importance, cotransfection of ribozyme ON abolished the mitogenic action of the conditioned medium from HepG2 cells transfected with apo(a) vector and DNA-based control ON.

**Discussion**

Lp(a) has been of interest in vascular biology because epidemiological studies have indicated it to be an independent risk factor for cardiovascular disease, for example, atherosclerosis and ischemic heart disease.1–6 Recent studies with transgenic technology showing that mice expressing apo(a) gene develop atherosclerosis have focused on the mitogenic action of apo(a) and Lp(a).13–15 Lp(a) and apo(a) have been thought to enhance proliferation of human VSMC in culture by inhibiting activation of plasminogen to plasmin because of high homology of apo(a) gene to plasminogen gene.8–10 Thus apo(a) gene develop atherosclerosis have focused on the mitogenic action of apo(a) and Lp(a).13–15 Lp(a) and apo(a) have been thought to enhance proliferation of human VSMC in culture by inhibiting activation of plasminogen to plasmin because of high homology of apo(a) gene to plasminogen gene.8–10 Thus apo(a) inhibited the activation of TGF-β, an autocrine inhibitor of VSMC growth, by plasmin through the blockade of the proteolytic activation of TGF-β.8–10 Therefore, an ideal therapeutic strategy for the treatment of patients with high Lp(a) concentration is to decrease Lp(a) concentration without any effects on plasminogen concentration. Although none of the reports mentioned the potential strategy related to this issue, ribozyme strategy can answer this difficult question. The present study demonstrated that ribozyme ON against apo(a) selectively inhibited Lp(a) production without affecting plasminogen production. The spec-

![Figure 3. A, Effect of ribozyme ON on plasminogen concentration in conditioned medium of HepG2 cells at 2 days after transfection as assessed by EIA. Control indicates HepG2 cells transfected with control vector; apo(a), HepG2 cells transfected with apo(a) vector and DNA-based control ON (DNA-RZ 151); ribozyme, HepG2 cells transfected with apo(a) vector and ribozyme ON (RZ 151). n=4 per group. B, Effect of ribozyme ON on mRNAs of apo(a) and plasminogen at 1 day after transfection. apo indicates untransfected HepG2 cells; apo+ribozyme, HepG2 cells cotransfected with apo(a) vector and ribozyme ON (RZ 151); apo+control, HepG2 cells transfected with apo(a) vector and DNA-based control ON (DNA-RZ 151); and NC, negative control (without RNA). This experiment was repeated 3 times. C, Effect of ribozyme ON on mitogenic action of Lp(a) on human aortic VSMC. Control indicates HepG2 cells transfected with control vector; apo(a), HepG2 cells transfected with apo(a) vector and DNA-based control ON (DNA-RZ 151); ribozyme, HepG2 cells transfected with apo(a) vector and ribozyme ON (RZ 151). n=6 per group. **P<0.01 versus control, #P<0.01 versus apo(a).
ificity of the inhibitory effect of ribozyme ON on Lp(a) production presented in this study was supported by several lines of evidence: (1) the ribozyme ON (RZ 151) inhibited Lp(a) production in vitro, whereas DNA control ON, mismatched ribozyme ON, and antisense ON did not, (2) ribozyme ON directed against apo(a) inhibited Lp(a) but not plasminogen production assessed by Western blotting, EIA, and RT-PCR, and (3) ribozyme ON inhibited Lp(a)–stimulated VSMC proliferation in vitro.

Besides ribozyme technology, as discussed earlier, antisense technology may also be useful. However, antisense technology may not be useful in this case for the following reasons: (1) it is difficult to select antisense sequences around ATG sites that are most commonly and effectively used as antisense sequences, because the structure of the apo(a) gene around ATG sites is completely identified to the plasminogen gene and (2) the antisense against apo(a) may inhibit plasminogen gene expression, in addition to apo(a). Moreover, the inhibitory effect of ribozyme on target gene expression theoretically is higher than antisense (passive versus active).19 Although the real therapeutic value of this ribozyme strategy against apo(a) requires further studies, our present study revealed one of potential strategies for the treatment of atherosclerosis. The potential targets for ribozyme-mediated cleavage include viral proteins (HIV, and so on), cell cycle regulatory proteins, transcriptional factors, and aberrant fusion proteins.19 High Lp(a) concentration may be one of ideal targets for ribozyme-mediated gene therapy.

Application of ribozyme technology to human gene therapy may require enhancement of the efficiency of cellular uptake and the stability of ribozyme ON, since ribozyme ON is easily degraded by nucleases because of its RNA backbone. To overcome these issues, in this study, we used a viral protein–mediated ON transfer technique that results in rapid cellular uptake and a 10-fold higher transfection efficiency of ON plasmid DNA than lipofection or passive uptake methods.24 In addition, we used ribozyme ON chemically modified with the phosphoribosyl backbone of RNA to phosphorothioate. The practical use of these ribozyme ON as therapy for atherosclerosis induced by high Lp(a) will depend on the development of a delivery system into the liver for long-term expression. Moreover, further studies are necessary to test the efficacy of ribozyme ON in vivo for the application to therapy. Overall, this study provides the first evidence that Lp(a) production can be prevented by ribozyme ON directed against apo(a) gene independent from plasminogen production, suggesting a novel therapeutic strategy for the treatment of cardiovascular diseases related to high Lp(a). The selective blockade of apo(a) is particularly attractive because the high homology of apo(a) to plasminogen causes difficulty in the development of drugs against Lp(a). We anticipate that the modification of ribozyme ON pharmacokinetics will facilitate the potential clinical utility of the ribozyme strategy.

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