Anti-miR-33 Therapy Does Not Alter the Progression of Atherosclerosis in Low-Density Lipoprotein Receptor-Deficient Mice

Tyler J. Marquart, Judy Wu, Aldons J. Lusis, Ángel Baldán

Objective—To determine the efficacy of long-term anti-miR-33 therapy on the progression of atherosclerosis in high-fat, high-cholesterol–fed Ldlr−/− mice.

Methods and Results—Ldlr−/− mice received saline, or control or anti-miR-33 oligonucleotides once a week for 14 weeks. The treatment was effective, as measured by reduced levels of hepatic miR-33 and increased hepatic expression of miR-33 targets. Analysis of plasma samples revealed an initial elevation in high-density lipoprotein cholesterol after 2 weeks of treatment that was not sustained by the end of the experiment. Additionally, we found a significant increase in circulating triglycerides in anti-miR-33–treated mice, compared with controls. Finally, examination of atheromatous revealed no significant changes in the size or composition of lesions between the 3 groups.

Conclusion—Prolonged silencing of miR-33 fails to maintain elevated plasma high-density lipoprotein cholesterol and does not prevent the progression of atherosclerosis in Ldlr−/− mice. (Arterioscler Thromb Vasc Biol. 2013;33:455-458.)

Key Words: ABCA1 ◼ atherosclerosis ◼ HDL ◼ miR-33 ◼ VLDL

Recenly, we and others reported on miR-33 (also known as miR-33a or miR-33a-5p), an intragenic miRNA encoded within sterol regulatory element-binding protein 2 (SREBP-2) that modulates the expression of several transmembrane transporters, that include ATP-binding cassette transporter, sub family A, member 1 (ABCA1); ATP-binding cassette transporter, subfamily G, member 1; ATP-binding cassette, sub-family B, member 11; ATPase aminophospholipid transporter, class I, type 8B, member 1.1–6 These studies suggested that miR-33 plays important roles in fine-tuning both high-density lipoprotein (HDL) and biliary metabolism. Indeed, we and others showed that short-term silencing of hepatic miR-33 with antisense oligonucleotides increases both HDL-cholesterol (HDL-c)1–5 and hepatic bile secretion.6 Additionally, treatment of mice with anti-miR-33 oligonucleotides led to increased reverse cholesterol transport in vivo.6,7 The reverse cholesterol transport pathway mobilizes extrahepatic cholesterol into HDL back to the liver, where both cholesterol and its metabolite bile acids are secreted into bile for final excretion through the feces. Hence, reverse cholesterol transport is regarded as atheroprotective and strategies that promote/accelerate the flow of cholesterol through this pathway, for example, raising HDL levels or promoting hepatobiliary removal of sterols, are predicted to be effective in reducing the risk of cardiovascular disease.8

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Collectively, the initial reports on miR-33 suggested that patients with hypercholesterolemia might benefit from a therapy that incorporated silencing of miR-33 (eg, anti-miR-33 oligonucleotides). In agreement with this idea, Rayner et al reported that anti-miR-33 oligonucleotides were able to increase HDL-c and accelerate the regression of atherosclerotic lesions in hyperlipidemic Ldlr−/− mice switched to standard chow diet.7 However, no reports are yet available regarding the impact of miR-33 silencing on the progression of atherosclerosis. Hence, our purpose was to determine the efficacy of long-term anti-miR-33 therapy on the development of arterial disease in high-fat, high-cholesterol–fed Ldlr−/− mice.

Materials and Methods

Male, 10-week-old Ldlr−/− mice (Jackson Laboratories) were maintained on a 12 hour/12 hour light/dark cycle with unlimited access to food and water, and injected intraperitoneally with 200 μL saline (n=5), or 7 mg/kg control (5′-TCCTAGAAAGAGTAGA; n=13) or anti-miR-33 (5′-TGCAACTACAATGCA; n=11) locked nucleic acid oligonucleotides (a kind gift from Miragen Therapeutics, Inc) once a week. After 2 weeks on chow, mice were switched to, and maintained on, a western diet (WD) containing 21% fat and 1.25% cholesterol (Research Diets D12108) for 12 weeks. Mice were fasted for 6 hours
Results

Anti-miR-33 treatment did not affect body weight (Figure 1A), although a trend was observed for accelerated weight gain in the anti-miR-33 group after 10 weeks of treatment. Analysis of plasma samples showed that both total cholesterol and HDL-c increased in the first 2 weeks of treatment with anti-miR-33 oligonucleotides compared with control treatments (Figure 1B and 1D). These data agree with previous reports\(^1\) and provide evidence that the anti-miR-33 treatment was efficacious. As expected, at the end of the WD feeding, circulating cholesterol levels increased in all animals (Figure 1C); however, no changes in HDL-c were noted between groups (Figure 1E). Furthermore, we noted that mice injected with anti-miR-33 oligonucleotides showed increased plasma triglycerides (TG), both on chow and after WD (Figure 1F and 1G). Fast performance liquid chromatography analysis of plasma samples confirmed the changes in HDL-c on chow, but not on WD, in the anti-miR-33 group (Figure I in the online-only Data Supplemental materials).
Concomitant changes in the levels of apolipoprotein A1 and apolipoprotein B48/100 were also noted in these plasma samples (Figure I in the online-only Data Supplement), further verifying the changes in HDL-c and TG. Collectively, these data suggest that the increase in HDL-c after miR-33 silencing is transient. This is also the first study to report changes in circulating TG after treatment with anti-miR-33 oligonucleotides. These latter results were unexpected, as a previous study showed a decline in plasma VLDL-TG in monkeys receiving anti-miR-33 treatment.9

One possibility to explain the absence of changes in HDL-c at week 12 in the anti-miR-33 group is that the bioavailability and potency of the oligonucleotides decline over time or in combination with the WD. Additionally, hepatic miR-33 levels are known to decrease in mice fed a WD,2,3 which might also compromise the success of the antisense treatment. However, RNA and protein analysis of the livers showed that miR-33 was indeed effectively silenced in the anti-miR-33 group, as levels of miR-33 were decreased ≈75%, whereas those of miR-33 targets (ABCA1; ATP-binding cassette, sub-family B, member 1; carnitine palmitoyltransferase 1a) were significantly increased in these mice compared with controls (Figure 1H and Figure IIA in the online-only Data Supplement). RNA and protein levels of other genes declined or remained unchanged in the same livers (Figure 1H and Figures I and IIA in the online-only Data Supplement), suggesting that the changes in several miR-33 targets were specific. It is important to stress that the changes in hepatic ABCA1 noted in the livers of anti-miR-33 mice after 12 weeks on WD were not paralleled by increased levels of HDL-c in plasma, as noted above. The reasons behind these latter paradoxical results remain to be elucidated. Further analysis revealed no significant changes in liver weight, and hepatic cholesterol or TG contents (Figure 1J–1L). However, hepatic free fatty acid levels were significantly reduced in the anti-miR-33 group (Figure 1M). This latter result could be explained by the significant increase in CPT1α or decrease in fatty acid synthase expression in the same livers (Figure 1H and I). Additionally, we measured the hepatic expression of miRNAs that are known to regulate sterol and TG metabolism.10 Data in Figure IIB in the online-only Data Supplement show that miR-378, miR-27a, and miR-122 (but not miR-758, miR-370, miR-335, and miR-125a-5p) were modestly elevated in mice receiving anti-miR-33 treatment. The functional consequences of deregulated expression of these latter miRNAs on TG and sterol homeostasis in mice receiving anti-miR-33 oligonucleotides are unknown. Nevertheless, taken together, the data suggest that the anti-miR-33 oligonucleotides were effective in silencing hepatic miR-33 at the end of the 12 weeks of WD feeding.

Finally, analysis of atherosclerotic lesions both in the aortic root and in en face preparations of the whole aorta revealed no significant differences between groups (Figure 2A–2D). Likewise, assessment of macrophage and collagen contents in aortic root sections of selected mice in each group with similar lesion sizes revealed no discernible differences between treatments (Figure 2B).

Discussion

This is the first report on the impact of long-term anti-miR-33 treatment on the progression of atherosclerosis in Ldlr−/− mice. Previous regression studies7 employed mice that received antimiRs for only 4 weeks, and no data are yet available regarding the continuing efficacy of the oligonucleotides in this regression model. Our data show conclusively that anti-miR-33 oligonucleotides, although effective in suppressing hepatic miR-33 expression and increasing the expression of hepatic miR-33 targets, did not raise HDL-c after mice were switched to the WD and did not provide atheroprotection.

Primates, but not rodents, express a second miR-33 gene (miR-33b) from an intron of SREBP-1. Notably, SREBP-1 and -2 are differentially regulated by hormones, dietary challenges, or statin treatment. In any case, the expression of miR-33a and miR-33b is expected to follow that of their corresponding host gene. Whether the 2-nucleotide mismatch (outside of the seed sequence) between miR-33a and miR-33b results in differential targeting remains to be established, although studies in human HepG2 cells11 and in nonhuman primates9 suggest that both miRNAs target the same set of genes with similar specificity. Nevertheless, anti-miR-33 oligonucleotides are expected to block the action of both miR-33a and miR-33b.9 A limitation of our study is that the lack of rodent miR-33b prevents the direct translation of our results in Ldlr−/− mice to humans. Indeed, in marked contrast to our study, the
longitudinal study in nonhuman primates did show a sustained elevation of HDL-c, as well as a decrease in TG. Whether the differences in cholesterol and TG metabolism between mice and primates in response to anti-miR-33 treatment are due to miR-33b remains to be established. Such discrepancies between rodents and primates thus raise an important concern regarding the direct translation of data from rodent models to human physiology and metabolic disorders. Further studies using humanized mice, in which a miR-33b transgene is inserted within an intron of SREBF-1, will help us address this important question.

The mechanism behind the loss of elevated HDL-c in the anti-miR-33 group remains to be elucidated. It is likely that the potential atheroprotective effect of anti-miR-33-based therapies rely, at least in part, in their ability to promote and sustain such an increase in HDL-c. The fact that hepatic ABCA1 expression is still induced after 14 weekly anti-miR-33 injections suggests the existence of homeostatic compensatory mechanisms controlling plasma HDL. Interestingly, Brewer et al reported that hepatic overexpression of ABCA1 results in enhanced atherosclerosis in Ldlr−/− mice, whereas van Eck et al showed that macrophage ABCA1 overexpression reduces atheromata in Ldlr−/− mice. It is conceivable that the lack of atheroprotection in our model is the result of both proatherogenic and antiatherogenic effects that follow long-term silencing of miR-33 both in the liver and in macrophages in the lesion. Remarkably, Rayner et al showed that the expression of ABCA1 was induced in macrophages recovered from atheromata in 2′-fluoro-methoxyethyl anti-miR-33-treated mice. Whether 2′F/MOE and locked nucleic acid oligonucleotides have different pharmacokinetic properties that result in distinctive bioavailability in plaques remains to be established.

To address the potential atheroprotective effect of anti-miR-33-based therapies, we transplanted apoE−/− mice with miR-33−/− mice or treatment with anti-miR-33 oligonucleotides are intriguing, and likely reflect the impact of miR-33 expression in several cell types (eg, hepatocytes, macrophages, endothelial, and smooth muscle cells). It is conceivable that anti-miR therapy could be effective in some, but not all, cell types where miR-33 is normally expressed. Tissue/cell-specific knockout mice for miR-33 will help us understand the relative contribution of hepatocyte and plaque (macrophage, endothelial, and smooth muscle cell) miR-33 to atherogenesis, and the antiatherogenic potential of anti-miR-33 oligonucleotides. It will also be important to establish whether different chemistries alter the bioavailability and potency of anti-miR-33 oligonucleotides in either liver or lesions.

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Disclosures

T.J.M. and Á.B. are pursuing a patent related to anti-miR-33 silencing.

References

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SUPPLEMENTAL MATERIALS AND METHODS

Plasma Lipid Analysis
Plasma samples were collected via superficial temporal vein bleeds at week 0 (2 weeks on chow) and week 12 (12 weeks on WD). Total cholesterol, HDL-cholesterol, and triglycerides were assayed enzymatically using Cholesterol E, HDL-Cholesterol E, and Triglycerides kits (Waco Chemicals). Plasma lipoprotein profiles were obtained by a modified Column Lipoprotein Profile (CLiP) method\(^1\). Briefly, 15 \(\mu\)L of plasma were diluted in 60 \(\mu\)L of saline, and 10 \(\mu\)L of this mixture was auto-injected into a Superose-6 column (GE Healthcare) using elution buffer (saline, 2 mM EDTA, 0.01% sodium azide, pH 7.4) at a flow rate of 0.6 mL/min at 40°C. The FPLC eluate was immediately mixed with cholesterol reagent (Thermo Scientific) at a flow rate of 0.3 mL/min, and incubated at 40°C in a 5 m KOT coiled reactor. The final mixture entered a capillary spectrophotometric detector set at 500 nm, and the profiles were collected in real time using LC Solution software (Shimadzu).

Liver Lipid Analysis
Tissue lipids were extracted into CHCl\(_3\) by a modified Folch method, resolubilized in water\(^2\), and quantitated using kits for cholesterol, triglyceride, or free fatty acids (Wako Chemicals).

RNA Analysis
RNA was isolated from livers with Trizol. Complementary DNAs (cDNAs) were generated from 1 \(\mu\)g of DNase1-treated RNA using MultiScribe Reverse Transcriptase (Applied Biosystems) and random hexamers. Real-time quantitative PCR was done using Power SybrGreen reagent (Applied Biosystems) in a LightCycler-480 (Roche). Primer sets are available upon request. Values were normalized to 36B4 and calculated using the comparative \(\Delta\Delta Ct\) method. The expression of miR-33 was normalized to U6, using MiRCURY miRNA assays (Exiqon).
**Protein Analysis**

Protein extracts were obtained from livers as described\(^3\). Forty micrograms of protein or 0.1 µL of plasma (in 20 µL saline) were resolved in 4–12% Bis–Tris gels (or in 3–8% Tris-Acetate gels for APOB), transferred to PVDF membranes, and probed with antibodies for ABCA1 (1:1,000; Novus, NB400-105), CPT1\(\alpha\) (1:2000; Proteintech, 15184-1-AP), FAS (1:1,000; Thermo, MA5-14887), PLIN-2 (1:2000; Abcam, ab37516), \(\beta\)-ACTIN (1:100; Sigma, A2066), APOA1 (1:1,000; Meridian, K23500R), APOB48/100 (1:2,000; Meridian, K23300R), and APOE (1:1,000: Meridian, K23100R) in TBS-Tween20 containing 4% non-fat dry milk. Immune complexes were detected with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibodies (1:10,000; SCBT).

**Morphometric and Histologic Analysis of Atherosclerotic Lesions**

Quantification of lesion areas in frozen sections of the aortic root and in *en face* preparations of the whole aorta was done as described\(^4\) by a single operator blind to the treatment the mice received. Cryosections were stained with a monoclonal rat anti-mouse CD68 antibody (1:400 dilution; AbD Serotec) and Masson’s trichrome to reveal macrophage and collagen content, respectively.

**Statistical Analysis**

Data are shown as mean ± s.e.m. Differences in hepatic mRNA expression, circulating lipid levels, and atherosclerotic lesion size between groups (saline vs. control oligonucleotide vs. anti-miR-33 oligonucleotide) were analyzed by one-way ANOVA. Differences in body weight over time were analyzed using repeated measures ANOVA with a Bonferroni post-hoc test. All statistical analyses were performed using SPSS version 18. Statistical significance was set at \(P \leq 0.05\)
SUPPLEMENTAL FIGURE LEGENDS

Figure I. Effects of anti-miR-33 therapy on circulating cholesterol and apoproteins. Mice were dosed as described in Fig.1, and bled at weeks 0 (Pre WD) and 12 (12 week WD). Plasmas were analyzed by FPLC to obtain cholesterol lipoprotein profiles (A, C), and by western blot to measure the abundance of apoproteins APOA1, APOB, and APOE (B, D).

Figure II. Expression of selected hepatic RNAs following anti-miR-33 treatment. The levels of particular mRNA (A) and miRNA (B) were analyzed in the livers of western diet-fed mice following treatment with saline, control or anti-miR-33 oligonucleotides, as described in Methods. Data are shown as mean ± s.e.m.; n=5; *P<0.05

SUPPLEMENTAL REFERENCES


Figure I

A. Cholesterol Profile Pre WD

B. Pre WD

C. Cholesterol Profile 12 Week WD

D. 12 Week WD
Figure II

A. Relative mRNA Expression

B. Relative miRNA Expression

miR-27a miR-122 miR-125 miR-335 miR-370 miR-378 miR-758

miR-27a miR-122 miR-35 miR-370 miR-378 miR-758

saline control anti-miR-33

* * *
Anti-miR-33 치료는 생쥐 죽상경화 병변 진행을 억제하지 못했다.

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Summary

배경
Anti-miR-33 치료가 고지방-고콜레스테롤 식이를 계속하는 LDL 수용체 유전자 결핍 생쥐에서 죽상경화를 억제할 수 있는가를 연구하였다.

방법 및 결과
LDL 수용체 유전자 결핍 생쥐에게 고지방-고콜레스테롤 식이를 제공하면서, 대조군, 생리식염수 투여군, anti-miR-33 투여군의 3군으로 배정하여 14주 동안 매주 투여하였다. Anti-miR-33 투여로 간의 miR-33 생산이 감소하고 miR-33 목표 단백 발현이 증가하였다. 혈중 HDL 콜레스테롤은 anti-miR-33 투여 2주 후에 유의하게 증가하였지만 이후에는 기저치로 회귀한 채 유지되었다. 중성지방은 miR-33 투여에 의해 유의하게 증가하였다. 하지만 miR-33 투여는 죽상경화반의 크기나 구성을 변화시키는 효과를 보이지 않았다.

결론
Anti-miR-33 투여는 고지방-고콜레스테롤 식이를 계속한 LDL 수용체 유전자 결핍 생쥐에서 HDL 콜레스테롤을 증가시키지 못했고, 죽상경화의 진행을 억제하는 효과를 보이지 않았다.

Commentary

miR-33은 HDL 콜레스테롤의 합성에 관여하는 여러 단계에 작용하여 HDL 콜레스테롤의 합성을 억제한다고 알려져 있다. 생쥐에서는 miR-33 (miR-33a, miR-33a-5p)가 단독으로 SREBP-2의 발현을 억제하여 ABCG1, ABCA1, ABCB11, CPT1-alpha 등의 발현을 억제하며, 인체에서는 miR-33a와 miR-33b가 각각 역할을 나누어 작용한다.

MicroRNA는 체내에서 존재하는 작은 RNA로서, 단백질로 해독 표현되지 않는 mRNA의 3’부분에 상보적으로 작용하여 해당 유전자 발현을 조절하는 RNA이다. miRNA는 생체의 다양한 유전자 발현을 조절하는 새로운 기전을 담당한다고 알려져 관심을 끌고 있다.

이 연구는 anti-miR-33을 장기간 투여한 최초의 연구이며, 기존 연구결과들과 달리 anti-miR-33의 효과가 긍정적으로 유의하게 증명되지 않았다는 점이 특징이다. 이는 miRNA를 조절하는 방법을 이용하여 동맥경화를 억제하고 치료하려는 최근의 연구 흐름에 매우 중요한 문제가 있음을 예보해주는 연구이다.

그 동안 LDL 수용체 유전자 결핍 생쥐에서 고지방식이를 정상 식이로 변경하면 죽상경화의 진행이 억제되며, 특히 이 과정에서 anti-miR-33을 투여하면 HDL 콜레스테롤이 유의하게 증가하고 또한 죽상경화 진행이
더 유의하게 억제된다고 보고되었다. 하지만 이 연구에서 anti-miR-33의 투여는 고지방·고콜레스테롤 식이를 계속한 LDL 수용체 유전자 결핍 생쥐에서 HDL 콜레스테롤을 증가시키지 못했고, 죽상경화의 진행을 억제하지 못하였다. 고지방 식이에서 정상 식이로 변경한 과거 연구와는 달리 이 연구에서는 고지방 식이를 계속한 점과, anti-miR-33을 4주 동안이 아닌 14주 동안 더 긴 시간 투여한 차이가 있다. 하지만 miR-33의 작용 기전이나 작용 결과에 대한 여러 과거의 연구결과들에 비추어볼 때, 이 연구에서 HDL 콜레스테롤의 증가가 나타나지 않은 점은 매우 특이한 결과이다. 저자들은 식사의 영향과 장기간 투여에 따른 anti-miR-33 효과 저하의 가능성을 언급하고 있다. 첫째로 고지방 식이로는 miR-33의 발현을 억제한다고 알려져 있기에, anti-miR-33의 효과가 적게 나타날 수 있으며, 둘째로 장기간 투여는 anti-miR-33의 생물학적 반감기를 짧게 하고 효과 감소성을 감소시킬 가능성이 있다. 하지만 저자들은 추가 분석한 결과에 따르면, anti-miR-33의 투여에 의하여 miR-33의 작용이 75% 감소하였고 이에 따라 miR-33의 목표 유전자들의 발현이 증가하여 간의 ABCG1, ABCA1, ABCB11, CPT1-alpha 발현이 유의하게 증가하였다. 따라서 연구 결과에 대한 이러한 추측은 옳지 않을 가능성이 있다. 이와 관련된 기전에 대한 추가적인 연구가 필요하다.
물론 ABCA1의 발현이 증가하면 죽상경화가 억제된다는 보고가 있지만, 오히려 죽상경화의 진행이 촉진된다는 연구 결과도 있다. 즉, ABCA1의 발현 증가 여부가 콜레스테롤 역수송 능력의 증가와 항상 일치하지는 않는다는 것을 알 수 있다. 따라서 이 연구에서 콜레스테롤 역수송 능력의 변화를 측정하였다면 이에 대한 설명에 도움이 되었을 것으로 생각한다. 또한 이 연구는 설치류의 miR-33에 대한 연구이기에 다른 연구대상에 이 결과를 확대 해석할 수는 없다.
이 연구에서 anti-miR-33 투여가 증상지방을 증가시킨 점, 그리고 ABCA1 증가에도 불구하고 HDL 콜레스테롤을 증가시키지 못하고 죽상경화의 진행을 억제하지 못한 기전에 대해서는 향후 추가적인 연구가 필요할 것이다.

REFERENCES