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Scavenger receptor BI is required for our previously published lipid nanoparticle-based siRNA approach for in vivo protein C silencing in mice

Submitted for publication.

To the editor,

Atherothrombosis, characterized by the development of a superimposed thrombus upon rupture or erosion of an atherosclerotic plaque, is the underlying cause of common acute cardiovascular events, such as myocardial infarction or ischemic stroke ¹. Although atherosclerosis development is widely studied in (genetically modified) mouse models, these atherosclerotic plaques do not spontaneously progress towards the atherothrombosis stage. For this purpose, we have recently developed the first spontaneous atherothrombosis mouse model. As previously reported in *Arteriosclerosis, Thrombosis and Vascular Biology* ², siRNA-mediated lowering of the natural anticoagulant protein C (*siProc*) induced the occurrence of atherothrombosis in the aortic root of hypercholesterolemic apolipoprotein E (APOE) knockout mice at one week after siRNA administration. However, spontaneous thrombus formation only occurred at a low incidence (12-25%) ^{2,3}, which highlights that further optimization of the model is necessary before we can identify conditions that affect the atherothrombosis incidence.

Platelets play an important role in the pathogenesis of atherothrombosis ⁴. In this context, mice lacking a functional high-density lipoprotein receptor, i.e. scavenger receptor BI (SR-BI) knockout mice, exhibit an interesting platelet phenotype. More specifically, SR-BI deficiency-associated hypercholesterolemia in plasma results in cholesterol accumulation in platelets, causing them to circulate in an activated state ⁵⁻⁷. Importantly, this activated platelet phenotype is associated with a higher susceptibility for FeCl₃ damage-induced arterial thrombosis in the carotid artery ⁵. Hence, it is of clear interest to study whether modifying SR-BI functionality can also increase the incidence of atherothrombosis in *siProc*-treated mice.

In order to determine if SR-BI deficiency increases the susceptibility for *siProc*-induced atherothrombosis, we challenged age- and sex-matched wild-type and SR-BI knockout mice with an atherogenic diet containing 15% fat, 1% cholesterol, and 0.5% cholic acid for 8 weeks before injection with *siProc* or a non-targeting control siRNA (*siNEG*). After 8 weeks dietary challenge, wild-type mice developed significant hypercholesterolemia (plasma total cholesterol level: 205±8 mg/dL; n=26) and early lesions in the aortic root (14±2x10³ μm²; n=24). In line with the notion that early lesions are not susceptible for atherothrombosis, no atherothrombosis was observed in the aortic root of *siProc*-treated wild-type mice despite a 50% reduction in hepatic mRNA levels of *Proc* (*siNEG*: 0.064±0.009 (n=7), *siProc*: 0.032±0.0005 (n=20); *p*<0.0001). We previously consistently observed an atherothrombosis incidence of 12-25% in APOE knockout mice ^{2,3}. However, the previously used APOE knockout mice had advanced aortic root atherosclerotic plaques (plaque size ~400-600x10³ μm²). Moreover, the extent of hypercholesterolemia in APOE knockout mice (~1000 mg/dL) was much larger than in the wild-type mice of the current study, possibly resulting in increased inflammatory and plaque destabilizing conditions. Dedicated studies on the effect of plaque size and hypercholesterolemia on atherothrombosis susceptibility are warranted.

Atherogenic diet-fed SR-BI knockout mice developed exacerbated hypercholesterolemia (plasma total cholesterol levels: 1549 ± 85 mg/dL; $n=29$). As a result, SR-BI knockout mice did develop advanced plaques in the aortic root with an average size of $425 \pm 21 \times 10^3 \mu\text{m}^2$ ($n=26$). Surprisingly, despite the advanced stage of the lesions, no aortic root atherothrombosis was observed in these animals. Analysis of *Proc* mRNA expression in livers of *siProc* treated SR-BI knockout mice revealed that this was probably due to an impaired hepatic targeting of the siRNA complex. Instead of a decrease, the relative expression of *Proc* actually tended to increase (0.064 ± 0.005 for siNEG ($n=6$), 0.083 ± 0.005 for *siProc* ($n=19$); $p=0.058$). Therefore, unfortunately no conclusions regarding the possible prothrombotic effects of SR-BI deficiency under conditions of *Proc* silencing can be drawn from this study.

In order to inhibit *Proc* production, we complexed our siRNA with commercially available InvivoFectamine™ 3.0 Reagent (Invitrogen). This reagent is specifically designed to target hepatocytes and has previously been shown to be highly effective and non-toxic when used for liver-targeted RNA interference⁸. Although the exact composition of this compound is not publicly known for competitive reasons, the supplier has indicated that it is an animal-origin free lipid nanoparticle⁹. Previous studies have described a role for APOE and the LDL receptor in the uptake of neutral, cationic and ionizable nanoparticles (liposomes) in mice^{10,11}. However, in our earlier studies InvivoFectamine™ 2.0 Reagent and InvivoFectamine™ 3.0 Reagent-complexed *siProc* silencing was successful^{2,3}. Moreover, transfection was also efficient in APOE*3Leiden.CETP mice (unpublished data). Our current study suggests that SR-BI is rather involved in the hepatic uptake of InvivoFectamine™ 3.0 Reagent-complexed siRNA. SR-BI is widely recognized as a multi-ligand scavenger protein, and it clears, among other things, native and modified lipoproteins¹², and anionic phospholipids¹³. Therefore, it is not unlikely that SR-BI is also directly mediating the uptake of InvivoFectamine™ complexed siRNA.

In conclusion, our studies show that SR-BI is required for an InvivoFectamine™ 3.0-based siRNA approach to silence Protein C in hepatocytes *in vivo*. Our studies highlight that caution is warranted in the design and interpretation of experiments involving liver-targeted siRNA in mouse models of disturbed scavenger receptor functioning.

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