Stroke

RESEARCH LETTER

Effects of Inhibition or Deletion of PCSK9 (Proprotein Convertase Subtilisin/Kexin Type 9) on Intracerebral Hemorrhage Volumes in Mice

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Anti-PCSK9 (proprotein convertase subtilisin/kexin type 9) antibodies lower low-density lipoprotein cholesterol levels and significantly reduce cardiovascular end points.[1] Safety concerns were raised as ultra-low levels of low-density lipoprotein cholesterol can be achieved. Low cholesterol levels are associated with an increased rate of intracerebral hemorrhage (ICH),[2] and hematoma growth was increased with low serum cholesterol.[3,4] The objective of this study was to test the effects of PCSK9 inhibition or genetic deletion on hematoma volume after experimental ICH (collagenase model) using 3 different strain of mice (ie, hypercholesterolemic, normocholesterolemic, PCSK9$^{-/-}$ versus controls). Experimental procedures were approved by official committees. The authors declare that all supporting data are available within the article and in the Data Supplement.

After 6 weeks of Western-type diet, APOE×3-Leiden CETP (cholesteryl ester transfer protein) high cholesterol animals treated with Anti-PCSK9 antibodies (CmAb1; 10 mg/kg SC every 10 days) showed significantly lower (−31%) cholesterol levels than vehicle controls. In contrast, cholesterol levels in CmAb1-treated wild-type mice were not significantly different versus controls. PCSK9$^{-/-}$ mice had −53% lower cholesterol levels compared with wild-type controls. APOE×3-Leiden CETP mice treated with CmAb1 had ≈1.5× larger ICH volumes compared with vehicle controls. Mortality trended to be higher in CmAb1-treated animals compared with vehicle controls (34%; not significant). In B6129SF1/J mice treated with CmAb1, there were no significant differences in ICH volume or mortality versus vehicle. Also, no significant difference was found in ICH volumes or mortality of PCSK9$^{-/-}$ mice compared with controls.

Our results suggest that high plasma cholesterol levels may have protective effects on hematoma expansion, while we did not find an independent effect of PCSK9 inhibition on ICH growth. Overall, our findings are in line with studies reporting an inverse association between cholesterol levels and ICH.[2] In a meta-analysis with different lipid-lowering drugs, lipid lowering was associated with a modestly increased risk of ICH in secondary prevention trials.[5] In contrast, this was not the case for PCSK9 inhibitors in randomized clinical trials.[1] We did not combine anti-PCSK9 antibodies with statins, unlike in clinical trials excluding a possible add-on effect. Furthermore, we included a mouse strain, whose serum lipid levels were not affected by anti-PCSK9 antibodies to differentiate potential pleiotropic effects from those induced by changes in cholesterol levels.

In conclusion, mice with ultrahigh cholesterol levels (APOE×3-Leiden CETP) had significantly smaller ICH volumes compared with mice with lower cholesterol levels after anti-PCSK9 antibody treatment. This was neither confirmed in PCSK9 knockouts nor in normocholesterolemic wild-type mice, which were not responsive to pharmacological cholesterol lowering by anti-PCSK9 antibody.

Key Words: animal ■ cholesterol ■ collagenase ■ hematoma ■ mice

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Nonstandard Abbreviations and Acronyms

ICH	intracerebral hemorrhage
PCSK9	proprotein convertase subtilisin/kexin type 9

ARTICLE INFORMATION

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Disclosures

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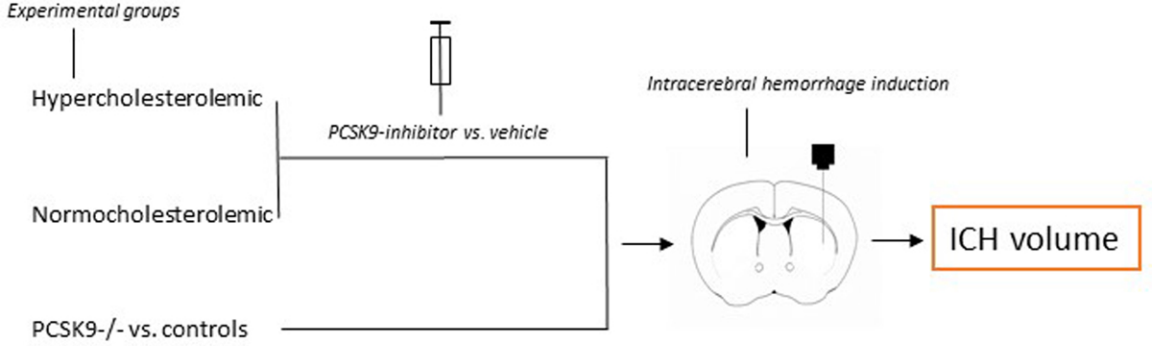

PSCK9 antibodies effectively lower cholesterol levels. Here, we tested whether treatment with PSCK9 inhibitors or its genetic deletion increases intracerebral hemorrhage (ICH) volumes in mice. We induced ICH by intrastriatal injection of collagenase VII-s in hypercholesterolemic, normocholesterolemic and PCSK9-/- mice (low cholesterol). **ICH volume was increased in hypercholesterolemic mice treated with PSCK9 inhibitors compared to vehicle-treated controls.** Additional experiments revealed no independent effect of pharmacological or genetical PCSK9 inhibition on ICH volume.

SUPPLEMENTAL MATERIAL

Methods

Animals, study design, and statistics

The authors declare that all supporting data are available within the article. Experimental procedures were approved by official committees. APOE*3Leiden.CETP or B6129SF1/J mice were fed 6 weeks of Western Type Diet (WTD) (1,25% cholesterol, SSNIFF Spezialdiäten GmbH) and randomized to treatment with fully human anti-PCSK9 antibody CmAb1 (PL-45134; 10mg/kg every 10 days by a blinded investigator) or vehicle (saline). PCSK9-/- mice and their respective controls (B6129SF1/J mice) received normal chow.

Intracerebral hemorrhage volume was compared in APOE*3Leiden.CETP mice (anti-PCSK9 antibody, n = 30; vehicle, n = 30), B6129SF1/J (anti-PCSK9 antibody, n = 20, vehicle, n = 20) and PCSK9-/- mice vs. B6129SF1/J-mice (n = 11/group). In a different set of animals, plasma cholesterol levels were measured (APOE*3Leiden.CETP / anti-PCSK9 antibody, n = 10; APOE*3Leiden.CETP / vehicle, n = 11; B6129SF1/J / anti-PCSK9 antibody n = 8; B6129SF1/J / vehicle, n = 8; PCSK9-/- n = 6, B6129SF1/J n = 6).

GraphPad Prism 7 was used for statistical analysis. Data are presented as mean ± standard deviation. Mann-Whitney U test or t-test were used to compare groups.

Cholesterol measurement

Plasma cholesterol levels were determined 6 weeks after the feeding period and anti-PCSK9 antibody or vehicle treatment started or immediately in the normal chow group (PCSK9-/- vs. B6129SF1/J). Samples of APOE*3Leiden.CETP mice were analyzed using a commercially available colorimetric cholesterol assay (Abcam). Samples of B6129SF1/J and PCSK9-/- mice

were analyzed using gas–liquid chromatography analysis. Cholesterol measurements were performed by an investigator blinded to treatment status.

Collagenase model of intracerebral hemorrhage

An investigator blinded to treatment status performed surgery. Mice were anesthetized with 1-2% isoflurane in 70% N_2O/29% O_2. Local anesthetic (bupivacaine) was applied. A heating pad was used to maintain body temperature. The following coordinates from the bregma were used to drill a small borehole in the skull: 0 mm anterior, 2.0 mm lateral. A needle (32 gauge, 0.5 µl microinjection needle, Hamilton 7000 series) was slowly lowered 3.5 mm in depth into the right striatum. Over a period of 5 minutes 0.5 µl containing 0.1 U of collagenase VII-s were slowly injected. The needle was left in place for 10 minutes and then slowly removed over 5 minutes. After suturing mice were returned to their cages.

Intracerebral hemorrhage volume

An investigator blinded to treatment status performed measurements of ICH volumes. Mice were deeply anesthetized (ketamine/xylacine) and then transcardially perfused with 30 ml of phosphate buffered saline (PBS). We performed two sets of experiments. Based on previous studies we chose a 24 h endpoint, however, mortality was high (36/62 animals). Therefore, in a second set we sacrificed animals at 6 h to reduce stress for animals (20/60 of APOE*3Leiden.CETP and all B6129SF1/J from the anti-PCSK9 antibody vs. vehicle experiment; mortality lowered to 22/60 animals). Importantly, hemorrhage growth has ceased by 6 h in our model and we did not find any differences between 6 and 24 hrs in this study.[1] Brains were collected. After homogenization (30 sec) of ipsilateral hemispheres in 3 ml PBS, ultrasound was applied for 1 minute. Samples were centrifuged (30 min, 13000 rpm at 4°C), and 25 µl of supernatant mixed with 100 µl of Drabkin's reagent. To measure hemoglobin,

absorption rates were measured at 540 nm using a plate reader. Hemoglobin concentration was converted into hematoma volumes calculated on basis of a standard curve.[2]

Results

Plasma cholesterol levels

APOE*3Leiden.CETP animals treated with anti-PCSK9 antibody showed significantly lower (-31%) total plasma cholesterol levels than vehicle treated animals (387±35 vs. 559±18 mg/dL, respectively; vehicle n = 11, anti-PCSK9 antibody n=10; p = 0.0002). In contrast, total plasma cholesterol levels in wild-type B6129SF1/J animals were not different in anti-PCSK9 antibody treated animals compared to controls (vehicle 197±12 mg/dL; anti-PCSK9 antibody 194±12 mg/dL; n = 8/group; p = 0.866). PCSK9 -/- mice (49±1 mg/dL; n=6) showed significantly lower levels of cholesterol (-53%) compared to their respective controls (B6129SF1/J 104±6 mg/dL; n=6; p<0.0022).

Intracerebral hemorrhage volume

APOE*3Leiden.CETP mice treated with anti-PCSK9 antibody had a mean hematoma volume of 19.6±2.3 µl while vehicle treated mice had a mean hematoma volume of 13.2±1.3 µl (n=30/group; p=0.041). This translates to an approximately 1.5–fold higher hematoma volume in animals treated with anti-PCSK9 antibody. Mortality was 20/30 in mice treated with anti-PCSK9 antibody and 15/30 in vehicle controls (p=0.19).

In B6129SF1/J mice no significant difference was found in hematoma volume and mortality of anti-PCSK9 antibody treated (17.6±1.3µl; n=20, mortality 9/20) vs. vehicle treated animals (17.8±1.9µl; n=20; p=0.91; mortality 6/20). Also, no significant difference was found in ICH-volumes of PCSK9-/-mice (10.1±2.3µl; n=11; mortality 2/11) compared to B6129SF1/J controls (11.3±2.7µl; n=11; p=0.95, mortality 6/11). There was no significant difference in mortality between treatment groups.

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*** Preclinical Checklist**

Preclinical Checklist: Prevention of bias is important for experimental cardiovascular research. ***This short checklist must be completed, and the answers should be clearly presented in the manuscript.*** *The checklist will be used by reviewers and editors and it will be published. See "Reporting Standard for Preclinical Studies of Stroke Therapy" and "Good Laboratory Practice: Preventing Introduction of Bias at the Bench" for more information.*

This study invovles animal models:
Yes

Experimental groups and study timeline

The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study:	Yes
An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated:	Yes
An overall study timeline is provided:	N/A

Inclusion and exclusion criteria

A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article:	Yes

Randomization

Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided:	Yes
Type and methods of randomization have been described:	Yes
Methods used for allocation concealment have been reported:	No

Blinding

Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible:	Yes
Blinding procedures have been described with regard to masking of group assignment during outcome assessment:	N/A

Sample size and power calculations

Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided:	

Data reporting and statistical methods

Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups:	Yes
Baseline data on assessed outcome(s) for all experimental groups have been reported:	N/A
Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms:	Yes
Statistical methods used have been reported:	Yes
Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures:	Yes

Experimental details, ethics, and funding statements

Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described:	
Different sex animals have been used. If not, the reason/justification is provided:	No

Statements on approval by ethics boards and ethical conduct of studies have been provided:	Yes
Statements on funding and conflicts of interests have been provided:	Yes

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