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Blood 142 (2023) 7119–7121

The 65th ASH Annual Meeting Abstracts

ONLINE PUBLICATION ONLY

801.GENE THERAPIES

rAAV8-PC Gene Therapy for Severe Protein C Deficiency Using a Novel Murine Model

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Introduction
Protein C (PC) is a vitamin K-dependent protein, synthesized in the liver. The PROC gene is located on chromosome 2 at position 2q13-14 and encodes 9 exons. Severe protein C deficiency (SPCD) is a rare devastating autosomal recessive disease that presents at birth with purpura fulminans (PF) and is associated with high rates of morbidity and mortality. As current management options are limited and burdensome, there is a large unmet need for new treatments including curative therapies. Because small increases in PC may significantly ameliorate the clinical presentation of SPCD and potentially prevent PF, we hypothesize that SPCD could be treated by PC gene therapy (GT).
Aim
To establish a murine model enabling the survival of SPCD mice and to evaluate PC expression after recombinant adeno-associated viral vector- PC (rAAV8-PC) gene therapy.
Methods
The study was conducted with C57Bl/6 PC heterozygous mice (a kind gift of Prof. Weiler, [Wisconsin]), severe hemophilia A (HA) mice and wild type C57Bl/6 mice. Genotyping was performed by PCR using appropriate primers for murine PROC and F8 genes.
rAAV8-PC was produced by transfecting HEK293 cells with PX-680 as rAAV helper, AAV8 and PC plasmid with an alpha-1 antitrypsin promotor. Extraction and purification were performed using a commercial kit. Viral concentration was determined by a designated commercial qPCR assay.
rAAV8 - PC (10^{12} /kg Vg of AAV8-PC) was injected via the tail vein to 5-8 weeks old mice.
Blood samples were collected from the facial vein into 1.5 ml tubes containing 0.109M sodium citrate at blood: anticoagulant ratio of 1:1 (vol/vol).
PC plasma levels were measured prior and following rAAV8-PC infusion using a commercial ELISA assay for PC antigen.
Thrombin generation was measured in plasma samples using a modification of the calibrated automated thrombinoscope assay.
Results
As expected, breeding of heterozygous PC mice yielded only 3/200 homozygous SPCD mice (PC levels= 0), that survived for less than 24 hours. Thus, GT for heterozygous PC mice was considered; however, PC plasma levels in heterozygous PC mice were not substantially different from those in WT mice (median levels in heterozygous PC and WT mice: 216 ng/ml vs. 190 ng/ml, respectively), which precluded this option. Hence, with the aim of increasing the survival rate and duration of homozygous SPCD mice, we decided to rebalance the severe thrombotic disorder of SPCD, by induction of a genetically engineered combination with the severe coagulopathy of Hemophilia. We established an alternative SPCD murine model by breeding severe HA mice with heterozygous PC mice. This approach yielded homozygous SPCD mice with severe HA (PROC-/F8-), exhibiting a significantly prolonged survival compared to homozygous SPCD mice with normal F8 levels (over 90% survived over 6 weeks). Genotyping and ELISA of PC levels confirmed PC homozygosity (null mutation) as well as severe HA.

Nineteen PROC-/F8- mice were injected with rAAV8-PC into the tail vein and followed for at least 12 weeks. Nine mice died during blood sampling at different time points and were excluded from further analysis. Individual responses to PC- GT were noted among the remaining 10 mice, reflected by non-uniform PC expression measured by ELISA (initial PC increase, time to peak and PC peak plasma levels). An increase in PC level was noted at week 2-5 post vector infusion, reaching PC peak level between week 2-10 post GT. Table 1 summarizes the individual patterns of 10 PROC-/F8-mice that underwent GT. The functional activity of PC in the murine plasma following GT was supported by thrombin generation assays. Analyses performed in PROC-/F8-mice before and after GT demonstrated endogenous thrombin potential (ETP) and peak hight decrease following GT, as compared to untreated PROC-/F8-mice.

Conclusion

We present here a novel murine model of combined PC and factor VIII deficiency that supports the survival of SPCD mice and enables PC gene therapy studies. Our results provide the first proof of concept that infusion of rAAV8-PC results in increased PC plasma levels in mice, and may serve as a basis for future studies investigating GT for SPCD. The concept of modulating a severe genetic prothrombotic disorder, which is nearly incompatible with life, by genetic combination with severe coagulopathy may offer a valuable tool enabling investigation and potential development of treatment modalities for the prothrombotic disorder.

Disclosures Levy-Mendelovich: *Pfizer:* Honoraria, Research Funding; *Novo nordisk:* Honoraria, Research Funding; *Roch LTD:* Honoraria. **Samelson-Jones:** *Biomarin:* Consultancy; *Genentech:* Consultancy; *Pfizer:* Consultancy, Honoraria; *GeneVentiv:* Current holder of *stock options* in a privately-held company; *Amarna:* Current holder of *stock options* in a privately-held company. **barazani-Brutman:** *Roch LTD:* Honoraria. **Nisgav:** *Mor LTD-owned patents:* Patents & Royalties: a co-inventor for Mor LTD-owned patents related to some studies in this Abstract.. **Livnat:** *MOR LTD-owned patents:* Patents & Royalties: a co-inventor for Mor LTD-owned patents related to some studies in this Abstract. **Kenet:** *pfizer, Anlyam, BPL, Bayer, Okpo, Pfizer, Shire , Roche:* Honoraria, Research Funding; *Roch LTD:* Honoraria.

Table 1: Outcomes of PROC-/F8-mice following rAAV8-PC gene therapy

Animal #	Time to initial plasma PC increase (weeks)*	Time of plasma PC peak (weeks)*	Plasma PC peak level (ng/ml)
1	3	3	2488
2	6	8	697
3	2	6	832
4	6	10	422
5	4	4	166
6	4	4	356
7	2	2	714
8	4	8	1329
9	5	5	785
10	3	6	835

*weeks were measured from the time of rAAV8-PC infusion

Figure 1

https://doi.org/10.1182/blood-2023-181140