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Complement C2 siRNA mediated therapy of myasthenia gravis in mice

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ABSTRACT

Activation of complement components is crucial in the progression and severity of myasthenia gravis and experimental autoimmune myasthenia gravis (EAMG). Mice deficient in complement component C4 or treated with monoclonal antibody to C1q are resistant to EAMG. In this study, we show that inhibition of complement cascade activation by suppressing the expression of a critical low-abundant protein, C2, in the classical complement pathway, significantly improved clinical and immunopathological manifestations of EAMG. Two weeks after a second booster immunization with acetylcholine receptor, when mice exhibit muscle weakness, i.p. injection of C2 siRNA significantly suppressed C2 mRNA in the blood cells and liver of EAMG mice. Treatment of EAMG mice with C2 siRNA, once a week for 5 weeks, significantly improved muscle strength, which was further evidenced by functional AChR preservation in muscle, reduction in number of C3 and membrane-attack complexes at neuro-muscular junctions in forelimb muscle sections, and a transient decrease in serum IgG2b levels. Our study shows for the first time that siRNA-mediated suppression of C2, a component of the classical complement system, following established disease, can effectively contribute to the remission of EAMG. Therefore, C2 siRNA mediated therapy can be applied in all complement mediated autoimmune diseases.

1. Introduction

Myasthenia gravis (MG) is a well-characterized complement and antibody-mediated autoimmune disease [1]. The disease occurs due to complement and auto-antibody-mediated deficit of acetylcholine receptor (AChR) at the neuromuscular junction (NMJ), preventing acetylcholine (ACh) from binding to the muscle postsynaptic AChR and resulting in ultimately impaired contractibility and muscle weakness [2–5]. An experimental model of MG, experimental autoimmune MG (EAMG) can be induced by immunizing C57BL6 (B6) mice with AChR in Complete Freund's Adjuvant (CFA) [6,7]. We and others have previously demonstrated that complement factors C1 to C9 are critically involved in EAMG and MG pathogenesis [8–11]. We have also shown genetic evidence for the involvement of the classical complement pathway (CCP) in the development of EAMG [8]. In both MG and EAMG, formation of the membrane attack complex (MAC), initiated by activated C3, co-localizes with AChR and causes severe structural injury of endplates and lyses the postsynaptic membrane that ultimately disperses and depletes AChR at NMJ [12].

Although MG patients derive benefit from current therapies, they are neither curative nor without side effects. We, therefore, aimed to treat EAMG targeting the CCP by using siRNAs, rather than a small molecule inhibitor or antibody, as these agents either form systemic immune complexes or other harmful by-products [13]. Protection from renal ischemia reperfusion injury has been previously shown by the *in vivo* silencing of C5a receptor or C3 in animal models [14,15]. However, a siRNA targeting the complement factors has never been attempted in the treatment of MG or other autoimmune disorders. We, therefore, intended to assess CCP inhibition as a potential therapy in antibody- and complement-mediated EAMG by delivering a non-vector based, chemically modified siRNA *in vivo*.

Of all the components of the complement system, the second component, C2, is an ideal candidate for siRNA-mediated gene silencing for two reasons. First, as C2 is expressed at a very low level, a huge quantity of siRNA is not required for C2 mRNA inhibition and thus any potential toxic/side effects can be avoided. Second, by targeting the CCP component, C2, the alternative pathway activated by microorganisms will be preserved and thus beneficial for host defense [16]. Moreover, C2 is a key protein that acts effectively to activate the complement cascade and the formation of MAC. In this study we show for the first time that short-term treatment of mice with established MG with C2-specific siRNA alone suppressed C2

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mRNA expression in blood cells and liver, improved MG symptoms, prevented deposition of MAC in NMJ, protected muscle AChR integrity and function, and enhanced longevity.

2. Materials and methods

2.1. Animals, EAMG model and clinical evaluation of disease

Eight-week-old B6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine). AChR from the electric organ of *Torpedo californica* was affinity purified from the neurotoxin affinity column [17,18]. B6 mice were immunized and boosted at 4 and 8 weeks with 20 μg of affinity purified Torpedo AChR emulsified in CFA (heat-killed *Mycobacterium butyricum*) [18]. All mice were housed in a barrier facility and maintained according to the Animal Care and Use Committee guidelines of the University of Texas Medical Branch. Grip strength of mice was quantified on a Dynamometer (Chatillion Digital Force Gauge, Columbus Instruments, OH) after a 2nd boost, prior to treatment and once a week following treatment with C2 siRNA or non-targeting (NT) control siRNA for 5 weeks [8].

2.2. In vitro validation of C2 mRNA inhibition by C2 siRNA

C2-specific siRNA (Silencer select mouse complement component 2, siRNA ID: s63158), NT control siRNA and Lipofectamine RNAiMAX for *in vitro* experiments were purchased from Invitrogen (Carisbad, CA). Mouse hepatoma AML-12 and culture medium F-12, nutrients and fetal bovine serum were obtained from ATCC (Manassas, VA).

2.3. C2 interference in vivo-strategy

For the *in vivo* experiments, an *in vivo* compatible, single siRNA duplex (predesigned siRNA, HPLC, id 63158) specific to target the C2 mRNA (NM_013484) and NT control siRNA were purchased from Invitrogen. All siRNAs were endogen free, HPLC purified, chemically modified to stabilize and abrogate any non-specific immune response and off-target effect *in vivo*. An *in vivo* delivery agent, invivofectamine (Invitrogen) was used to conjugate siRNA. C2 siRNA and NT control siRNA were dissolved in RNAse-free and endotoxin-free water at 3 μg/μl. As per the manufacturer's protocol, siRNA, complexion buffer and invivofectamine were used to make a complex that was further dialyzed in PBS and then diluted in PBS (50 μg/200 μl per mouse) for injection. C2 siRNA was also tested in a non-complex form dissolved in PBS and injected i.v. or i.p. at 50 μg in 200 μl per mouse. A smart pool C2 siRNA (Dharmacon, CO) conjugated with polymer (Transit *in vivo* gene delivery system) or TransIT LT1 from Mirus Biocorp (WI) was also tested for all *in vivo* and *in vitro* experiments.

All mice at the level of grade 1 and grade 2 were randomly assigned to two equal disease groups ($n = 10$ per group) to receive either C2 siRNA or NT control siRNA treatment. Two weeks after the 2nd booster immunization, siRNAs were injected i.p. into mice once a week for 5 weeks. A set of untreated, CFA or CFA-AChR-immunized mice were also kept as negative and positive controls in this experiment. Mice were bled thrice: 4 days prior to first treatment, once at 3 days post-first treatment (from 4 mice per group) and at termination (all mice, 5 days after 5th treatment). Serum (separated from blood cells at each time), liver, triceps muscles, and carcasses were stored frozen (−80 °C). In a parallel experiment, EAMG mice treated with C2 siRNA and control siRNA ($n = 3$) were sacrificed at 3

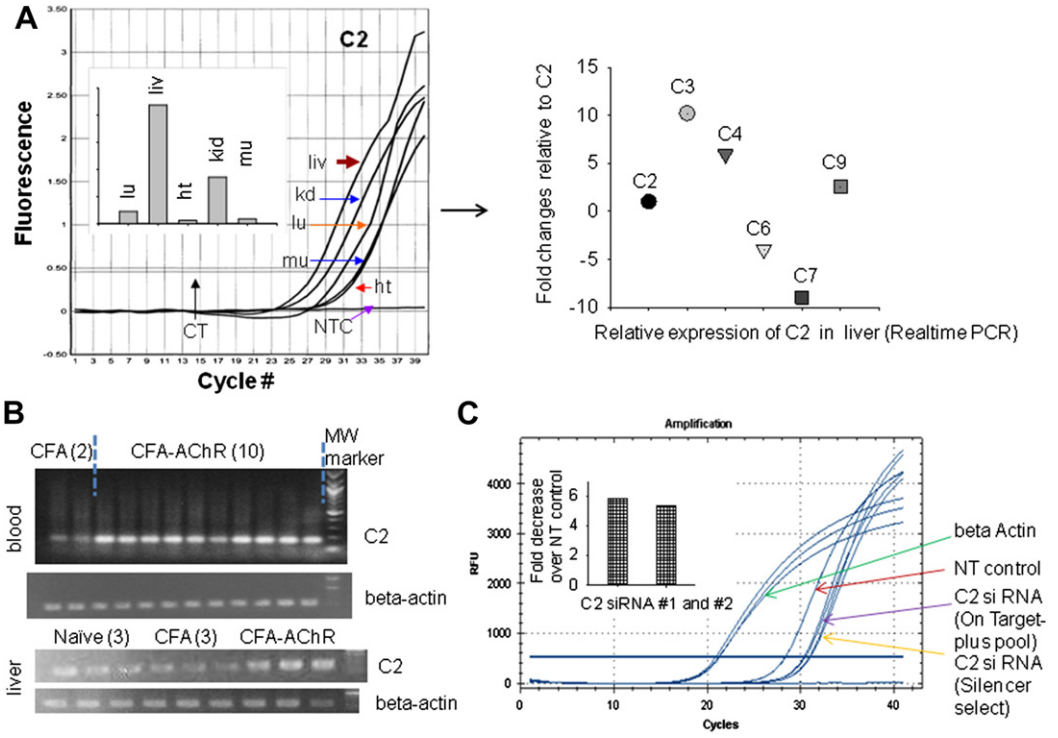

Fig. 1. R-PCR quantitation to determine C2 levels in mouse tissues, blood cells and C2 knock down in mouse hepatoma cells. (A) Left: C2 mRNA in mouse liver (liv), lung (lu), heart (ht), kidney (kd) and skeletal muscle (mu). Amplification plot indicates a higher amount of C2 cDNA/mRNA in the liver in equal amounts of starting template (cDNA) from different tissues (normalized with beta actin). NTC = no template control. Right: Fold changes in expression of complement genes relative to C2 (Real-time PCR). Error bars are too small to be seen. (B) C2 mRNA levels in blood cells and liver. Elevated levels of C2 mRNA (RT-PCR product is 192 base pairs) was evident in blood cells (top 2 panels) and liver (bottom 2 panels) of CFA-AChR immunized EAMG mice relative to CFA immunized or naïve mice following 2 booster immunizations. (MW = Molecular weight marker). (C) siRNA mediated C2 knockdown in AML-12 cells. Either vehicle (transfection reagent) or C2-specific siRNA or non-targeting control (NT) at 50 nM were added to AML-12 cells and harvested 72 h later to perform analysis of C2 mRNA inhibition relative to controls. Real-time PCR reveals 5.5- to 6-fold inhibition of C2 mRNA level (relative to NT control) with either single siRNA (#2) or pooled siRNA (#1). Each result is a representative of 3 independent experiments. CT: cycle threshold.