Virus receptor trap neutralizes coxsackievirus in experimental murine viral myocarditis

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Abstract

Objective: The coxsackie and adenovirus receptor (CAR) and the decay-accelerating factor (DAF) are receptors for coxsackievirus B3 (CVB3), which is known as the major cause of human viral myocarditis. We investigated the potential for therapeutic use of soluble virus receptor fusion proteins.

Methods: We designed and generated a novel virus receptor trap (hCAR–hDAF:Fc) consisting of both CVB3 receptors and the Fc portion of human IgG1 and evaluated its antiviral effects in experimental CVB3 myocarditis.

Results: Among four soluble virus receptor fusion proteins (hCAR:Fc, hDAF:Fc, hCAR–hDAF:Fc and hDAF–hCAR:Fc), hCAR:Fc and hCAR–hDAF:Fc in the supernatant of transfected cells neutralized echovirus, adenovirus, and various serotypes of CVB in a dose-dependent manner. Both soluble viral receptor proteins bound to the VP0 and VP1 capsid proteins of CVB3. The in vivo efficacy of viral receptor proteins was evaluated by intramuscular injection of plasmid (hCAR:Fc or hCAR–hDAF:Fc) followed by electroporation in a murine model of CVB3 myocarditis. Serum levels of the virus receptor proteins increased relative to baseline values from day 3 and peaked on day 14 at 12.9-fold for hCAR:Fc and 7.1-fold for hCAR–hDAF:Fc. The 3-week survival rate was significantly higher in hCAR–hDAF:Fc-treated mice (61%) than in hCAR:Fc-treated mice (29%) and in controls (15%; p<0.05). Myocardial inflammation, fibrosis, and myocardial virus titers were all significantly reduced in the hCAR:Fc and hCAR–hDAF:Fc groups compared to the controls.

Conclusion: Our soluble virus receptor trap, hCAR–hDAF:Fc, attenuated viral infection, myocardial inflammation, and fibrosis, resulting in higher survival rates in mice with coxsackieviral myocarditis. Furthermore, it consists exclusively of human components, and we demonstrated that this soluble virus receptor trap may be used as a potential candidate for a novel therapeutic agent for the treatment of acute viral myocarditis during the viremic phase.

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1. Introduction

Coxsackievirus B (CVB) is the most common of the pathogens that cause human viral myocarditis, which leads to severe inflammation, a heightened immune response, and is often fatal [1–3]. CVB requires the coxsackie and adenovirus receptor (CAR) and the decay-accelerating factor (DAF, CD55) for attachment to cells and internalization [4–7].

Receptor trap therapy, which uses soluble cytokine receptors or viral receptors that block the action of cytokines or the intracellular entrance of virus, has been
recently developed for the treatment of autoimmune disease or viral infection [8–13]. Therefore, we reasoned that it might be possible to block CVB infection with a trap consisting of both CVB-binding receptors. We designed a previous reported hDAF:Fc, hCAR:Fc [9,10] and novel virus receptor traps, hCAR–hDAF:Fc and hDAF–hCAR:Fc which contain two distinct binding sites, from the extracellular domains of hCAR and hDAF. Because both binding sites of two receptors occur in a separate chain, they might together facilitate high-affinity binding and therefore constitute potent antiviral effectors with therapeutic potential. We evaluated the antiviral effects of virus receptor trap hCAR–hDAF:Fc using cultured cells and an experimental animal model of viral myocarditis.

2. Methods

2.1. Preparation of plasmid DNA

cDNA encoding the soluble extracellular domains of human CAR and DAF was cloned by reverse transcription-polymerase chain (RT-PCR) reaction using total RNA prepared from HeLa cells. The following primers were used for CAR: 5′-AATTGGATCCATGGCGCTCCTGCTGTGCTT-3′, 5′-AATTGAATTCAGTTAGACGCAACA-3′. The following primers were used for DAF: 5′-AATTGAATTCATGACCGTCGCGCGGCCGA-3′, 5′-AATTGAATTCGCATTCAGGTGGTGGGCC-3′.

After confirmation of the sequences of the PCR products, CAR and DAF cDNA were cloned into an mammalian expression vector (pCK:Fc) in frame and upstream from cDNA encoding the human IgG1 Fc region, resulted in the pCK–hCAR:Fc, pCK–hDAF:Fc, pCK–hDAF–hCAR:Fc and pCK–hCAR–hDAF:Fc plasmids [14]. The plasmids were purified using EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA), and stored at −20 °C before use (Fig. 1).

2.2. Immunoblot for expression of virus receptor proteins in vitro

The expression of target proteins was confirmed by immunoblotting. Briefly, 2 μg of plasmid DNA was transfected into 293T cells and the supernatants were harvested at 72 h after transfection. Aliquots (10 μL) of supernatant were treated with SDS sample buffer, and proteins were separated using SDS-polyacrylamide gel (8%) electrophoresis. The gels were then probed with anti-human IgG antibodies conjugated with horseradish peroxidase (Sigma, St. Louis, MO) and visualized with an ECL system (Amersham, Piscataway, NJ). The concentrations of soluble virus receptor proteins in the supernatants of the transfected cells were quantified with a human IgG enzyme-linked immunosorbent assay (ELISA) kit (Bethyl Laboratories, Montgomery, TX).

2.3. In vitro antiviral assay

HeLa cells were infected with various enteroviruses (at a multiplicity of infection, MOI=1) that had been preincubated with various concentrations of four different virus receptor proteins for 30 min before infection. After 24 h of incubation, 15 μL of the cell proliferation reagent supplied with Cell Counting Kit 8 (CCK8, Dojindo Lab, Tokyo, Japan) was added, and the cells were incubated for further 2 h. Absorbance at 450 nm was measured with an ELISA reader. In preliminary experiments, maximal concentration of hDAF–hCAR:Fc in the supernatants was below 5 ng/mL which is less than detectable range of human IgG ELISA kit. Furthermore, hDAF–hCAR:Fc in that concentration could not neutralize CVB3 (105 plaque-forming units, PFU) and hDAF:Fc (150 ng/mL) could not neutralize CVB3 neither. Therefore, we compared antiviral effects of hCAR:Fc and a novel virus trap, hCAR–hDAF:Fc, through further experiments. Viruses were preincubated with both virus receptor traps (0–150 ng/mL) for 30 min at room temperature and HeLa cells were infected by these viruses (MOI=1). At 24 h later, absorbance at 450 nm was measured with an ELISA reader using CCK8. Non-infected HeLa cells were used as control and set arbitrarily to 100. Data are presented mean±S.E.M. from three independent experiments.

Adenovirus type 5 also has been known using CAR as the receptor. HeLa cells were infected with adenovirus-GFP (type 5, MOI=1) that had been preincubated with 150 and 300 ng/ml concentrations of hCAR:Fc and hCAR–hDAF:Fc proteins for 30 min before infection. Cellular lysates were collected from adenovirus-GFP infected HeLa cells at 24 h postinfection, and Western blot was performed using a
GFP polyclonal antibody. GFP expression \((n=4)\) were quantitated by densitometric analysis using imageJ (version 2.2.4) and normalized to control levels (sham-infected cells without virus receptor protein), which was arbitrarily set to 1.0.

2.4. Gene transfer by intramuscular DNA injection followed by electroporation

The protocols used in this study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). All procedures were reviewed and approved by the Animal Care and Use Committee of the Samsung Medical Center. Six-week-old inbred male BALB/c mice were anesthetized using a mixture of ketamine (100 mg/kg) and xylazine (5 mg/kg). An aliquot of 20 μL of plasmid DNA in distilled water (20 μL) was injected into the anterior tibialis muscle of each mouse through a 27-gauge needle. An aliquot of 20 μL was injected into the anterior tibialis muscle of each mouse through a 27-gauge needle. Distilled water (20 μL) was injected into the anterior tibialis muscle of each mouse through a 27-gauge needle.

2.5. Murine viral myocarditis model

CVB3 was derived from the infectious cDNA copy of the cardiotropic H3 strain (CVB3-H3) [18]. Three days after electroporation, mice were infected intraperitoneally with 10⁶ PFU of CVB3-H3. Mice were euthanized, and sera and organs were collected on days 3, 7, and 14. These mice \((n=3)\) for each day) were excluded from the survival analysis.

2.6. Histopathological analysis and organ virus titers

The apical parts of the hearts were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin–eosin or picrosirius red. Sections were graded for inflammation by two different persons, using a semi-quantitative scale of 0 to 4 [18]. Myocardial fibrosis was evaluated by computer-assisted analysis of areas stained with picrosirius red (Image-Pro Plus, Media Cybernetics, San Diego, CA). The basal parts of the hearts were homogenized in culture medium, and the supernatant virus titers were determined by PFU assay [14,18].

2.7. Immunoprecipitation to identify CVB3 capsid proteins binding sites of virus traps

We investigated whether the soluble virus traps, hCAR:Fce and hCAR–hDAF:Fc, could bind specifically to CVB3 viral capsid proteins. The coding sequences of two parts of the CVB3 capsid proteins, VP0 and VP1, were cloned into the BamHI and EcoRI restriction sites of the His-tagged expression vector, pcDNA4/HisMax C (Invitrogen, Carlsbad, CA), and then, both capsid proteins were expressed as His-tagged proteins in 293T cells. Aliquots (200 μL) of cell lysate from plasmid-transfected 293T cells were incubated with purified hCAR:Fc and hCAR–hDAF:Fc proteins (150 ng/mL) for 1 h at 4 °C. Cell lysate from untreated 293T cells was used as the control. Immunoprecipitation was performed with protein G agarose (20 μL, Santa Cruz Biotechnology, Santa Cruz, CA) in a total volume of 700 μL binding buffer (20 mM Tris–HCl, pH 8.0; 200 mM NaCl; 1 mM EDTA, pH 8.0; 0.5% NP-40) for 18 h at 4 °C with shaking. Co-precipitation was analyzed by immunoblotting using anti-His Ab (Invitrogen, Carlsbad, CA).

2.8. Dual immunofluorescent staining and transmission electron microscopy (TEM) of infected HeLa cells

The CVB3-trapping effect of the viral receptor proteins was further investigated at the cellular level by monitoring the entry of the CVB3 virus into the intracellular compartment. Briefly, HeLa cells on cover slips were infected for 30 min with CVB3 (MOI=100) that had been pre-incubated with 150 ng/mL of hCAR:Fc or hCAR–hDAF:Fc for 30 min before infection. The cells were fixed with 4% paraformaldehyde for 15 min, quenched with 50 mM NH₄Cl for 10 min, and then permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min. Cells were washed, blocked with PBS containing 2% bovine serum albumin (BSA), and incubated with anti-VP1 monoclonal antibody (1:200, NCL-ENTERO, Novocastra Laboratories, UK) for 40 min. The cover slips were washed three times with PBS, and then incubated for 30 min with Alexa Fluor 488 (1:100; Molecular Probes, Eugene, OR) and Alexa Fluor 594-phallolidin (1:200, anti F-actin, Molecular Probes). The cover slips were then washed and mounted onto slide glasses with fluorescent mounting medium. Cells were examined using a confocal microscope (LaserSharp, Bio-Rad, Hercules, CA). TEM was performed with the same condition of immunofluorescent stain. Confluent HeLa cells were cultured in 100 cm² dish. HeLa were infected for 8 h with CVB3 (MOI=10) that had been pre-incubated with 150 ng/mL of hCAR:Fc (2.5 nM) or hCAR–hDAF:Fc (1 nM) for 30 min before infection. Following 8 h of infection, the cells were washed three times with phosphate buffer saline (PBS) and were detached by Tris–EDTA solution. Detached cells were fixed in 1% glutaraldehyde for 4 h and, after washing in PBS containing 4.5% sucrose for 15 min (3×5 min), they were post-fixed in 1% osmium...
tetroxide (OsO₄) in PBS for 1 h. Afterwards they were embedded in Epon 812 and the Epon blocks were cut out with ultrathin sections (79–90 nm thick). Sections were stained with anti-VP1 monoclonal antibody and detected by gold-particle. Stained sections were examined using an electron microscope (Hitachi H7100, Tokyo, Japan).

Fig. 2. *In vitro* antiviral assay. (A) Expression of hCAR-Fc and hCAR–hDAF-Fc in cDNA transfected 293T cells is identified by immunoblot. F and P denote virus receptor fusion proteins with or without transmembrane domains, respectively. Since both virus receptor proteins with or without transmembrane domain had similar antiviral effects with same concentrations, the P structure was used in all subsequent experiments. (B) Cell survival assay revealed that both hCAR-Fc and hCAR–hDAF-Fc increased the survival of HeLa cells infected with various serotypes of CVB or echovirus 7 in a dose-dependent manner. Non-infected HeLa cells were used as control and set arbitrarily to 100. The numbers on the horizontal axis indicate CVB serotypes. Values are mean±S.E.M. from three independent experiments. (C) The dose-dependent neutralization capacity of virus receptor traps on CVB3-H3 was also validated by plaque-forming assay. The novel virus trap, hCAR–hDAF-Fc was more potent to neutralize CVB3 virus than hCAR-Fc in same concentrations.

Fig. 3. Virus traps neutralized the adenovirus-GFP (type 5) infection. HeLa cells were infected with adenovirus-GFP that was preincubated with both virus receptor traps (150 and 300 ng/mL) for 30 min at room temperature. Cellular lysates were collected at 24 h postinfection. hCAR-Fc or hCAR–hDAF-Fc significantly reduced the expression of GFP, which was measured by the number of GFP positive cells (A), and Western blot using anti-GFP polyclonal antibody hCAR-Fc or hCAR–hDAF-Fc significantly reduced expression of GFP, hCAR–hDAF-Fc neutralizes adenovirus at a 40-fold lower at the same dose (300 ng/mL) of hCAR-Fc (B). Data are presented mean±S.E.M. from three independent experiments.
Fig. 4. The effects of expressed virus receptor proteins in a murine coxsackieviral myocarditis model. (A) Expression of virus receptor proteins after intramuscular injection followed by electroporation. The serum levels of the virus receptor traps increased from day 3 and peaked at day 14. On day 14, serum levels of hCAR:Fc increased 12.9-fold (85.1 ± 1.0 ng/mL) and hCAR–hDAF:Fc increased 7.1-fold (46.8 ± 1.8 ng/mL) compared with the baseline (6.6 ± 1.2 ng/mL). Data are presented mean±S.E.M. from three independent experiments. (B) The 3-week survival rates were higher in mice treated with the virus receptor proteins. (hCAR–hDAF:Fc, 61%; hCAR:Fc, 29%; controls, 15%; p < 0.05). (C) Expressed virus receptor proteins attenuated myocardial inflammation and myocardial fibrosis. Myocardial inflammation (a–f: hematoxylin–eosin staining) and fibrosis (g–i: picrosirius red staining) were significantly reduced in the treatment groups. (D) Computer-assisted quantization of the areas of fibrosis showed significantly reduced myocardial fibrosis in the treatment groups and less fibrosis in hCAR–hDAF:Fc group than in hCAR:Fc group (p < 0.05). (E) Heart virus titers on day 3 and day 7 were significantly lower in the treatment groups than in the control (p < 0.05).
2.9. Statistics

Data are presented as mean±S.E.M. Numerical parameters were evaluated by Student’s *t*-test or analysis of variance (ANOVA). Survival rates were analyzed by the Kaplan–Meier method. Differences were considered significant at two-tailed *p*<0.05.

3. Results

3.1. In vitro expression and efficacy of virus receptor dimers

*In vitro* expression and secretion of transfected virus receptor proteins was confirmed by immunoblotting (Fig. 2A). All four soluble virus receptor proteins with or without VP0 or VP1 were expressed as His-tagged proteins in 293T cells. Purified soluble hCAR:Fc and hCAR–hDAF:Fc proteins were added to the cell lysate, and untreated cell lysate served as a negative control. After immunoprecipitation with protein G agarose, His-VP0 and His-VP1 were detected by anti-His antibody by immunoblot. The lysates of VP0-His and VP1-His transfected cells were used as a positive control, and the precipitates of virus receptor proteins without VP0 or VP1 were used as negative controls as shown in the lanes of VP0− and VP1−. (B) Intracellular entrance of CVB3 is blocked by hCAR:Fc and hCAR–hDAF:Fc. HeLa cells were fixed and immunostained at 30 min after CVB3 infection as a positive control (MOI = 100). The second panel of TEM column showed intracellular VP1 proteins were identified by primary anti-VP1 antibody and secondary Alexa Fluor 488 (green color) and gold particle for TEM (black spot, arrow). In contrast, the third and fourth panels did not show any intracellular VP1 in the HeLa cells infected with CVB3 that had pre-incubated with hCAR:Fc or hCAR–hDAF:Fc for 30 min before infection. Cells were counterstained with anti F-actin antibody (Alexa Fluor 594, red color). Immunostained scale bar = 10 μm, TEM × 100,000.
without transmembrane domain (F or P in Fig. 2A) could be detected in the supernatants after transfection. However, maximal concentration of hDAF–hCAR:Fc in the supernatants was below 5 ng/ml which is less than detectable range of human IgG ELISA kit. Moreover, hDAF–hCAR:Fc in that concentration could not neutralize CVB3, and hDAF:Fc (150 ng/ml) could not neutralize CVB3 neither (data not shown). Both virus receptor proteins with or without transmembrane domain had similar antiviral effects with same concentrations. Therefore, further experiments were performed using hCAR:Fc and a novel virus trap, hCAR–hDAF:Fc without transmembrane domain. Cell survival assays revealed that both hCAR:Fc and hCAR–DAF:Fc protected HeLa cells from cytopathic effects induced by various serotypes of CVB (types 1–6) or echovirus 7 in a dose-dependent manner (Fig. 2B and C).

Both hCAR:Fc and hCAR–hDAF:Fc significantly reduced adenovirus-GFP (type 5) infection when adenovirus infectivity was measured by the numbers of GFP positive cells (Fig. 3A), hCAR–hDAF:Fc neutralized adenovirus at a 40-fold lower with the same concentration (300 ng/ml) of hCAR:Fc when the infection rates were estimated by the GFP protein expression in Western blot (Fig. 3B).

The antiviral effect of our novel viral trap, hCAR–hDAF:Fc was more potent to neutralize CVB1, 2, and 5 virus (Fig. 2B) and adenovirus-GFP (Fig. 3) than hCAR:Fc in the same concentrations (150 and 300 ng/ml).

3.2. In vivo expression and effects of virus traps on a myocarditis model

After intramuscular plasmid injection followed by electroporation, the serum levels of the virus receptor proteins increased from day 3 and peaked at day 14. At peak, the concentration of hCAR:Fc was 12.9-fold greater (85.1 ± 1.0 ng/ml) than the baseline value (6.6 ± 1.2 ng/ml), and that of hCAR–hDAF:Fc was 7.1-fold greater (46.8 ± 1.8 ng/ml) (Fig. 4A). All electroporated mice without CVB3 infection survived more than 3 weeks before being sacrificed.

Only 15% of infected untreated mice, which consisted control group, survived until the end of the 3-week study period. However, 29% of hCAR:Fc mice (p = 0.013 versus control) and 61% of hCAR–hDAF:Fc mice (p < 0.001 versus control) survived at 3 weeks after infection (Fig. 4B).

3.3. Histopathological evaluation of inflammation and fibrosis

Myocardial inflammation on day 7 was markedly less in hCAR:Fc and hCAR–hDAF:Fc mice compared to controls (1.0 ± 0.5, 1.0 ± 0.5, 3.0 ± 0.5, p < 0.05 by ANOVA) (Fig. 4C). The area of myocardial fibrosis on day 14 significantly decreased in hCAR–hDAF:Fc mice (control, 11.6 ± 1.0%; hCAR:Fc, 5.8 ± 0.4%; hCAR–hDAF:Fc, 3.0 ± 0.2%; p < 0.05 by ANOVA) (Fig. 4D). The viral titers in the hearts also significantly decreased in the treatment groups than in the control group on day 3 (control, 6.7 ± 0.1; hCAR:Fc, 5.6 ± 0.1; hCAR–hDAF:Fc, 6.1 ± 0.1 log_{10} PFU/heart; p < 0.05 by ANOVA). Heart viral titers on day 7 were much less than those on day 3 in all groups, but those in the treatment groups were still significantly less compared to control group (control, 2.4 ± 0.2; hCAR:Fc, 0.0 ± 0.1; hCAR–hDAF:Fc, 1.7 ± 0.2 log_{10} PFU/heart; p < 0.05 by ANOVA) (Fig. 4E).

3.4. hCAR:Fc and hCAR–hDAF:Fc proteins bind directly to viral capsid proteins, and block intracellular entry of CVB3

Next, we investigated the specific viral capsid protein-binding sites of our viral receptor proteins. Immunoblot analysis revealed that VP0 and VP1 capsid proteins, which are expressed as His-tagged proteins in 293T cells and examined after immunoprecipitation with hCAR:Fc or hCAR–hDAF:Fc, is strongly detected by anti-His monoclonal antibody (Fig. 5A). The lysates of VP0-His and VP1-His transfected cells were used as a positive control and the precipitates of virus receptor proteins without VP0 or VP1 were used as negative controls (Fig. 5A, lanes of VP0– and VP1–). These results demonstrated that soluble virus receptor proteins bind directly to the VP0 and VP1 capsid proteins of CVB3, resulting in neutralization of CVB3 infectivity.

To confirm whether our viral receptor proteins could inhibit the intracellular entry of CVB3, we detected VP1 viral capsid protein in the CVB3 infected HeLa cells using immunofluorescent stain and TEM. Cells infected with wild type CVB3 showed the immunostained VP1 proteins in the cells. In contrast, cells infected with pre-incubated CVB3 with virus receptor proteins did not show any intracellular VP1 proteins (Fig. 5B). These results suggested that both hCAR:Fc and hCAR–hDAF:Fc can trap viral particles and block the entry of virus into the cells.

4. Discussion

Our study showed that the soluble virus receptor proteins, produced by transfection of plasmids, were able to neutralize many different serotypes of coxsackieviruses in vitro. Moreover, the expressed soluble virus receptor proteins exhibited a therapeutic effect on the in vivo murine coxsackieviral myocarditis model. Expressed hCAR:Fc and hCAR–hDAF:Fc in the sera of mice inhibited primary and/or secondary infection in the heart, decreased myocardial inflammation, prevented subsequent fibrosis, and improved the survival rate.

It is well known that CVB3 requires CAR and DAF for attachment to the cell and internalization [5]. Recently, DAF has known to be essential for primary virus binding and for internalization using caveolin [7]. Co-expression of DAF in Chinese hamster ovary cells expressing CAR aggravated the cytopathic effects of CVB3 [19], and monoclonal antibodies
to CAR or DAF successfully blocked virus infection in susceptible cells \textit{in vitro} and \textit{in vivo} [6,20]. Similarly, previous reports showed soluble virus receptor proteins that expressed CAR or DAF also attenuated virus infection and tissue inflammation [9,10]. Furthermore, simultaneous administration of antibody to CAR and DAF had a synergistic inhibitory effect on CVB3 [21]. Therefore, a soluble viral receptor trap consisting of both CVB-binding receptors (hCAR–hDAF:Fc) might effectively block CVB infection because the presence of two different binding sites in this virus receptor trap may facilitate high-affinity binding. It has been reported that the effective concentrations of virus traps to neutralize $10^4$ PFU of CVB3 was 75 $\mu$g/ml (75,000 ng/ml) for hCAR and to 150 $\mu$g/ml (150,000 ng/ml) for hDAF [9,10,22]. In contrast, only 150 ng/ml of hCAR–hDAF:Fc was sufficient to neutralize $10^4$ PFU of CVB3 in our experiments, demonstrating that our novel virus trap hCAR–hDAF:Fc has higher affinity to bind CVB3 than hCAR:Fc or hDAF:Fc. As shown in Fig. 2B, both virus receptor trap in our experiment could neutralize all serotypes of CVB, particularly our novel virus receptor trap hCAR–hDAF:Fc, was significantly increased cells survival at CVB1, 2, and 5 infection, which have been known to use both CAR and DAF as its receptors [20]. In a recent report [22], the concentrations of hCAR:Fc required to neutralize $10^4$ PFU of CVB5 was more than 10 nM (600 ng/ml). Therefore, it may be possible that the concentration (150 ng/ml) of hCAR:Fc was not enough to neutralize CVB5 in our experiment. In previous report [10], the concentration of hCAR:Fc to neutralize CVB3 was 150 $\mu$g/ml (2.5 $\mu$M), however, in our experiment 150 ng/ml (1 nM) of hCAR–hDAF:Fc was sufficient to neutralize $10^4$ PFU of CVB3 (Figs. 2C and 5), demonstrating that our novel virus trap has higher affinity to bind CVB3 than hCAR:Fc.

Intermittent direct administration of the soluble viral receptor proteins, CAR or DAF, suppressed coxsackieviral myocarditis and pancreatitis [9,10]. However, the effect on survival or the \textit{in vivo} presence of viral receptor proteins was not validated. Therefore, our strategy was to design a simple single-injection viral receptor trap using cDNA, and cloned into a mammalian expression vector, and then electroporation to enhance gene expression [15–17]. This resulted in the sustained \textit{in vivo} expression of viral receptor trap protein, which peaked at day 14. In addition, we also found that the serum concentration of virus receptor protein decreased at day 3. This was coincident with the time at which the CVB3 viral titer began to decline. The serum concentration of virus receptor proteins recovered after day 3, when the viral titer began to decline [9,10]. These data suggested that virus receptor trap plasmid injection followed by electroporation was increased a half life [23] of virus receptor proteins in mice serum, which can continuously block of virus re-infection at day 7 or more.

Also, we found that hCAR–hDAF:Fc neutralizes adenovirus at a 40-fold lower dose than the dose of hCAR:Fc required eliciting the same effect (Fig. 3). In addition, hCAR–hDAF:Fc trap showed better \textit{in vivo} efficacy, with significantly less inflammation and fibrosis, and lower mortality than did hCAR:Fc protein, despite its lower concentration in serum. Our results suggest that the combination of two distinct virus receptors in our novel virus receptor trap, hCAR–hDAF:Fc, may act synergistically to create a more powerful virus-blocking effect than the single virus receptor protein, hCAR:Fc. Direct binding of soluble CAR protein to CVB3 was recently reported, however, the binding site of CAR was not identified yet [11]. The three-dimensional structure of the virus receptor complex consisting of CAR-CVB3 and DAF-echovirus has been determined, and suggests that viral capsid proteins VP2 and VP3 are binding sites for DAF [24,25]. To identify the fundamental mechanism responsible for the virus-blocking effect of soluble viral receptor trap protein, we investigated the direct binding site of CVB3 capsid proteins. The specific binding of both hCAR:Fc and hCAR–hDAF:Fc to VP0 and VP1 viral capsid proteins was clearly confirmed by immunoprecipitation and immunoblotting. Although both of our virus traps could bind directly to the VP0 and VP1 capsid proteins, we could not identify the exact mechanism whether the protection of hCAR–hDAF:Fc protein is due to blocking virus or to other immunological effects. Furthermore, it has been known that the endothelial cell DAF is up-regulated by TNF-α and VEGF, and DAF inhibits complement binding [26]. And the levels of CAR expression is regulated by inflammatory cytokines such as TNF-α and IFN-γ in endothelial cells [27]. These data support that our soluble
The virus receptor trap may have some interactions with inflammatory cytokines that has expressed during early phase of viral myocarditis. However, in this experiment, the interactions between virus traps and cytokines had not been studied in vivo study.

Current management of human viral myocarditis depends on supportive therapy for systolic dysfunction [1]. Inhibition of viral infection, a major contributor to early direct myocardial damage, may be very effective during the viremic phase [28]. Because of the rapid deteriorating and fatal nature of human viral myocarditis, blocking viral infection and/or neutralizing the virus particles in the sera should be initiated as soon as possible. We have shown that 150 ng/ml of our novel virus receptor trap, hCAR–hDAF:Fc was enough to neutralize CVB3 in vitro, and that hCAR–hDAF:Fc can be effectively transfected into skeletal muscle by electroporation in vivo and the expressed hCAR–hDAF:Fc could reduce mortality in a murine viral myocarditis model. Although hCAR–hDAF:Fc consists exclusively of human components, it is very difficult to express the virus receptor traps at the right time and with the proper concentrations by the electroporation, and the injection of purified virus traps seems not to be practical. Further experiments are needed to determine the effective concentrations using purified, autologous but modified virus trap proteins and to identify the systemic adverse effects including autoimmunity. Nevertheless, in this study, we demonstrated that our novel virus receptor trap, hCAR–hDAF:Fc may be used as a potential candidate for novel therapeutic agent for the treatment of acute viral myocarditis during early viremic phase.

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