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Expression of coxsackievirus and adenovirus receptor (CAR)-Fc fusion protein in *Pichia pastoris* and characterization of its anti-coxsackievirus activity

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ABSTRACT

Coxsackievirus and adenovirus receptors (CARs) are the common cellular receptors which mediate coxsackievirus or adenovirus infection. Receptor trap therapy, which uses soluble viral receptors to block the attachment and internalization of virus, has been developed for the inhibition of virus infection. In this study, we have constructed a pPIC3.5 K/CAR-Fc expression plasmid for the economical and scale-up production of CAR-Fc fusion protein in *Pichia pastoris*. The coding sequence of the fusion protein was optimized according to the host codon usage bias. The amount of the CAR-Fc protein to total cell protein was up to 10% by 1% methanol induction for 96 h and the purity was up to 96% after protein purification. Next, the virus pull-down assay demonstrated the binding activity of the CAR-Fc to coxsackievirus. The analyses of MTT assay, immunofluorescence staining and quantitative real-time PCR after virus neutralization assay revealed that CAR-Fc could significantly block coxsackievirus B3 infection *in vitro*. In coxsackievirus B3 infected mouse models, CAR-Fc treatment reduced mortality, myocardial edema, viral loads and inflammation, suggesting the significant virus blocking effect *in vivo*. Our results indicated that the *P. pastoris* expression system could be used to produce large quantities of bioactive CAR-Fc for further clinical purpose.

1. Introduction

Coxsackievirus B (CVB) and adenovirus (AdV) are the most common pathogens that can cause viral myocarditis. However, both CVB (all six serotypes) and AdV (serotypes 2 and 5), which belong to divergent virus families, rely on coxsackievirus and adenovirus receptors (CARs) for cell attachment and internalization (Yanagawa et al., 2004). In addition to acting as the viral receptors, the CARs have been implicated in other distinct biological roles, such as cell adhesion proteins (Bruning and Runnebaum, 2003) and signal transduction molecules (Hauwel et al., 2005). CAR is a 46-kDa integral membrane protein that possesses two extracellular immunoglobulin-like domains (D1 and D2), a transmembrane domain, and a cytoplasmic domain (Jiang and Caffrey, 2007). The major structure which mediates virus infection is the extracellular domain: the N-terminal D1 domain, which is responsible for the binding to the canyon structure of CVB capsid or the fiber knob protein of AdV (Pinkert et al., 2009).

Recently, receptor trap therapy, which uses the soluble viral receptors to block the attachment and internalization of virus, has been developed as a treatment for virus infection (Asher et al., 2005; Goodfellow et al., 2005; Pinkert et al., 2009). Previous studies have shown that the soluble CAR protein expressed in the prokaryotic expression system could suppress CVB3 infection *in vitro* and reduce the myocardial CVB3 titer *in vivo* (Dörner et al., 2006, 2004). CARs, which are coupled to human IgG1 Fc fragments and expressed in a CHO expression system have demonstrated the protective efficacy against CVB3 infection *in vitro* and against myocardial and pancreatic injury *in vivo* (Lim et al., 2006; Yanagawa et al., 2004). But these expression systems are insufficient for large scale production of highly bioactive proteins with some unresolved issues, *i.e.*, the refolding of inclusion bodies, post-translational modification of proteins, elimination of bacterial endotoxins (*Escherichia coli*); the high cost of large-scale production, difficult manipulation, efficiency and safety issues (mammalian cells) (Daly and Hearn, 2005; Pinkert et al., 2009).

Therefore, to achieve scale-up production of this functional virus receptor protein in *P. pastoris* GS115 strain, a CAR-Fc fusion gene, which includes the extracellular domains of human CAR and the IgG1 Fc fragment, was designed. To facilitate the expression of the fusion protein in *P. pastoris*, the coding sequence of the

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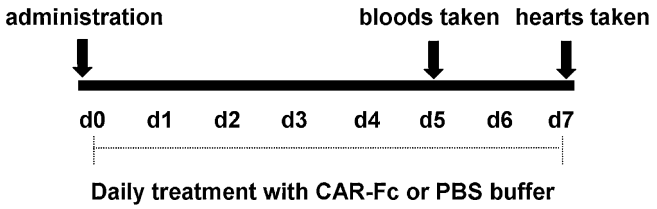

Fig. 1. The CAR-Fc treatment scheme and time line. Mice were divided into 4 groups. The virus-infected mice were intraperitoneally injected with CVB3 on day 0, followed by intravenous injection of PBS daily. The mice in CAR-Fc treated group were intravenously injected with CAR-Fc concurrent with CVB3, and were treated with CAR-Fc daily until the day of sacrifice. The mice in the normal and CAR-Fc control group were intravenously injected with PBS or CAR-Fc buffer respectively for 6 days.

recombinant protein was optimized according to the host codon usage bias. And the antiviral activity of the recombinant protein was evaluated using cultured cells and CVB3 infected mouse models.

2. Materials and methods

2.1. Strains, plasmids, media and animals

Details for strains, plasmids, media and animals used in this report were listed in supplementary materials.

2.2. Codon optimization of CAR and IgG1 Fc gene sequences

To facilitate the expression of CAR-Fc fusion protein, the genes coding (approximately 1.35 kb) for the extracellular domains of human CAR (GenBank accession no. NM_001338) and the IgG1 Fc fragment (h-CH2-CH3 moieties, GenBank accession no. AF237583) were codon optimized according to the *P. pastoris* codon usage bias.

2.3. Construction, transformation and expression of recombinant yeasts

The detailed procedures of construction of the recombinant plasmid pPIC3.5 K/CAR-Fc, expression of the CAR-Fc and protein purification and identification were included in supplementary methods.

2.4. Virus pull-down assay

An equal amount of 5 μg of CVB3 and purified CAR-Fc diluted in 0.01 M PBS (2 mM DTT, 0.1 mM PMSF, pH 7.4) were incubated at 4 °C for 2 h. And then 10 μl protein A-agarose (Santa Cruz, USA) was added to the mixture and incubated overnight at 4 °C on a rocker platform. Precipitates were subjected to western blot with mouse anti-CVB3 VP1 and successively with goat anti-human IgG antibodies (Zhongshan, China).

2.5. Virus neutralization assay with CAR-Fc in vitro

The virus neutralization effect of CAR-Fc *in vitro* was investigated with MTT assay and immunofluorescence staining of virus particles according to the protocols described in supplementary methods.

2.6. Virus infection in vivo

Eight-week-old BALB/c mice were randomly assigned to one of four groups; each group consisted of 10 mice. The detail of the administration schedule was presented in Fig. 1. To obtain more specimens, tissues of the dying mice were harvested before they died.

Table 1
Primer sequences used for PCR amplification.

Primer	Sequence
CAR forward primers (CAR FP) [a]	TATA**TACGTA**ACCATGTTGTCCATCACTACTCCAG
Fc reverse primers (Fc RP) [b]	TATTTATT**GCGGCCGC**TCACTTACCTGGGGACAAGG
5′AOX primer	GACTGGTTCCAATTGACAAGC
3′AOX primer	GCAAATGGCATTCTGACATCC
CVB3 (VP1 region) forward primer	AACTCAGGTGCCAAGCGGTATGCTG
CVB3 (VP1 region) reverse primer	TTGGTGTGTTAGGATCTGTGC
β-Actin forward primer	CGGGAAATCGTGCGTGAC
β-Actin reverse primer	GAAGGAAGGCTGGAAGAGTG

[a] The *SnaB* I restriction site is shown in bold, and the Kozak sequence is underlined.
[b] The *Not* I restriction site is shown in bold, and the stop codon is underlined.

2.7. Inhibition of CVB3 replication by CAR-Fc in vitro and in vivo

The inhibition of CVB3 replication by CAR-Fc *in vitro* and *in vivo* was measured with quantitative RT-PCR, as described in supplementary methods.

2.8. Myocardial viral titer assay

To detect the myocardial viral titer in the CVB3-infected and CAR-Fc treated mice, the heart tissues were homogenized in serum-free DMEM, followed by 10-fold serial dilution. The 50% tissue culture infective dose (TCID50) was calculated by the Reed–Muench method (Reed and Muench, 1938). The virus titer was expressed as $\log_{10}$ TCID50/mg tissue.

2.9. Myocardial lesion and serum zymogram evaluation

Hearts were embedded in Optimal cutting temperature (OCT) compound and snap frozen in liquid nitrogen. The frozen section was fixed in ice cold acetone and stained with hematoxylin and eosin. The percent area of cellular inflammation and myocardial necrosis was calculated by the method described else-where (Hua et al., 2009). To further measure the severity of myocardial damage, the serum contents including creatine kinase (CK), creatine kinase-MB fraction (CK-MB), lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and α-hydroxybutyrate-dehydrogenase (α-HBDH), were measured by automatic biochemistry analyzer Hitachi 7170S (Hitachi, Japan).

2.10. Statistical analysis

Data are presented as mean ± SEM as indicated in the figure legends. Statistical analysis was performed by student's *t*-test or ANOVA followed by Fisher's least significance difference test (LSD). A value of $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. Construction and screening of recombinant yeasts

As compared with the original genes of CAR and Fc, the rare codons such as AGG, CGA, CGT (coding for arginine); CCG, CCC (coding for proline); CTC (coding for leucine) and ACG (coding for threonine) were optimized according to the host codon usage bias (supplementary Fig. S1 and Table S1). The pPIC3.5 K/CAR-Fc was constructed (the diagram was shown in supplementary Fig. S2a) and identified by PCR and sequencing (data not shown). The positive transformants confirmed by PCR using 5′AOX/3′AOX primers (Table 1), produced a 1.57-kb product containing a 1.35-kb CAR-Fc coding sequence and 220-bp flanking sequence from pPIC3.5 K