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Co-Expression of Human Complement Regulatory Proteins DAF and MCP with an IRES-Mediated Dicistronic Mammalian Vector Enhances Their Cell Protective Effects

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Abstract—C3 convertase regulatory proteins, decay accelerating factor (DAF, CD55) and membrane cofactor protein (MCP, CD46), have complementary function and transfected into non-human cells might confer protection against human complement. This may be an effective strategy to alleviate C-mediated cell damage by combining the two activities. In this study, we constructed a dicistronic mammalian expression vector pcDNA3-MCPIRESDAF using the internal ribosomal entry sites (IRES) of the encephalomyocarditis virus (EMCV), and stable cell lines were obtained by G418 screening. Integration of extraneous genes was identified by PCR. RT-PCR and Western blotting analysis demonstrated that the EMCV IRES allowed for efficient co-expression of hMCP and hDAF in NIH3T3 cells stably transfected with pcDNA3-MCPIRESDAF. Human complement-mediated cytolysis assays showed that co-expressed DAF and MCP proteins could provide more significant protection against complement-mediated cytolysis than either hMCP or hDAF alone. These results suggest that DAF and MCP synergize the actions of each other, and the IRES-mediated polycistronic vector should improve the efficiency and effectiveness of multi-gene delivery. The pcDNA3-MCPIRESDAF vector has potential therapeutic value for effectively controlling complement activation, thereby increasing the possibility of inter-species transplantation.

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Key words: complement regulatory proteins, internal ribosome entry site (IRES), dicistronic expression vector, co-expression, hyperacute rejection

The activation of complement encompasses a series of initiation, amplification, and lytic steps and their discrete reaction, which is normally regulated by complement regulatory proteins (CRPs), such as the decay accelerating factor (DAF, CD55) and the membrane cofactor protein (MCP, CD46) [1, 2]. DAF acts by dissociating the C3 and C5 convertases in both the classical and alternative pathways through its ability to bind C4b and C3b [3, 4], while MCP serves as a cofactor for the factor I-mediated cleavage of C3b and C4b [5]. These two membrane regulators complement each other in function. Therefore, transfer and coordinated expression of the two human regulatory protein molecules is an important requirement for alleviating C-mediated cell damage.

In recent years, polycistronic vectors have been applied to obtain complex gene transfer and therapy effects in a range of gene therapy protocols, including immune gene therapy of cancer [6-9]. The internal ribosome entry site (IRES) derived from the 5′-nontranslated regions of the encephalomyocarditis virus (EMCV) genome has high translation efficiency and is functional in a variety of cultured cell types, so EMCV IRES vector is widely used to express multiple proteins from a sin-

Abbreviations: CMV) cytomegalovirus; CRP) complement regulatory protein; DAF) decay accelerating factor; DMEM) Dulbecco's modified Eagle's medium; EMCV) encephalomyocarditis virus; IRES) internal ribosome entry site; MCP) membrane cofactor protein; MTT) 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NHS) normal human serum.

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gle transcriptional unit in gene therapy and gene transfer experiments [10-12].

In the present study, we constructed the eukaryotic expression vector pcDNA3-MCPIRESDAF using the IRES elements from EMCV, which was designed to simultaneously express human MCP and DAF of hCRPs within the same cell. We assessed the synergistic action of human MCP and DAF to determine the effective combination for avoiding complement-mediated cytolysis toward xenogeneic cell membrane.

MATERIALS AND METHODS

Cell culture. Mouse fibroblast cells (NIH3T3) were obtained from the Chinese Type Culture Collection (Wuhan, P. R. China) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated fetal calf serum (FCS, 10% v/v). Cultures were maintained at 37°C in the presence of 5% CO_2.

Vector construction. The construct pcDNA3-MCPIRESDAF was made using standard recombinant DNA techniques [13]. The vector pcDNA3-MCP has been previously described [14]. It contains the *hMCP* gene with its own CMV promoter and a selectable neomycin marker gene. The vector pcDNA3-MCPIRESDAF was made in several steps. The 649 bp IRES of ECMV was amplified by PCR using the vector pIRES2-EGFP (BD Biosciences Clontech, USA) as templates, with an *Xho*I site at the 5′ end and a *Spe*I site at the 3′ end. The 1684 bp hDAF cDNA was PCR-amplified containing a *Spe*I site at 5′ end and an *Apa*I site at 3′ end using plasmids pcDNA3-DAF as templates. They were digested with appropriate restriction enzymes and purified using standard procedures. The resulting hDAF and IRES fragments were then subcloned into the pcDNA3-MCP vector opened by *Xho*I and *Apa*I.

Transfection and selection of stable transfectants. NIH3T3 cells were transfected with pcDNA3, pcDNA3-DAF, pcDNA3-MCP, or pcDNA3-MCPIRESDAF using the calcium phosphate precipitation method [13]. The transfected NIH3T3 cells were maintained in DMEM plus 10% FCS in an atmosphere of humidified 5% CO_2/95% O_2. Stable transfectants were selected in 400 μg/ml G418 (Gibco-BRL, USA). To verify the stable integration of these extraneous genes, the total genomic DNA was extracted from the transfected clones and used as templates for PCR.

RT-PCR analysis of coordinated expression of human MCP and DAF in stable transfectants. The expressions of transfected genes in NIH3T3 cell transfectants were demonstrated by RT-PCR. Total RNA was isolated from transfected NIH3T3 cells using Trizol® LS Reagent (Gibco-BRL) and treated with RNase-free DNase I (Promega, USA). First strand cDNA was synthesized using a reverse transcriptase (RT) according to the manufacturer's instructions (Gibco-BRL). Expression of hDAF was measured by RT-PCR using the following primers: Fw 5′-CGACTAGTGCGTCCTTGTTCTAACCCG-3′, Rv 5′-ACTGGGCCCTTGCTCTGTTGACATTCC-3′. The expected PCR product was 1336 bp long. The primers used to detect the expression of hMCP were: Fw 5′-CATATGACAGCGTCTTCCGC-3′, Rv 5′-CAGCTGCATTCATGAGAGTG-3′. The expected product was a 1233 bp fragment. The IRES primers Fw 5′-ACTCAGATCTCGAGCTCAAGCTTCG-3′ and Rv 5′-CCACTAGTGTGGCCATATTATCATC-3′ were used to detect IRES expression at the mRNA level and the expected product was a 649 bp fragment. The primers used to detect the whole ORF and IRES were: Fw 5′-CATATGACAGCGTCTTCCGC-3′, Rv 5′-ACTGGGCCCTTGCTCTGTTGACATTCC-3′. The expected PCR product was 3218 bp long.

Western blot analysis. Cells were harvested, lysed, and boiled in Laemmli buffer [15]. On tenth of each lysate was loaded and migrated by SDS-PAGE (10%), and then analyzed by Western blotting [16]. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (TTBS) (10 mM Tris, 0.15 M NaCl, 0.1% Tween 20) for 1 h at 37°C, incubated with anti-hMCP mouse mAb E4.3 (Antibody Diagnostica Inc., USA) or anti-hDAF mouse mAb BRIC216 (IBGRL, UK) at dilution 1 : 100 in TTBS for 2 h at 37°C, washed three times for 10 min each in TTBS, and then incubated for 1 h with rabbit anti-mouse horseradish peroxidase-conjugated antibodies (Zhongshan Biotechnology, P. R. China) at room temperature. The color reaction was carried out by incubation in 0.1 M Tris-HCl, pH 6.8, containing 0.5 mg/ml diaminobenzidine (Sigma, USA) and 0.01% H_2O_2.

Human complement-mediated cytolysis assays. NIH3T3 cell transfectants were incubated with the anti-NIH3T3 anti-serum at a dilution of 500 : 1 and 25 and 50% normal human serum (NHS) as a source of complement. Amelioration of complement-mediated lysis by the transfectant molecules was determined by an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay [17]. NIH3T3 cell transfectants were seeded onto microtiter plates at $1 \cdot 10^4$ cells/well in 100 μl DMEM containing 10% FCS, and were incubated for 16 h with 5% CO_2 at 37°C. The target NIH3T3 cells were incubated in 25 and 50% NHS at 37°C for 1 and 5 h in 96-well flat-bottomed microplates, and then the supernatant was discarded. The NIH3T3 cells were washed twice in 100 μl PBS. Cell viability was analyzed by a colorimetric assay (MTT assay), in which the complete solubilization of the purple formazan crystals was measured based on the spectrophotometric absorbance of the samples measured using a microtiter plate (ELISA) reader with a test wavelength of 570 nm. Cell viability was determined by the following formula: percent viability = (absorbance in the sample/absorbance in the negative control) × 100%. As a negative control, mouse serum was used instead of NHS.

Trypan blue exclusion was also employed for assessment of C-mediated NIH3T3 cell cytotoxicity.

Statistical analysis. All data are shown as the mean ± standard deviation (SD) of the mean. Group comparisons were performed with one-factor analysis of variance and paired T-test when appropriate. Data with calculated P values of <0.05 were considered to be significant.

RESULTS AND DISCUSSION

Transfection of NIH3T3 cells with vector pcDNA3-MCPIRESDAF. To express high levels of hMCP and hDAF simultaneously, recombinant expression vector pcDNA3-MCPIRESDAF was constructed. The structure of the dicistronic expression vector is shown in Fig. 1a. The pcDNA3-MCPIRESDAF vector was amplified in *Escherichia coli* DH5α. DNA sequencing confirmed the polycistronic vector was constructed successfully. The double hCRPs were co-expressed in NIH3T3 cells by transfection, followed by selection of stable transfectants in the presence of G418. As shown in Fig. 1b, integration of human MCP into the chromosomal DNA of transfected cells was ascertained by an MCP-specific 1223 bp (lane *12*), an EMCV IRES-specific 649 bp (lane *8*), and a DAF-specific 1330 bp (lane *4*) DNA fragment when the total genomic DNA of pcDNA3-MCPIRESDAF transfectants were used as templates. In contrast, nonspecific product was detected when the total genomic DNA of NIH3T3 pcDNA3 transfectants (lanes *3*, *7*, and *11*) or NIH3T3 (lanes *2*, *6*, and *10*) was the template.

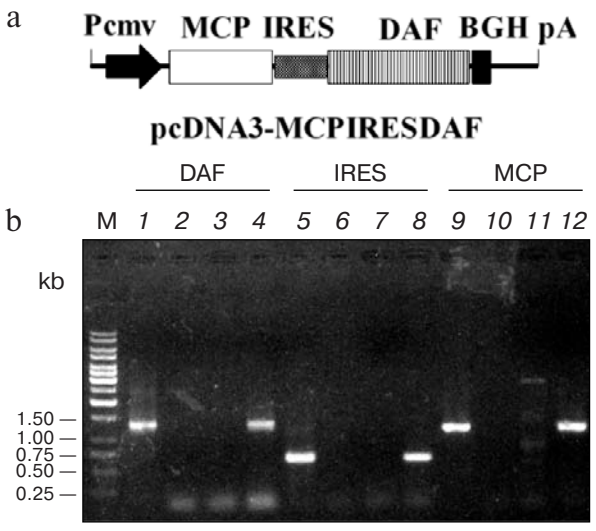

Fig. 1. a) Schematic representation of the pcDNA3-MCPIRES-DAF dicistronic plasmids used for this work. The cDNAs encoding human MCP and DAF separated by the EMCV IRES were placed under the control of the CMV promoter (PCMV). The plasmid also contained the neomycin gene (BGH) for selection of transfected cells and polyadenylic acid (pA). b) PCR analysis of the integration of human MCP, DAF, and EMCV IRES on the stable pcDNA3-MCPIRESDAF transfectants. The templates used for PCR were: *1*, *5*, *9*) DNA of the plasmid pcDNA3-MCPIRESDAF as positive controls; *2*, *6*, *10*) genomic DNA of NIH3T3 cells; *3*, *7*, *11*) genomic DNA of NIH3T3 pcDNA3 transfected cells; *4*, *8*, *12*) genomic DNA of NIH3T3 pcDNA3-MCPIRESDAF transfected cells; M, 1 kb DNA ladder marker.

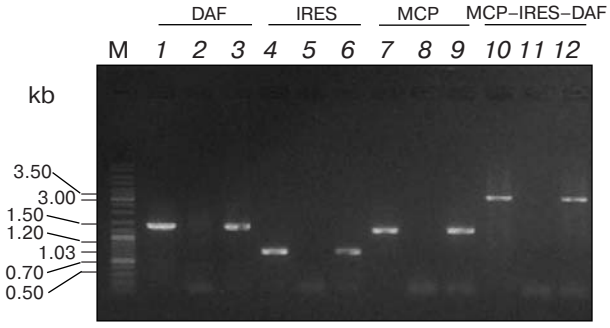

Fig. 2. RT-PCR analysis of human MCP and DAF cDNAs and EMCV IRES reveals mRNA expression in stably transfected NIH3T3 cells. The templates used for PCR were: *1*, *4*, *7*, *10*) DNA of plasmid pcDNA3-MCPIRESDAF as positive controls; *2*, *5*, *8*, *11*) cDNA of NIH3T3 pcDNA3 transfected cells; *3*, *6*, *9*, *12*) cDNA of NIH3T3 pcDNA3-MCPIRESDAF transfected cells; M, GeneRuler™ DNA Ladder Mix.

Co-expression of human MCP and DAF in mammalian cell lines. To ascertain whether hDAF, IRES, and hMCP were co-expressed at the mRNA level in the stable pcDNA3-MCPIRESDAF transfectants, RT-PCR was carried out with primers specific for the three sequences. As shown in Fig. 2, with a 1223 bp DNA fragment specific for human MCP (lane *9*), 649 bp PCR products for IRES of EMCV (lane *6*), 1330 bp DNA products specific for human DAF (lane *3*), and 3218 bp PCR products for human MCP cDNA, the EMCV IRES and human DAF cDNA (lane *12*) were detected in the transfectant pcDNA3-MCPIRESDAF, while no specific PCR products were observed in pcDNA3 control transgenic samples.

The EMCV IRES in an expression vector permits the translation of two open reading frames from one mRNA in the transfected cells. To verify this, Western blotting analysis of cell lysates from pcDNA3-MCPIRESDAF, pcDNA3-MCP, pcDNA3-DAF, and pcDNA3 transfectants under reducing conditions was carried out using mouse monoclonal antibodies specific for human DAF and MCP, respectively. The results in Fig. 3 show that a 75-kD band for human DAF and 68-kD (upper) and 45-kD (lower) bands for MCP, respectively, were present in the lysate. In contrast, no specific bands for human MCP or DAF were observed in the vector control-transfected cells. These results demonstrated that human MCP and DAF molecules were expressed simultaneously on the NIH3T3 cells stably transfected with pcDNA3-MCPIRESDAF.

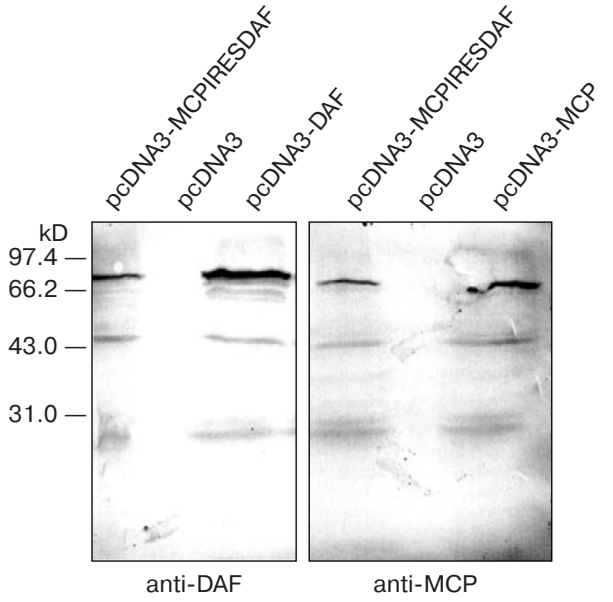

Fig. 3. Western blot analysis confirms the co-expression of human DAF and MCP proteins in pcDNA3-MCPIRESDAF transfectant. Extracts of pcDNA3, pcDNA3-MCP, and pcDNA3-DAF and pcDNA3-MCPIRESDAF transfectants were electrophoresed by SDS-PAGE on a 10% gel and blotted onto nitrocellulose membrane. Blots were probed with monoclonal anti-MCP or anti-DAF antibodies and horseradish peroxidase (HRP)-linked secondary antibody.

Xenogeneic host cells are more protected from human complement-mediated cytolysis by cooperation of human DAF and MCP. To assess the efficacy of our combinatorial genetic approach in NIH3T3 cells, the sensitivity to C-mediated cytolysis was compared between those transfected with the vector pcDNA3, single DAF or MCP, and double (DAF + MCP) CRPs when challenged with human serum. As a result, cellular damage of the NIH3T3 was stronger in those samples incubated with 50% NHS than those incubated with 25% NHS, but without any significant difference. NIH3T3 incubated for 5 h with 25 and 50% NHS showed stronger cell injury (73.5 ± 1.7 and 76.2 ± 1.0%) than those incubated for 1 h (68.8 ± 2.1 and 73.3 ± 0.6%, respectively), with no significant difference (Fig. 4). For these results, 25% NHS was used for further experiments. NIH3T3 cells stably transfected with pcDNA3-MCPIRESDAF showed cellular viabilities 57.0 ± 0.5%, each showing a significant difference when compared with groups 2 (control vector pcDNA3), 3 (single MCP), and 4 (single DAF) ($P < 0.01$, $P < 0.01$, and $P < 0.05$, respectively), which showed cellular viabilities 31.1 ± 2.1, 36.1 ± 1.1, and 47.2 ± 1.9%, respectively (Fig. 5). In addition, NIH3T3 cells stably transfected with single CRP or double CRPs, in groups 3 (single MCP), 4 (single DAF), and in group 5 (MCP + DAF) showed a significant difference when compared with group 2 ($P < 0.05$, $P < 0.05$, and $P < 0.01$, respectively) (Fig. 5). Similar results were obtained using Trypan blue staining (data not shown). These results demonstrated that human MCP and DAF could protect the host cells from C-mediated cytolysis after transfection of NIH3T3 cells, and that both proteins together work better than either one alone. Our results using IRES mediated co-expression are thus consistent with earlier findings using co-transfection of different plasmids to express both proteins [14, 18, 19]. The combined application of different hCRPs is promising for achieving total complement inhibition and high-level co-expression of multiple hCRPs, which is necessary in gene therapy to resist xenograft hyperacute rejection [18-20].

To bring together the complementary activities of these two regulatory molecules, DAF and MCP, a membrane-bound bifunctional chimeric complement inhibitor was created that possessed both factor I cofactor activity and decay accelerating activity [21-23]. The soluble MCP–DAF hybrid (CAB-2) was also developed, which possessed both membrane cofactor and decay accelerating activities and inactivated both classical and alternative C3 and C5 convertases *in vitro* as measured by assays of inhibition of cytotoxicity and anaphylatoxin generation [24]. However, the fusion molecule of different natural proteins represents novel epitopes, which is likely to trigger an immune response; therefore, the potential immunogenicity may limit its clinical applications.

Transgenic animals expressing multiple hCRPs have been generated by crossbreeding of single transgenic animal lines [25, 26] or by co-microinjection of separate constructs for hCRPs [18, 27]; however, the screening procedures are expensive, tedious, and time-consuming.

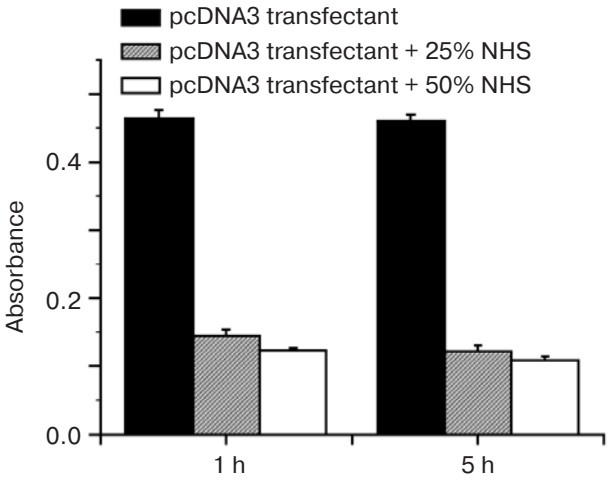

Fig. 4. Analysis of cellular damage of mouse fibroblast NIH3T3 cells transfected with pcDNA3 with normal human serum (NHS) in the MTT assay. Fibroblast NIH3T3 cells were incubated for 1 or 5 h in the presence of 25 or 50% NHS. The MTT assay was used to assess the cellular damage to NIH3T3 cells caused by human complement activation.

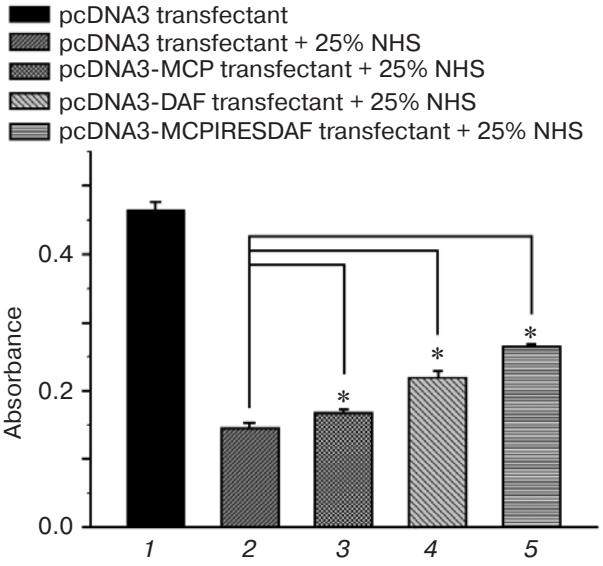

Fig. 5. Co-expressing human DAF and MCP fully protects NIH3T3 cells from lysis by human complement. NIH3T3 transfectants (pcDNA3, pcDNA3-MCP, pcDNA3-DAF, and pcDNA3-MCPIRESDAF) were incubated in the absence or presence of 25% NHS for 1 h. Cellular damage of the NIH3T3 transfectants was assessed using the MTT assay.

EMCV IRES-mediated polycistronic expression vectors are often used in gene therapy for strategies in which several genes need to be expressed [6, 7]. However, there have been few reports on their possible use for hCRPs in xenogeneic cells or animals utilizing this strategy, which should be advantageous as a potential therapeutic strategy to inhibit C-mediated hyperacute rejection.

In this context, we used EMCV IRES to construct a dicistronic vector pcDNA3-MCPIRESDAF, which was expected to highly express hCRPs MCP and DAF at a constant ratio in transfected cells. The co-expression of human MCP and DAF from such a polycistronic transcription unit at both mRNA and protein levels was confirmed using RT-PCR and Western blotting. These results are consistent with previous reports showing that the EMCV IRES-dependent second gene can be efficiently expressed [10-12]. Human complement-mediated cytolysis assays showed that co-expression of human DAF and MCP provides more powerful protection compared to human MCP or DAF expressed alone in NIH3T3 cells. All these results suggest that human DAF and MCP can cooperate and confer synergism by combining the two activities in close proximity to each other. It seems clear that the combination of human DAF and MCP would be important for protection of xenotransplanted organs from complement-mediated attack. Similar to other researchers, we postulate that EMCV IRES could be a useful tool for improving the efficiency of gene transfer.

In conclusion, the IRES-mediated expression vector provides a potent means for simultaneous transfer of multiple hCRPs genes into cultured cells and animal models, which has potential application of preventing hyperacute rejection in further clinical trials.

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