Protective Effects of Different Combinations of Human *MCP*, *DAF*, and *CD59* on Complement-Dependent Cytolysis in NIH 3T3 Cells

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Abstract

Objectives: **To analyze the protective effects against complement-mediated cytolysis of the *MCP*, *DAF*, and *CD59* human complement regulatory proteins, alone and in combination, on NIH 3T3 mouse fibroblast cells.**

Materials and Methods: **We constructed 3 double- and 3 single-human complement regulatory protein plasmids (pIRES-h*MCP*-h*DAF*, pIRES-h*MCP*-h*CD59*, pIRES-h*DAF*-h*CD59*, pIRES-A-h*MCP*, pIRES-B-h*DAF*, and pIRES-B-h*CD59*). The plasmids were transfected into NIH 3T3 cells, and stable transfectants were obtained by treatment with 200 kg/m3 G418 for 2 weeks. Normal human serum (50%) as a source of complement was added to the culture medium of stable transfectants. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to analyze the protective ability of different human complement regulatory protein plasmids on complement-dependent cytolysis.**

Results: **The viability of double-human complement regulatory protein stable transfectants was significantly higher than that of single-human complement regulatory protein stable transfectants ($P < .05$). Among the double-transfectants, cells expressing pIRES-h*MCP*-h*DAF* and pIRES-h*MCP*-h*CD59* survived better than cells expressing pIRES-h*DAF*-h*CD59* (91.75% ± 3.30% and 84.88% ± 2.36% vs 66.19% ± 6.52%; $P < .05$). Among the single-transfectants, cells expressing pIRES-A-h*MCP* or pIRES-B-h*DAF* survived better than cells expressing pIRES-B-h*CD59* or pIRES empty vector (53.76% ± 3.84% and 56.32% ± 2.83% vs 43.28% ± 0.96% and 40.27% ± 1.11%; $P < .05$).**

Conclusions: **These results suggest that the *MCP+DAF* and *MCP+CD59* combinations could be more effective than *DAF+CD59* in protecting the NIH 3T3 cells from injury caused by complement-dependent cytolysis, whereas *MCP* or *DAF* alone is stronger than *CD59* alone in inhibiting membrane attack complex formation.**

Key words: *Xenotransplantation, Immune rejection, Human complement regulatory proteins, Coexpression*

Introduction

The increasing demand worldwide for donor organs has led to a renewed interest in xenotransplantation. Pigs are considered the best donor choice for organ transplant in humans. However, porcine organs are rapidly damaged by hyperacute rejection after transplant. This process is caused by naturally occurring human antibodies binding to the carbohydrate epitope Galα1,3Gal (Gal) on pig vascular endothelium, with subsequent activation of the complement system.[1-5] In theory, there are 2 major methods to control hyperacute rejection. One is to knock out the α-1,3-galactosyltransferase that expresses a Gal epitope, and the other is to introduce human complement regulatory proteins (hCRPs) into the pig. Three major types of hCRPs are *DAF*, *MCP*, and *CD59*, which are expressed mainly by endothelial cells in mammals. They inhibit

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complement activation via different mechanisms in the complement activation cascade.[6-8] For example, *MCP* binds to complement activation products C3b and C4b, accelerating factor I-mediated inactivation of C3b and C4b[9-11]; *DAF* inhibits the formation of C3 and C5 convertases; and *CD59* inhibits formation of the membrane attack complex. Theoretically, concomitant expression of multiple hCRPs might inactivate complement more effectively than expression of a single hCRP. In transgenic pigs, longer survival of kidney xenografts was obtained with simultaneous expression of *DAF* and *CD59* compared to *DAF* expression alone.[12] It has been suggested that concomitant expression of multiple CRPs in xenogeneic organs can effectively inhibit hyperacute rejection. Therefore, it is important to identify methods for achieving multiple and simultaneous expression of CRPs in xenogeneic porcine organs.

The internal ribosome entry site (IRES) derived from the 5′-nontranslated region of the encephalomyocarditis virus exerts high-efficiency protein translation and is functional in a variety of cell culture systems. Vectors containing IRES have been shown to permit simultaneous overexpression of 2 genes in tandem from a single bicistronic RNA transcript.[13, 14] To investigate the protective effects of hCRPs, alone or in combination, on complement-dependent cytolysis, we used a bicistronic pIRES vector to construct pIRES-h*MCP*-h*DAF*, pIRES-h*MCP*-h*CD59*, pIRES-h*DAF*-h*CD59*, pIRES-A-h*MCP*, pIRES-B-h*DAF*, and pIRES-B-h*CD59* plasmids. Viability of NIH 3T3 cells stably transfected with these plasmids was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Materials and Methods

Plasmid construction and preparation

Total RNA was extracted from human umbilical vein endothelial cells with an RNAsimple Kit (Tiangen Biotech Co, Ltd, Beijing, China). Quality and quantity of the RNA were determined by gel electrophoresis and spectrophotometry. The first cDNA chain was synthesized with 0.4 μM oligo(dT),[18] 1 μg total RNA, 1 × buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM $MgCl_2$), 1.2 mM dNTPs, 1.6 U RNase inhibitor, and 8 U Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) in a 25-μL reaction volume. The reverse transcription condition was 25°C for 10 minutes, 37°C for 60 minutes, and inactivation by heating at 95°C for 5 minutes. Specific primers for plasmid construction were designed with the use of Oligo 6.0 software and synthesized by Sangon Biotech Co, Ltd (Shanghai, China) (Table 1). Amplification reactions contained 1 μL cDNA, 1× buffer (10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl), 0.2 mM dNTPs, 0.4 μM of each primer, and 1 U *Taq* DNA polymerase (Sangon Biotech Co, Ltd). The amplification condition was 95°C for 5 minutes, followed by 35 cycles of 95°C for 20 seconds, 60°C for 30 minutes, 72°C for 90 seconds, and a final extension at 72°C for 10 minutes. Polymerase chain reaction (PCR) fragments were purified with a TIANgel Midi Purification Kit (Tiangen Biotech Co, Ltd) and cloned into the pMD-18T vector (Takara Biotech, Co, Ltd, Dalian, China). The targeted fragment containing the extended 5′-sequence was cut out with desired restriction enzymes and then ligated to pIRES digested with the same restriction enzymes. Positive clones were verified by sequencing. Plasmids for transfection

Table 1. Primers for vector construction.

Gene	Accession number	Insert site	Sequence (5'→3')	Fragment size (bp)
h*MCP*	NM_002389		F: *TA*GCTAGCATGGAGCCTCCCGGCC	
			R: *TG*CTCGAGTCAGAGAGAAGTAAATTT	1194
		MCSA: *Nhe*I/*Xho*I		
h*DAF*	NM_000574		F: *TA*GCTAGCATGACCGTCGCGCGG	
			R:*TA*CTCGAGCTAAGTCAGCAAGCCCA	1155
h*DAF*	NM_000574		F: *TA*TCTAGAATGACCGTCGCGCGG	
			R: *CT*GTCGACCTAAGTCAGCAAGCCCA	1155
		MCSB: *Xba*I/*Sal*I		
h*CD59*	NM_000611		F:*TA*TCTAGAATGGGAATCCAAGGAGG	
			R:*CT*GTCGACTTAGGGATGAAGGCTCC	395

Italicized sequences denote protected base pairs; underlined sequences denote restriction enzyme sequences.

were extracted from positive bacteria with an EndoFree Plasmid Kit (Tiangen Biotech Co, Ltd).

Cell culture and transfection

The NIH 3T3 mouse fibroblast cell line was obtained from the Cell Bank, Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco modified Eagle medium (DMEM) (Invitrogen Corporation, Carlsbad, CA, USA [also Gibco]) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sangon Biotech Co, Ltd). Culture conditions were maintained at 37°C in a humidified atmosphere with 5% CO_2. Cells were seeded onto 24-well plates (Corning Inc., Lowell, MA, USA) at a density 1×10^5 cells/well in 500 μL DMEM with 20% FBS and without antibiotics. At 90% to 95% confluence, cells were transfected with Lipofectamine 2000 (Invitrogen) and 1 μg plasmid DNA in Opti-MEM I (Invitrogen) according to the manufacturer's protocol. The pEGFP-N1 plasmid was used as a reporter plasmid to monitor transfection efficiency, and the empty pIRES plasmid was used as negative control. Cells were selected 36 hours posttransfection by treatment with 200 κg/m^3 G418 (Sangon) for 2 weeks.

Real-time quantitative polymerase chain reaction

Primers for h*MCP*, h*DAF*, h*CD59*, and mouse β-*actin* (mβ-*actin*) were designed by combining the capabilities of Primer Express software v3.0 with Oligo 6.0 (Table 2). All primer pairs spanned at least 1 intron of genomic DNA so as to distinguish, by size, real-time PCR products from PCR products of residual genomic DNA that might exist in our RNA samples. Total RNA was isolated from NIH 3T3 cells stably transfected with different plasmids with an RNAsimple Total RNA Kit (Tiangen Biotech Co, Ltd), and then treated with DNAse I (Sigma-Aldrich, St. Louis, MO, USA) to eliminate genomic DNA contamination before reverse transcription. The first cDNA strand was synthesized according to the method described for plasmid construction. Real-time quantitative PCR was carried out in a Bio-Rad iQ5 Quantitative PCR System (Bio-Rad Laboratories, Inc, Hercules, CA, USA) using the machine's default cycling protocol. The 20-μL reaction volume contained 10 μL SYBR Green Master Mix (×2) (Bio-Rad Laboratories, Inc), 1.0 μL of each primer (1 μM), 1.0 μL cDNA or plasmid containing mouse β-*actin*, and 7 μL double-distilled water. A negative control containing double-distilled water instead of cDNA was included in all PCR reactions. Amplified products were assessed according to the melting curve profile, visualized by ethidium bromide staining of agarose gels, and verified by sequencing. Standard curves were generated by gradient dilution of plasmid containing the desired gene or the mβ-*actin* gene. Data were calculated as the average of triplicate experiments, and expression of each target gene was normalized to that of the mβ-*actin* gene.

Table 2. Primers for real-time polymerase chain reaction.

Gene	Accession number	Sequence (5'→3')	Fragment size (bp)
hMCP	NM_002389	F: CATACATGGCTACCTGTCT	
		R: CATCTGATAACCAAACTCGT	121
hDAF	NM_000574	F: TTCTCATGTAACACAGGGTA	
		R: CTCTGCACTCTGGCAAC	103
hCD59	NM_000611	F: AGCTGGGTTACAAGTGTATAA	
		R: CTTGCAGCAGTAGTACGTTA	106
mß-actin	NM_007393.2	F: CCAACACAGTGCTGTCTG	
		R: ACAGAGTACTTGCGCTCA	133

Human complement-mediated cytolysis

Damage to NIH 3T3 cells caused by human complement activation was evaluated by MTT assay. A total of 1×10^4 NIH 3T3 cells were seeded in each well of a 96-well plate (Corning Inc) and cultured at 37°C in a humidified atmosphere with 5% CO_2. When the cells reached 70% to 80% confluence, the medium was replaced with DMEM containing no (control) or 50% NHS as a source of complement, and the cells were cultured for another 60 minutes. The supernatant was then discarded, and the cells were washed twice with D-Hank solution. Complement-mediated cytolysis in the presence of different transfectants was determined by quantitative colorimetric assay with an MTT kit (Nanjing KeyGen Biotech Co Ltd, Nanjing, China). The assay was carried out according to the manufacturer's instructions, and the absorbance of completely solubilized purple formazan crystals was measured in a Tecan Infinite 200 microplate reader (Tecan Group Ltd, Mannedorf, Switzerland). Experiments were repeated 6 times for each NIH 3T3 transfectant. Cell viability was expressed as the ratio of the signal from treated cells (with 50% NHS) to the signal from untreated cells (no NHS). The wavelength used to measure formazan absorbance was 570 nm, and the reference wavelength was 655 nm.

Statistical analyses

Results of the MTT assay were analyzed with the SAS 8.02 analysis of variance program (SAS Institute Inc, Cary, NC, USA). Statistical significance was set at $P \leq .05$.

Results

Human complement regulatory protein recombinant plasmids

We constructed 6 hCRP plasmids, including *MCP+DAF*, *MCP+CD59*, and *DAF+CD59* double-hCRP plasmids simultaneously expressing 2 genes in a bicistronic transcript, as well as MCP, *DAF*, and *CD59* single-hCRP plasmids. The structure of each recombinant plasmid is depicted in Figure 1. We investigated the expression of these hCRPs in NIH 3T3 cells, with the empty pIRES plasmid as a control. Results confirmed that IRES could drive double or single gene expression, consistent with previous reports.[15, 16]

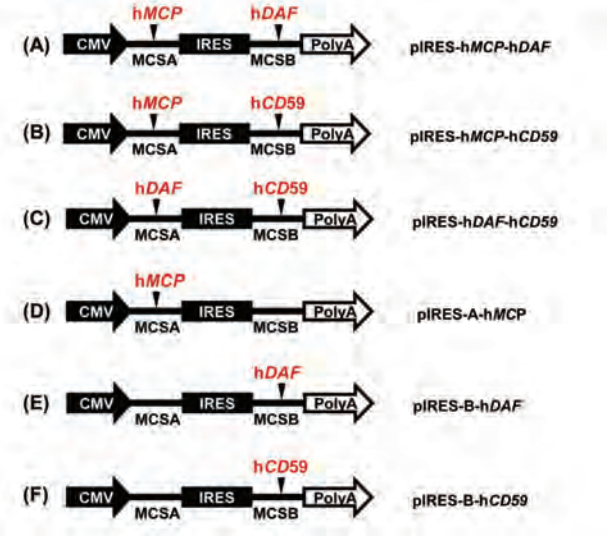

Figure 1. Schematic of plasmid constructs. Transcription of the target genes was driven by a *Cytomegalovirus* or an internal ribosome entry site (IRES) promoter.

Stable transfection in NIH 3T3 cells

The pEGFP-N1 plasmid was used as a positive control for transfection. We investigated the expression of green fluorescent protein (GFP) in NIH 3T3 cells 36 hours after transfection under an inverted fluorescence microscope. Results showed that our transfection conditions and system worked well. The use of 200 kg/m^3 G418 was confirmed effective for the selection of stable transfectants in preliminary experiments. The mRNA levels of hCRPs expressed in each experimental group were determined by real-time PCR (Figure 2). Expression of h*MCP* showed no significant differences among pIRE-h*MCP*-h*DAF*, pIRE-h*MCP*-h*CD59*, and pIRE-A-h*MCP* stable transfectants ($P > .05$). However, h*DAF* mRNA expression was greater in pIRE-h*DAF*-h*CD59* and pIRE-B-h*DAF* stable transfectants than in pIRE-h*MCP*-h*DAF* transfectants ($P < .05$). There were no significant differences in h*CD59* expression among pIRE-h*MCP*-h*CD59*, pIRE-h*DAF*-h*CD59*, and pIRE-B-h*CD59* stable transfectants ($P > .05$).

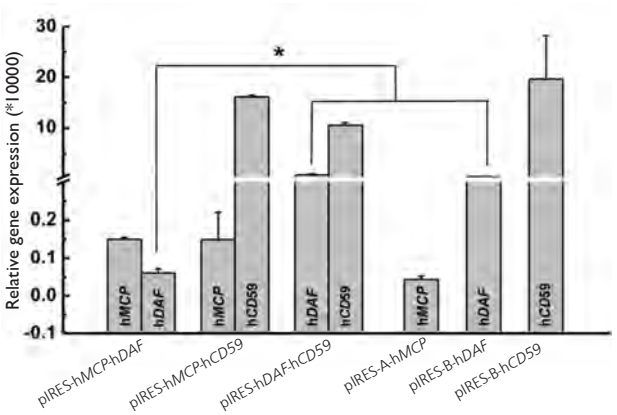

Figure 2. Relative expression profiles of human complement regulatory proteins by transfection with different plasmids. The relative expression levels of each target gene, normalized to that of β-*act*in expression, are shown. Data are presented as mean ± SEM. The asterisk at the top of each bar denotes significant difference in the same gene among groups. Statistical significance was set at $P \leq .05$.

Human complement-mediated cytolysis of NIH 3T3 cells

Most human cells express 2 or more hCRPs that can act collaboratively to inhibit complement activation by different mechanisms. To assess the protective effects of different combinatorial plasmids, we evaluated the sensitivity to complement-mediated cytolysis among NIH 3T3 cells stably transfected with the pIRES empty vector and single (h*MCP*, h*DAF*, or h*CD59*) or double (h*MCP*+h*DAF*, h*MCP*+h*CD59*, or h*DAF*+h*CD59*) plasmids in response to no or 50% NHS. Results showed that the cellular damage was obviously more severe in cells incubated with 50% NHS compared to no NHS (supplementary data). Among the double-hCRP stable transfectants, pIRES-h*MCP*-h*DAF* and pIRES-h*MCP*-h*CD59* cells survived significantly better than pIRES-h*DAF*-h*CD59* cells (91.75% ± 3.30% and 84.88% ± 2.36% vs 66.19% ± 6.52%; $P < .05$) (Figure 3). Among the single-hCRP stable transfectants, pIRES-

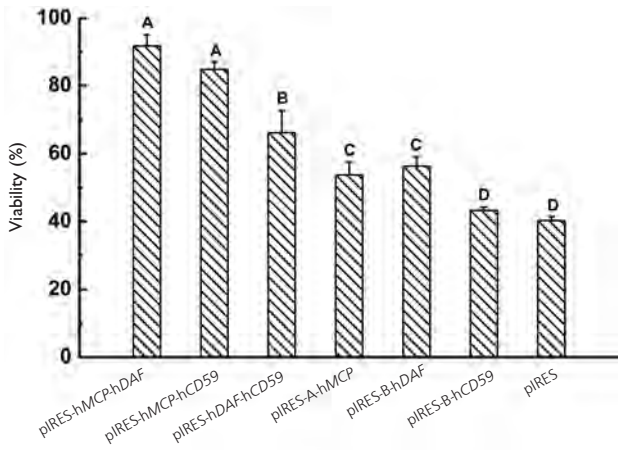

Figure 3. Viability of NIH 3T3 cells transfected with different plasmids or with empty vector control. Data are presented as means ± SEM. Transfection was repeated 6 times for each group. The letter on the top of each bar indicates the statistical result; different letters indicate statistical significance ($P \leq .05$), whereas the same letter indicates lack of statistical significance.

A-h*MCP* and pIRES-B-h*DAF* cells survived better than pIRES-B-h*CD59* or empty vector pIRES cells (53.76% ± 3.84% and 56.32% ± 2.83% vs 43.28% ± 0.96% and 40.27% ± 1.11%; $P < .05$). In addition, the viability of each of the double-hCRP stable transfectants was greater than that for each of the single-hCRP stable transfectants ($P < .05$). Similar results were obtained with trypan blue staining (data not shown).

Discussion

Our present results indicate that *MCP, DAF*, and *CD59* exerted an additive or collaborative effect in inhibiting the formation of the membrane attack complex. In the classic pathway of complement activation, antibody interacts with complement component 1q (C1q), forming the membrane attack complex, which consists of C5b/C6/C7/C8/C9. This results in cell leakage or lysis. In this process, *MCP, DAF*, and *CD59* proteins act in different ways and at various stages of complement activation. For example, *MCP* is a cofactor of factor I-mediated cleavage of C3b and C4b, *DAF* prevents the assembly of C3 and C5 convertases and accelerates the decay of C3 convertase, whereas *CD59*, as a membrane regulator, prevents the binding of C9 to the C5b/C6/C7/C8 complex in the terminal pathway (ie, membrane attack complex formation on the cell membrane).[11, 17, 18] Similar speculation has been made in several studies.[15, 19, 20] However, our present results differ from those of others,[11, 16] in that *MCP+DAF* exerted an inhibitory effect on complement-mediated cytolysis, similar to *MCP+CD59* but more potent than *DAF+CD59*. Because expression levels of hCRPs in cells might influence their function, we used real-time PCR to determine mRNA levels of *MCP, DAF*, and *CD59*, which was not done in other studies.[11, 16] In addition, our results showed that *MCP* or *DAF* alone was more effective than *CD59* in protecting NIH 3T3 cells from complement-mediated cytolysis, suggesting the possibility that complement activation might be regulated by a nonclassic pathway, such as that involving cell-surface receptors C1q, C3a, C5a, and iC3b. It is also possible that antibody-independent complement activation also occurs by the lectin and/or alternative pathways.[11]

In conclusion, our present results suggest that *MCP+DAF* and *MCP+CD59* combinations might be more effective than *DAF+CD59* in protecting NIH 3T3 cells from injury induced by complement-dependent cytolysis, whereas *MCP* or *DAF* alone might be more potent than *CD59* alone in inhibiting membrane attack complex formation.

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