Protection of xenogeneic cells from human complement-mediated lysis by the expression of human DAF, CD59 and MCP

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Abstract

CD59 and membrane cofactor protein (MCP, CD46) are widely expressed cell surface glycoproteins that protect host cells from the effect of homologous complement attack. cDNAs encoding human CD59 and MCP cloned from Chinese human embryo were separately transfected into NIH/3T3 cells resulting in the expression of human CD59 and MCP protein on the cell surface. The functional properties of expressed proteins were studied. When the transfected cells were exposed to human serum as a source of complement and naturally occurring anti-mouse antibody, they were resistant to human complement-mediated cell killing. However, the cells remained sensitive to rabbit and guinea pig complement. Human CD59 and MCP can only protect NIH/3T3 cells from human complement-mediated lysis. These results demonstrated that complement inhibitory activity of these proteins is species-selective. The cDNAs of CD59 and MCP were also separately transfected into the endothelial cells (ECs) of the pigs transgenic for the human DAF gene to investigate a putative synergistic action. The ECs expressing both DAF and MCP proteins or both DAF and CD59 proteins exhibited more protection against cytolysis by human serum compared to the cells with only DAF expressed alone. ß 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

The human complement system is part of the immune system involved in self–non-self recognition and destruction [1]. Its activation results in the formation of the C3/C5 convertases, which cleave C5 to initiate the formation of membrane-attack complexes (MAC or C5b-9). In the formation of MAC different steps can be discerned: the formation of C5b6, semistable C5b-7, and C5b-8, which is in fact a C9 convertase. To protect themselves from damage by these fortuitously deposited products host cells express several membrane-bound proteins, which are known as regulators of complement activation (RCA) [2]. Their activities are focused at two critical steps in the complement pathway. Decay-accelerating factor (DAF, CD55), MCP and complement receptor 1 (CR1) act at the level of the C3/C5 convertases. They inhibit C3/C5 convertase formation. DAF accelerates decay dissociation of the C3 and C5 convertases and decreases the formation of convertases [3]. MCP binds to the complement activation products C3b and C4b and serves as a cofactor for the factor-I-mediated cleavage of C3b and C4b [4,5]. This group is focused at the C3 convertase stage of the complement cascade, providing critical control of the deposition of C3 that can lead to clearance or lysis of the marked particle or cell. Control of lytic MAC formation (the terminal complement pathway) on host cell membranes is provided by two further proteins: a 65-kDa homologous restriction factor or C8-binding protein [6] and a more recently discovered 20-kDa protein designated CD59 [7]. They interfere with C9 polymerization by C5b-8. In the case of CD59, a widely distributed cell surface glycoprotein, this is achieved through specific interaction with the C9 polymerase (C5b-8), which prevents the formation of...
The five regulatory proteins are widely distributed, being found on most blood cells and tissue exposed to the bloodstream [9]. Originally they were discovered on circulating blood cells and have been most intensively studied either on red cells (DAF and CD59) or lymphocytes (MCP). Both CD59 and DAF are anchored in the cell membrane via a glycosylphosphatidylinositol (GPI) moiety and these two proteins along with other GPI-anchored molecules are deficient in the blood cells of patients with paroxysmal nocturnal hemoglobinuria [10]. The deficiency of GPI-anchored complement regulators in these individuals is causally related to an increased susceptibility of their blood cells to lysis by homologous complement. In addition to their expression on blood cells, however, and consistent with their general role in protecting host cells from complement-mediated damage, DAF, MCP and CD59 are now known to be widely distributed on a variety of adult tissues [11].

An important feature of membrane-bound complement-regulatory proteins is their species-selective inhibitory activity [12,13]. Here, we demonstrate directly the protective role of human CD59 and MCP after expression on mouse fibroblasts. The cDNAs of CD59 and MCP were transfected into NIH/3T3 cells and the cells were exposed to human serum. Only those cells on which CD59 and MCP were expressed were protected from lysis by human complement. The cDNAs of CD59 and MCP were also transfected into endothelial cells (ECs), which were from pigs transgenic for the human DAF gene. From our results it appeared that those cells with two proteins expressed were more protected than cells with only one protein expressed. These biological functions of the expressed CD59 and MCP could be of interest for tumor, transplant, and reproductive immunologists. For example, the incorporation of human RCAs into a xenogeneic organ by transgenesis should protect the transplanted organ or tissue from lysis, which is called complement-mediated hyperacute (transplant) rejection in a human in vitro model for xenotransplantation with pig hearts [14].

2. Materials and methods

2.1. Construction of expression plasmids

The human CD59 cDNA and MCP cDNA stretches containing the complete encoding region were obtained by PCR using the total mRNA of Chinese human embryo as substrate [15]. Both full-length cDNAs were subcloned into a mammalian expression vector pCDNA3 between a CMV promoter and a BGH-polyadenylated sequence (Invitrogen, Carlsbad, CA, USA). CD59 cDNA inserted into the EcoRI-NotI site of pCDNA3 was called pCDNA3CD59, MCP cDNA inserted into the EcoRV-XhoI site of the pCDNA3 was called pCDNA3MCP. The two plasmids pCDNA3CD59 and pCDNA3MCP were transformed into Escherichia coli DH5α and isolated, characterized and amplified using standard techniques. Sequences of the two genes were confirmed by DNA sequencing.

2.2. Cells and transfection

NIH/3T3 cells (mouse fibroblasts; Chinese Center Type Culture Collection, Wuhan, PR China) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with heat-inactivated 10% fetal bovine serum. The calcium-phosphate co-precipitation method was employed to transfect the NIH/3T3 cells as reported by Sambrook et al. [16]. Cells were grown to 5×10⁵ cells per 60 mm diameter dish 1 day before transfection. About 8.8 μg of plasmid DNA dissolved in 220 μl of distilled water was used for transfection. The DNA, 250 μl of 2×Hanks’ buffered salt solution (HBS) and 31 μl of 2 mol l⁻¹ CaCl₂ were mixed for 30 min and were added dropwise to the cell monolayer. The cells were incubated for 24 h and then fed again with fresh medium. The transfected clones were selected in the presence of 400 μg ml⁻¹. G418 (Gibco BRL, Grand Island, NY, USA). Thereafter, the medium was replaced every 3 days with fresh complete medium containing G418. The cultures were incubated at 37°C in a humidified atmosphere containing 5% (V/V) CO₂. After 2 weeks G418-resistant colonies were isolated and expanded in tissue culture plate and flasks. To verify the integration of the two genes, the chromosomal DNA of the three transfected clones were isolated and used as template for PCR.

ECs were isolated from the aorta of the pigs transgenic for the human DAF gene (Biotechnology Research Center, Hubei Academy of Agricultural Sciences, Wuhan, PR China). The ECs were established in DMEM containing 10% fetal calf serum. The plasmid DNA was transfected into 70–80% confluent ECs using Dosper liposomal transfection reagent according to the manufacturer’s instructions (Boehringer Mannheim). One day before transfection, approximately 3×10⁵ cells were seeded per well of a 6-well culture plate (35 mm) in 2 ml of the culture medium without serum. On the day of transfection, 1–2 h before adding the transfection complex, culture medium was replaced with 1 ml of fresh medium without serum. About 4 μg of plasmid DNA dissolved in 50 μl HBS and 10 μl Dosper dissolved in 50 μl HBS were mixed gently and incubated at room temperature for 20 min. After the formation of the transfection complex, 100 μl of DNA/ liposome complex was pipetted in drops around the medium. Cultures were incubated for an additional 6 h at 37°C in a humidified atmosphere containing 5% (v/v) CO₂. Finally, the transfection medium was replaced by 3 ml fresh, serum-containing culture medium. A complement-dependent cytotoxicity assay was executed 48 h after starting the transfection experiment.
2.3. Complement-dependent cytotoxicity assay

Complement-mediated cell lysis was determined by microscopic examination following trypan blue staining. Equal volumes of cells and different serum dilutions were incubated for 60 min at 37°C. Viabilities were determined by dye exclusion and the percentage cytotoxicity was calculated. Normal human serum (NHS) contains naturally occurring antimouse antibody and complement. NHS loses its complement activity upon heat inactivation (40 min at 56°C). For cytotoxicity assays, each condition was performed in duplicate or triplicate and each experiment was performed three or four times.

3. Results

3.1. Transfection of NIH/3T3 cells by three recombined expression vectors

0.42-kb human CD59 cDNA and 1.2-kb MCP cDNA containing the full coding region were obtained by reverse transcription PCR from total mRNA of Chinese human embryo. The sequence data indicated that CD59 cDNA was identical to the natural one including a 128-aa open reading frame. MCP cDNA including a 369-aa open reading frame was one of the eight iso-forms which have been reported before [17]. It contains exon 9 encoding its Ser/Thr-rich domain and exon 14 encoding its cytoplasmic tail, which is why they were called ST CYT2 iso-forms. We constructed mammalian expression vectors containing human CD59 and MCP under the control of a CMV promoter. We ended up with three recombined expression vectors: pCDNA3, pCDNA3CD59, and pCDNA3MCP, and transfected them into NIH/3T3 cells. After transfection, three stable clones: NIH/3T3 pCDNA3, NIH/3T3 CD59 and NIH/3T3 MCP, were obtained by G418 selection.

Integration of the two genes in the chromosomal DNA of transfected cells was confirmed by visualization of the 0.42-kb and 1.2-kb PCR products when the chromosomal DNAs of NIH/3T3 CD59 and NIH/3T3 MCP cells were used as templates. In contrast, no PCR product was seen if the chromosomal DNA of NIH/3T3 pCDNA3 cells was the template (Fig. 1).

3.2. Lysis of NIH/3T3 pCDNA3 cells by NHS

The function of human complement was measured by testing the sensitivity of NIH/3T3 pCDNA3 cells to complement-mediated cytotoxicity upon exposure to human serum. NHS served as a source of complement and naturally occurring anti-mouse antibody. Incubation with NIH results in a quick cell lysis while the heat-inactivated NHS containing little or no complement hardly influenced the viability of NIH/3T3 pCDNA3 cells (Fig. 2).

3.3. Protection of murine cells from human complement-mediated lysis by expression of human CD59 and MCP

The function of human CD59 and MCP on NIH/3T3 cells was measured by testing the sensitivity of NIH/3T3 cells to complement-mediated cytotoxicity upon exposure to human serum. Three transfectants, NIH/3T3 pCDNA3
cells expressing no protein, NIH/3T3 CD59 cells expressing human CD59, and NIH/3T3 MCP cells expressing human MCP, were incubated with increasing dilutions of human serum and cell cytotoxicity was then monitored by trypan blue staining. A protective effect was observed as shown in Fig. 3. Under identical conditions, the control NIH/3T3 pCDNA3 cells were effectively lysed by human complement. However, in contrast to NIH/3T3 pCDNA3 cells, NIH/3T3 CD59 and NIH/3T3 MCP cells expressing human CD59 and MCP protein, respectively, were protected from lysis by human complement. In this system, we found that about 20-fold higher concentrations of human serum were necessary to lyse 40% of the NIH/3T3 CD59 and NIH/3T3 MCP cells as compared to non-transfected control cells. NIH/3T3 CD59 cells expressing human CD59 were almost totally resistant to lysis by 20% human serum. There was also a trend toward more lysis (less protection) in the cells expressing MCP than in the CD59 transfectants, whose lytic curves were similar.

3.4. Species specificity of NIH/3T3 CD59 cells and NIH/3T3 MCP cells to complement-mediated lysis

The expression of human CD59 and MCP on NIH/3T3 cells protected them from lysis by human complement. This confirmed that heterogeneously expressed CD59 or MCP is responsible for providing the observed protection from complement-mediated lysis. Fig. 4 further reveals a pattern of species-selective activity for human CD59 and MCP. Human CD59 and MCP effectively protected NIH/3T3 cells from lysis by human complement, but not by guinea pig or rabbit complement. Both NIH/3T3 CD59 cells and NIH/3T3 MCP cells were lysed by guinea pig and rabbit serum complement to essentially the same extent as control cells. The three transfected cell lines almost all retained about 40% viability when they were treated

Fig. 3. Protection of NIH/3T3 cells from lysis by human complement by expressing human CD59 and MCP. Three types of transfected cells, NIH/3T3 CD59 cells (■), NIH/3T3 MCP cells (▲), and NIH/3T3 pCDNA3 cells (●), were incubated with increasing concentrations of human serum, and viabilities were determined by dye exclusion with trypan blue and the percentage cytotoxicity was calculated. The figure shows representative data from three different experiments.

Fig. 4. Sensitivity of transfecant cells to human, rabbit, and guinea pig complement. Three transfected cells, NIH/3T3 pCDNA3 cells (A), NIH/3T3 CD59 cells (B), and NIH/3T3 MCP cells (C), were exposed to different concentrations of either human (●), rabbit (▲) or guinea pig serum (■), and viabilities were determined by dye exclusion with trypan blue and the percentage cytotoxicity was calculated. The figure shows representative data from three different experiments.
with 50% rabbit serum. However, when the cells were incubated with 50% guinea pig serum, about 50% of the cells survived.

3.5. Expression of two proteins, CD59 and DAF, or MCP and DAF, in pig ECs confer more protection from human complement-mediated lysis

After we transfected three recombined expression vectors, pCDNA3, pCDNA3CD59 and pCDNA3MCP, into ECs from a pig transgenic for the human DAF gene, we got five kinds of pig cells to be tested: ECsN which are from normal pigs, ECsDAF which are from pigs transgenic for hDAF, ECsDAF pCDNA3, ECsDAF pCDNA3CD59, and ECsDAF pCDNA3MCP. The double capacity of human DAF and CD59 or DAF and MCP to protect transfected pig ECs was assessed by cytotoxicity assays in which cells were incubated with human serum and the death rates of the cells were detected by trypan blue staining. The ECsN were lysed by human complement. In contrast, the ECsDAF were protected from lysis by human complement. CD59, MCP and DAF conferred roughly equivalent complement-protective effects when expressed alone or combined. In the last four conditions, they abrogated 60–80% of cytotoxicity even in the presence of 50% human serum. The ECsDAF pCDNA3MCP cells expressing both human DAF and MCP proteins exhibited more protection against cytolysis by human serum comparable to ECsDAFpCDNA3 cells with DAF expressed alone. Compared with only DAF-expressing ECs, the death rates of the ECs with DAF and MCP coexpressed declined from 30% to 18%, respectively, when they were treated with 50% human serum. The ECsDAF pCDNA3CD59 cells expressing both DAF and CD59 proteins were even a little more effectively protected from complement-dependent cytolysis than ECsDAF pCDNA3MCP cells (Fig. 5).

4. Discussion

Full-length cDNA clones of CD59 and MCP were isolated from human embryos. To determine whether CD59 and MCP generated on heterogeneous cells by transfection retain their capacity to inhibit human complement, NIH/3T3 cells transfected with CD59 and MCP cDNA were incubated with human serum as well as guinea pig and rabbit serum. Since the transfected cells were resistant to human complement, but not to guinea pig and rabbit complement, CD59 and MCP generated on the transfectants retained their species specificity. Species specificity might, therefore, be conferred by some specific amino acid sequences in the molecule [18]. The results described in this report may have an important ramification for transplantation immunology. The critical shortage of human donor organs has generated interest in porcine to human xenotransplantation. Xenotransplantation – the transplantation of organs and tissues between animal species – would supply an unlimited number of organs to meet people’s need. However, organ transplantation between distantly related species results in hyperacute rejection of the transplant, which consist of a violent immune response involving the complement system in the first place. Hyperacute rejection results from the deposition of preformed (natural) antibodies that activate complement on the luminal surface of the vascular endothelium, leading to vessel occlusion and graft failure within minutes to hours. In various animal models, either or both complement pathways may be involved [19]. It is a primary barrier to organ transplantation between disparate species such as pig and primate. Complement attack is due to the antibody-mediated or otherwise initiated activation of the complement cascade, but this phenomenon is physiologically subjected to negative regulation by a set of species-specific proteins, known as RCAs, such as DAF, MCP and CD59. This kind of endogenous membrane-as-
sociated complement inhibitor normally protects ECs from autologous complement. However, these molecules are species-restricted and are therefore ineffective in inhibiting activated xenogeneic complement. So, our results show that we can modulate hyperacute rejection by expressing human complement-regulatory proteins in transgenic animals based on the hypothesis that the complement-regulatory proteins act in a relatively species-specific manner such that the pig equivalents of the human complement-regulatory proteins do not function effectively against human complement. The NIH/3T3 cells expressing CD59 or MCP were of particular interest because exposure of NIH/3T3 cells to NHS may be similar to what occurs during transplantation of a discordant xenograft. Each NHS tested contained complement and naturally occurring anti-mouse antibody. We have shown that heterogeneous expression of human CD59 and MCP on NIH/3T3 cells affords a measurable level of protection against human complement-mediated lysis.

DAF, CD59 and MCP have different functions in complement activation. They act in a different way and at various stages of complement activation. DAF prevents the assembly of C3 and C5 convertases and accelerates the decay of C3 convertases. MCP is a cofactor of the factor I-mediated cleavage of C3b and C4b, while CD59 controls the terminal complement pathway–MAC formation on host cell membranes. Most human cells express two or more of these regulators of complement activation and these proteins could act in an additive or synergistic fashion. Certainly they can act synergistically since CD59, MCP and DAF use different mechanisms for inhibiting complement activation. In the present study, we demonstrate that xenoendothelial cells expressing two proteins were more effectively protected from complement-dependent cytolyis than those cells expressing only a single inhibitory protein. We found out that the protective effect of DAF and CD59 is better than that of DAF and MCP. The reason may be that the restriction of lysis by DAF and MCP is at about the same stage of complement activity. They all inhibit C3/C5 convertase formation and MCP is just one of the cofactors of factor I, a protein that accelerates the cleavage of C3b to iC3b. In contrast, CD59 is involved in the control of the final stage of complement-mediated lysis preventing successful channel formation by MAC. This protection can readily be distinguished from those acting earlier in the complement sequence by the ability to inhibit reactive or ‘innocent-by-stander-cell’ lysis. The protection by both DAF and CD59 was not only restricted in the earlier stage but also in the lytic stage and none of them needed cofactor. So, the coexpression of human MCP, CD59 or DAF on a transplanted mammalian organ may protect the xenograft from attack by the human complement system and thus may prolong graft survival by circumventing human-complement attack [20]. We are now at the stage of generating pigs transgenic for DAF, MCP or CD59 in order to produce animals whose organs may be suitable for transplantation into humans.

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References


