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Regulation of Complement-Mediated Swine Endothelial Cell Lysis by Herpes Simplex Virus Glycoprotein, gC1[1]

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Herpes simplex virus type 1 (HSV-1) encodes several immuno-regulatory proteins that allow it to escape from the human immune system. The regulatory function of a HSV-1 glycoprotein gC (HSV-gC1) molecule on complement-mediated swine endothelial cell (SEC) lysis was investigated. The HSV-gC1 gene was obtained by the PCR method from the HSV-1 genome. The complement-regulatory function of this molecule was analyzed by cytotoxicity assay, using Chinese hamster ovarian tumor (CHO) cell and SEC transfectants and six human serum samples. FACS and Western blot analysis revealed the expression of the HSV-gC1 molecule on the transfectants. The CHO cell transfectants showed significant resistance to cell lysis by the sera that did not contain the anti-HSV-gC1 antibody. The SEC transfectants, however, showed a marked resistance to cell lysis in all cases. The introduction of a viral immune regulator such as HSV-gC1 into the swine cell provides a new approach for successful xenotransplantation.

Key words: complement regulatory protein, HSV-gC1, swine endothelial cell, xenograft.

The current shortage of donor organs and the increasing need for them have led to a revival of interest in the area of xenotransplantation (*1*). Although many trials are currently underway to produce transgenic pigs that express human complement-regulatory proteins (CRP), such as membrane cofactor protein (MCP: CD46), decay accelerating factor (DAF: CD55) and CD59, hyperacute rejection and acute vascular rejection continue to be critical barriers to successful xenotransplantation (*2–7*).

Having co-evolved for millions of years with their natural hosts, viruses have developed many strategies to evade host immune responses. In particular, DNA viruses have adapted to host immune systems by capturing and modifying host genes that are able to affect the host immune response, for example, by regulation of antigen presentation, inhibition of apoptosis, interruption of complement activation, and the secretion of cytokines or cytokine antagonists. Such viral immunomodulatory proteins are frequently quite efficient in evading host immunity (*8*).

Herpes simplex virus type 1 (HSV-1) encodes several immune modulators, such as the HSV-1 glycoprotein C (HSV-gC1). HSV-gC1 accelerates the decay of the alternative pathway C3 convertase (C3bBb). In addition, it binds complement components C3 and C3b, and then blocks the interaction of C5 and properdin with C3b. These processes interfere with the amplification of both the classical and alternative complement pathways (*9*).

In the present study, in a search for a new tool for xenotransplantation instead of human CRP (*10–12*), we examined the function of a viral transmembrane-type complement regulator in the downregulation of complement-mediated cytolysis.

MATERIALS AND METHODS

Cell Culture—The SEC line MYP30 was cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) with L-glutamine and kanamycin/amphotericin (*13*). Chinese hamster ovarian tumor (CHO) cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in Ham's F12 medium containing 10% FBS with L-glutamine and kanamycin/amphotericin. Cultures were maintained in a 5% CO_2/95% air atmosphere at 37°C.

Constructon of Plasmids—For the cloning of the HSV-gC1 gene, two oligonucleotide primers to the NH_2-terminal and COOH-terminal of the HSV-gC1 gene were synthesized: 5′HSV, 5′-CCC GTT AAC AAG CTT GGG GAG GCG TCG GGC ATG-3′; 3′HSV, 5′-CCC CTC GAG ACC AAA CTA TAT AGA TAT TAA AAA GG-3′. The coding sequence of the HSV-gC1 gene was amplified from the HSV-1 genome by the PCR method. The reaction product was treated with restriction enzymes, Hpa I and Xho I, and subcloned into the mammalian expression vector pCXN2, in which the transcription of the inserted cDNA is driven by a β-actin promoter and a cytomegalovirus enhancer (*14*). As a control, the cDNA of DAF (*15, 16*) was also subcloned into pCXN2.

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Abbreviations: CRP, complement-regulatory proteins; DAF, decay accelerating factor ; HSV, Herpes simplex virus; gC, glycoprotein C; FBS, fetal bovine serum; CHO, Chinese hamster ovarian tumor cell; SEC, swine endothelial cell; LDH, lactate dehydrogenase; mAb, monoclonal antibody; NHS, normal human serum.

The nucleotide sequence was confirmed by use of an ABI 310 autosequencer (Applied Biosystems, Foster City, CA).

Transfection of the Constructed cDNA—The cDNAs of HSV-gC1 and DAF were introduced into CHO cells by electroporation and into MYP-30 cells by use of lipofectamine (Gibco, Rockville, MD) (*12*). Transfected cells were maintained in complete medium for several days in an atmosphere of humidified 5% CO_2 at 37°C, then transferred to complete medium containing 1.0 mg/ml G418 (Gibco, Rockville, MD) for CHO cells and 0.7 mg/ml for MYP-30 cells for selection.

Flow Cytometry—Expression of the constructs was confirmed by flow cytometry. Transfected cells (1×10^6) were incubated with 1 μg of a mouse monoclonal antibody (mAb) MC28 (anti–HSV-gC1 molecule) (Serotec, Raleigh, NC) for 30 min at 4°C, then with 1.25 μg of a fluorescein isothiocyanate (FITC)-labeled rabbit anti–mouse IgG antibody (ICN, Costa Mesa, CA) as a second antibody for 30 min at 4°C. Stained cells were analyzed using an FACS calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Naive MYP-30 and CHO cells were used for control.

Lactate Dehydrogenase (LDH) Assay—This assay was performed as described previously, using a Kyokuto MTX-LDH kit (Kyokuto, Tokyo). The transfected cells were plated at a concentration of 2×10^4 per well in a 96-well tray 1 day before assay. After 15 h, the plates were incubated with 20 and 40% normal human serum (NHS) diluted in serum-free medium for 2 h at 37°C, and the released LDH was determined. The spontaneous release of LDH activity from target cells was less than 5% of the maximal release of LDH activity, which was determined from the complete lysis by sonication. For naive and transfected CHO cells, an LDH assay was performed with 20 and 40% NHS diluted in serum-free medium which contained anti–CHO cell antiserum. Antiserum against the CHO cells were prepared from a rabbit, which had been immunized with CHO cells.

Immunoblotting—The protein contents of transfectant and naive cell lysates were quantified by the BCA method (Pierce, Rockford, IL), and 40 μg aliquots of the obtained proteins were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (*17*). The separated proteins were then electrophoretically transferred onto a nitrocellulose membrane (Schleicher & Schuell, Germany). The membrane was blocked in 5% skim milk in Tris-buffered saline/0.05% Tween 20 (TBST) for 1 h at 25°C, then incubated in 1% bovine serum albumin (BSA)/0.5% skim milk/TBST with mouse anti–HSV-gC1 monoclonal antibody for 1 h at 25°C. After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibody, and the signal was developed using an ECL detection system (Amersham, Uppsala, Sweden).

Statistics—Data are presented as the mean ± SEM. The Student-*t* test was used to ascertain the significance of differences within groups. Differences were considered statistically significant at the level of $p < 0.05$.

RESULTS

Cell Surface Expression of HSV-gC1—The expression vector containing HSV-gC1 or DAF was transfected into the CHO and SEC cells, and the expression levels of these molecules were determined by flow cytometry. Typical flow

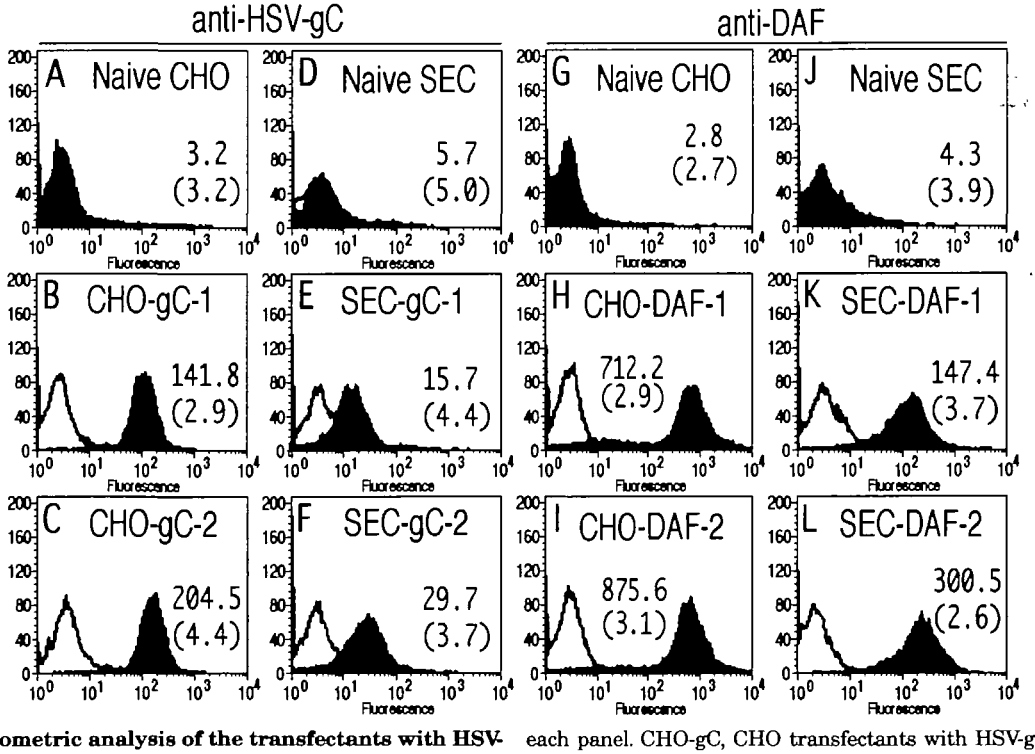

Fig. 1. **Flow cytometric analysis of the transfectants with HSV-gC1.** The FACS profiles of the cell surface expression were analyzed by anti–HSV-gC1 or anti–DAF mAb (closed histogram) and control isotypic Ab (open histogram). The mean shift values are indicated in each panel. CHO-gC, CHO transfectants with HSV-gC1 gene; CHO-DAF, CHO transfectants with human DAF gene; SEC-gC, SEC transfectants with HSV-gC1 gene; SEC-DAF, SEC transfectants with human DAF gene.

cytometric histograms for the transfectants are shown in Fig. 1. The CHO cell transfectants (CHO-gC) expressed HSV-gC1 molecule more easily than the SEC cell transfectants (SEC-gC).

Human Complement-Regulatory Function of HSV-gC1 on the CHO Cell Transfectants—The inhibitory effect of the HSV-gC1 molecule on complement-mediated cell lysis was examined using several transfectants with the molecule and six independent normal human serum samples (NHS) obtained from healthy volunteers. As a whole, CHO-gC showed significant resistance to 20% NHS in the cases of NHS#1 to NHS#4, although CHO-gC-1 did not show a statistically significant difference from naive CHO cell in the case of NHS#2 and NHS#4, because of the low level of the HSV-gC1 expression (Fig. 2A). On the other hand, DAF appeared to be quite effective even for 40% NHS (Fig. 2B). We next performed the LDH assay without rabbit anti-CHO cell antibody in order to analyze differences between these sera. NHS#5 and NHS#6 significantly activated the complement-mediated cytolysis of the CHO-gC transfectant but not the naive CHO cell (Fig. 2, C and D).

Human Complement-Regulatory Function of HSV-gC1 on the SEC Transfectants—In contrast to the CHO cell transfectants, the individual NHS samples showed various cytolytic activities to the naive SEC. While the naive SEC was easily lysed by human complement, the SEC-gC transfectants showed significant resistance to the complement-mediated cell lysis in all cases (Fig. 3, A and B).

Western Blot Analysis of the Transfectant with Individual Serum—To better understand these results, Western blotting analysis was performed with the cell lysates from the transfectants, using a mouse anti–HSV-gC1 antibody, MC28, as a first antibody, and a horseradish peroxidase–conjugated second antibody.

Anti–HSV-gC1 antibody showed a signal band of 80 kDa, which was consistent with the expected molecular mass of the HSV-gC1 molecule in CHO cells and the SEC transfectants (Fig. 4A). NHS#5 and NHS#6 contained the anti–HSV-gC1 antibody, but NHS#1 to #4 did not (Fig. 4, B and C, data relative to NHS#1 to #3 and NHS#5 are not shown).

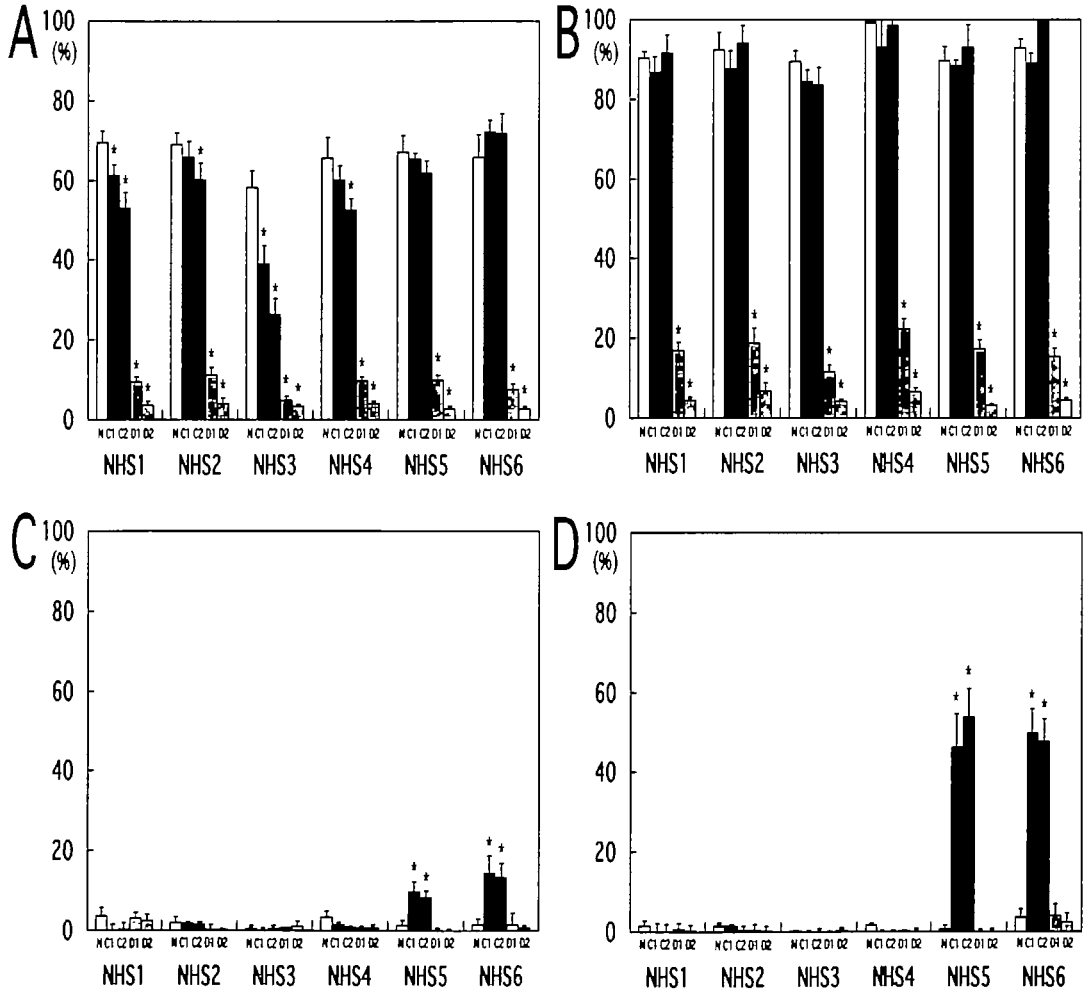

Fig. 2. **Complement-mediated cytotoxicity assay of the CHO cell transfectants with HSV-gC1 gene.** Complement-mediated cytotoxicity assay with the CHO-gC and CHO-DAF transfectants in the presence (A and B) (n = 6) or absence (C and D) (n = 4) of rabbit anti–CHO cell Ab as complement activator. 20% NHS (A and C) or 40% NHS (B and D) was used as a source of human complement. The results are presented as a percent of specific lysis. The asterisks indicate significant difference from naive cells ($p < 0.05$). Values are presented as mean ± SEM. N, naive CHO; C1, CHO-gC1; C2, CHO-gC-2; D1, CHO-DAF-1; D2, CHO-DAF-2.

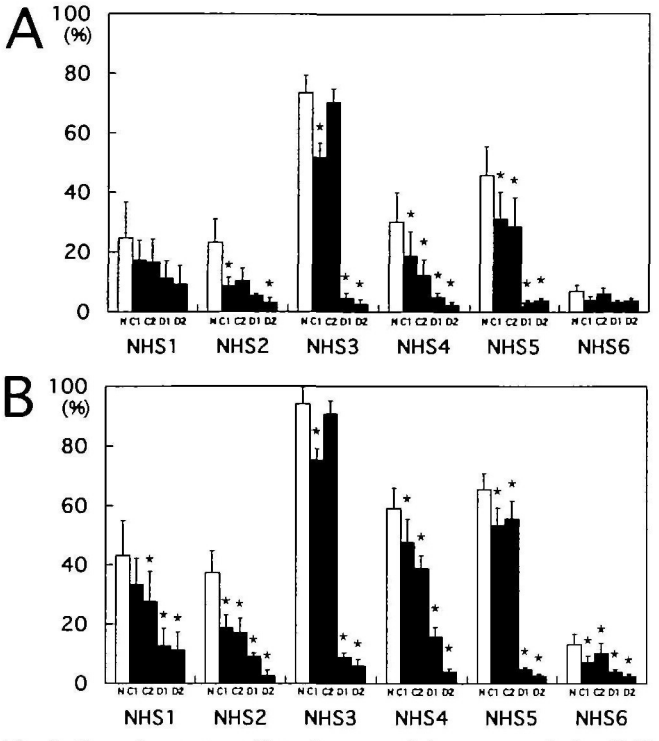

Fig. 3. **Complement-mediated cytotoxicity assay of the SEC transfectants with HSV-gC1 gene.** Complement-mediated cytotoxicity assay with the SEC-gC and SEC-DAF transfectants is indicated (A and B) ($n \geq 7$). 20% NHS (A) or 40% NHS (B) was used as a natural antibody and a source of human complement. The results are presented as a percent of specific lysis. The asterisks indicate significant difference from naive cells ($p < 0.05$). Values are presented as mean ± SEM. N, naive SEC; C1, SEC-gC1; C2, SEC-gC-2; D1, SEC-DAF-1; D2, SEC-DAF-2.

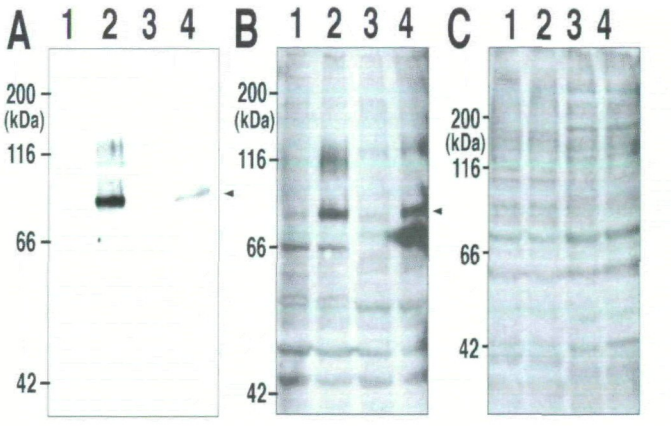

Fig. 4. **Western blot analysis of the transfectants with HSV-gC1 gene.** Western blot analysis was performed to detect the anti–HSV-gC1 Ab in the individual NHS. Anti–HSV-gC1 mAb, MC28, was used as a positive control (A). Typical results of positive and negative staining of HSV-gC1 by NHS#6 and NHS#4 sera are shown: NHS#6 reacted with HSV-gC1 protein (B), but NHS#4 did not (C). Arrowheads indicate the signal of HSV-gC1 protein. Lane 1, naive CHO; lane 2, CHO-gC-2; lane 3, naive SEC; lane 4, SEC-gC-2.

DISCUSSION

A comparison of the complement-regulatory function of gC1 and DAF revealed that it may be difficult to compare the efficacy of these molecules based on the results presented here, since their levels of expression in the SEC transfectants were quite different. In our previous study, DAF appeared to be the most effective molecule for downregulating complement-mediated SEC lysis, for the decay accelerating function of the assembled C3 convertases, C4b2a and C3bBb (*13*). However, it is incapable of cleaving the C4b and C3b once they become attached to SEC, and incapable of preventing the generation of C4a and C3a anaphylatoxins (*18*). During transplantation experiments of the transgenic pig with DAF to the monkey, large amounts of not only C1 but also C4b and C3b were present in the graft (*19, 20*). As a result, more complete complement regulation becomes necessary from numerous points of view in order to regulate AVR (*7*).

gC1 may be inferior to DAF in regulating the complement activity on the SEC surface. However, gC1 plays many roles in the complement regulation, as indicated in "INTRODUCTION." Therefore, it will be helpful to cover DAF function for achieving a more strict complement regulation on a xenograft.

The contrasting profiles of NHS#1-4 and NHS#5-6 in their complement-dependent cytotoxicity toward the CHO cell transfectants appears to be mainly caused by the presence of anti–HSV-gC1 antibodies, which could enhance antibody-mediated complement activation and/or block the complement regulatory function of gC1. The donors of NHS#5 and NHS#6 are patients with long-standing, latent orofacial infections. Eberle et al. reported that approximately 50% of the patients with such latent infections have the anti–HSV-gC1 Ab (*21*). The result from the CHO cell transfectants suggests the potential disadvantage of utilizing viral immune regulators for xenotransplantation. On the other hand, the SEC-gC transfectants showed significant resistance to the complement-mediated cell lysis in all cases.

The HSV-gC1 molecule is a highly glycosylated protein with numerous *O*- and *N*-linked oligosaccharides. The structure of these oligosaccharides varies among the infected host cell types, and the sialic acid in particular modifies the activity of HSV-gC1 (*22*). In addition, HSV gC-1 is associated with calnexin, a membrane-bound chaperone, in the endoplasmic reticulum (*23*). Species differences in terms of the glycosylation and/or protein folding might explain the difference in the inhibitory profile to human serum between the CHO-gC and SEC-gC transfectants.

Recently, several groups reported the utilization of the viral immunoregulatory proteins in swine cells, for example, vaccinia virus soluble complement control protein (VCP) and the poxvirus cytoplasmic apoptosis inhibitor CrmA (*24, 25*). These molecules are different from the cell surface proteins, such as the HSV-gC1 molecule. However, they also might have limited applicability for xenotransplantation.

Collectively, in spite of the possibility of the limited applicability, a viral transmembrane-type immunoregulator such as HSV-gC1 provides a new approach for evading the host immune reaction in swine-to-human xenografts. In the present study, we provided the first report on the utilization of viral transmembrane-type complement regulators for xenotransplantation.

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