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Effect of the sugar chain of soluble recombinant CD59 on complement inhibitory activity

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Abstract A soluble recombinant CD59#77 (rCD59#77), consisting of 77 amino acids starting from the N terminus of membrane-bound CD59, was prepared using a gene expression system in CHO cells. The rCD59#77 preparation was composed of glycosylated and non-glycosylated forms (G and NG forms). Unexpectedly, NG form was 7 times more potent than G form in complement inhibitory activity. Postulating that sialic acids on G-form molecules make it difficult for rCD59#77 to access nascent membrane attack complexes on the cell surface, the sialic acids were removed by neuraminidase treatment. However, the inhibitory activity was not changed. Next, one of two putative N-glycosylation sites was mutated by substituting Gln[18] for Asn[18]. The mutant, designated rCD59#77(N/Q), had no sugar moiety and was as active as the NG form of rCD59#77. These results suggest that the bulky sugar moiety at Asn[18] is not necessary for the complement-inhibitory activity of rCD59 and actually hampers that function.

Key words: CD59; Complement; Glycosylation; Membrane attack complex

1. Introduction

CD59 is a membrane glycoprotein of 18–21 kDa which is anchored to the cell membrane via the glycosyl phosphatidyl inositol (GPI) moiety. It inhibits formation of the complement membrane attack complex (MAC), which injures or activates the host cells in inflammatory and autoimmune diseases [1,2]. CD59 interacts with both the C8 and C9 components of complement during MAC assembly on the cell surface, interfering with additional incorporation of C9 into the intermediate complex [3,4]. It was first isolated from erythrocyte membrane [5] and then found on various kinds of cells [6,7].

Patients with paroxysmal nocturnal hemoglobinuria (PNH) are deficient in CD59, and their erythrocytes are highly susceptible to complement attack [8]. It has been shown that CD59 is essential for protection of not only erythrocytes but also nucleated cells against complement attack [9,10]. Matsuo et al. recently found that a monoclonal antibody capable of inhibiting the activity of rat CD59 reduced tissue injury in rat complement-mediated experimental glomerulonephritis [11]. In a clinical study, myocardial infarction decreased CD59 molecules and caused MAC formation at the infarcted lesions [12].

We have cloned and sequenced human CD59 cDNA using mRNA from peripheral blood mononuclear cells [13]. It encodes a polypeptide composed of 128 amino acids (a.a.) containing a signal sequence of 25 a.a. at the amino terminus and a signal sequence of 26 a.a. for GPI anchoring at the carboxyl terminus [13,14]. The mature molecule consists of 77 a.a. anchored with GPI on the cell surface [14]. There are two putative N-glycosylation sequences (Asn–X–Ser/Thr) at a.a. positions 8–10 and 18–20 of the mature molecule [15]. It was recently reported that urinary CD59 has fucosylated biantennary and triantennary oligosaccharide chains with variable sialylation, which is linked to Asn[18] [16].

Ninomiya et al. [17] used N-glycanase to remove the N-linked sugar moiety from human CD59 purified from erythrocytes and found that the deglycosylated CD59 was one-tenth as active as the original molecule in inhibiting MAC formation. In addition, CHO cells transfected with cDNA of human CD59 were cultured in the presence of 1-deoxymannojirimycin, which inhibited conversion from high-mannose to complex glycoproteins, made the cells more susceptible to injury by complement [17]. In contrast to these findings, it was recently reported that mouse fibloblast transfectants expressing a mutant CD59 lacking the sugar chain were more resistant to complement attack than transfectants expressing the wild-type CD59 [18]. Thus, the significance of the sugar chain remains to be elucidated in connection with the functional properties of CD59.

In this study, we prepared human rCD59#77 and a mutant rCD59#77(N/Q) lacking a sugar chain by using a CHO cell expression system and elucidated the significance of the sugar chain in the complement-inhibitory function. Our results suggested that the bulky sugar at Asn[18] hampers CD59 function.

2. Materials and methods

2.1. Assay of inhibition of reactive hemolysis

Human factors B and D were kindly provided by Dr. Y. Nakano, Showa University (Tokyo, Japan). Cobra venom factor (CVF) and human complement components C5 and C6 were purchased from Quidel (San Diego, CA). C5b6 complex was prepared by the method described previously [19], with a minor modification. Briefly, 2.5 μg of CVF, 10 μg of factor B and 10 μg of factor D were mixed in 40 μl of phosphate-buffered saline (PBS) containing 50 mM $MgCl_2$. The mixture was incubated at 37°C for 15 min to form CVF–Bb complexes, and then allowed to react with 100 μg of C5 and 100 μg of C6 in 240 μl of PBS at 37°C for 15 h. The C5b6 complex thus prepared was stored at −80°C until use. A hemolysis assay mixture contained 25 μl of 1×10^9/ml guinea pig erythrocytes (Egp), 25 μl of 2.5% human serum, 25 μl of CD59 solution and 25 μl of the C5b6 complex diluted

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Abbreviations: rCD59#77, a soluble recombinant CD59#77; rCD59#77(N/Q), a mutant with substitution of Gln for Asn[18]; Asn[18], Asn at the 18th a.a. position; MAC, membrane attack complex; G and NG forms, glycosylated and non-glycosylated forms of rCD59#77; GPI, glycosyl phosphatidyl inositol; a.a., amino acids; CHO, Chinese hamster ovary

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320-fold in a total of 100 μl of 25 mM veronal buffer (pH 7.4) containing 146 mM NaCl, 10 mM EDTA and 0.1% gelatin (EDTA–GVB). After incubation at 37°C for 30 min and centrifugation, the optical density of the supernatant was measured at 414 nm. The percentage of hemolysis was calculated relative to the control. Complement inhibitory activities were expressed as the percentages of reduction in hemolysis relative to that observed in the absence of CD59.

2.2. cDNA constructs

A plasmid of pEBD vector was constructed by insertion of an expression unit of the dehydrofolate reductase (*DHFR*) gene as a selection marker into a unique AatII site of pEF-BOS (kindly provided by Dr. S. Nagata, Osaka Bioscience Institute, Osaka, Japan [20]). To construct a truncated CD59#77 cDNA lacking 26 a.a. residues in the carboxyl terminus, CD59#77 cDNA fragment was amplified with Taq polymerase in the presence of oligonucleotides P1, 5′-TCTAGAGCGGCCGCTCCCCATCCGCTCAAGCAGGCCACCA-TGGGAATCCAAGGAGGGTCT-3′ and P2, 5′-TCTAGACTC-GAGCTATTAATTTTCAAGCTGTTCGTTAA-3′ as probes and pGEM352-3, which had the full length of CD59 cDNA [13] as the template. After digestion with *Xba*I, the cDNA fragment was inserted into the *Xba*I site of pEBD.

To construct a plasmid, i.e., pCD59#77(N/Q), expressing CD59#77(N/Q), in which Gln is substituted for Asn^{18}, a two-step polymerase chain reaction (PCR) was carried out. An N-terminal half cDNA fragment containing Gln at position 18 was initially amplified by PCR with oligonucleotides N/Q and P1 as primers. Oligonucleotide N/Q, 5′-GAGACACGCGTCAAAATCAGATGAACATTG-GACGGCTGTTTT-3′, which corresponds to a.a. positions 14 to 30, includes a Gln codon at the 18th a.a. and a restriction enzyme *Mlu*I recognition site. A C-terminal half cDNA fragment was also amplified by PCR with oligonucleotides Mlu, 5′-TCTGATTTT-GACGCGTGTCTCATTACCAAA-3′, containing *Mlu*I site and P2 as probes. After cleavage with *Xba*I and *Mlu*I, both cDNA fragments were inserted into the *Xba*I site in pEBD to obtain pCD59#77(N/Q).

2.3. Expression and purification of rCD59#77 and rCD59#77 (N/Q)

For preparation of these protein molecules, CHO cells lacking the *DHFR* gene were transfected with the expression vectors described above with LipofectAMINE (Gibco BRL) according to the manufacturer's protocol. At 72 h after transfection, the medium was changed from αMEM supplemented with 10% fetal calf serum to ribonucleoside- and deoxyribonucleoside-free αMEM supplemented with 10% fetal bovine serum dialyzed against PBS. The transfectants were pooled and then selected in the same medium containing methotrexate, added in stepwise increments from 1 μM to 1 mM. The cells showing the highest expression were cloned by limiting dilution. The expression levels of rCD59#77 and rCD59#77(N/Q) were about 50 mg/l and 20 mg/l, respectively. The culture supernatants were centrifuged at 10000×*g* for 15 min, and the rCD59#77 and rCD59#77(N/Q) proteins were purified with a monoclonal antibody against CD59 as described previously [21].

2.4. Separation of G and NG forms of rCD59#77

To separate the G and NG forms of rCD59#77, 1 mg of the rCD59#77 obtained as described above was dissolved 2 ml of 20 mM Tris-HCl (pH 8.0) and applied to a DEAE-5PW column (Tosoh Co., Ltd., Tokyo, Japan). The bound proteins were eluted with a linear gradient of NaCl from 0 to 0.25 M in 20 mM Tris-HCl (pH 8.0). The NG form was eluted at 0.08 M NaCl, while the G form was recovered in four fractions of 0.11, 0.13, 0.135 and 0.14 M NaCl. Since the complement-inhibitory activities of those four fractions were very close, they were combined and used as the G form in further experiments.

2.5. Removal of sugar chains

For removal of the N-linked sugar chain, 2 μg of rCD59#77 was heated at 95°C for 3 min in 15 mM sodium phosphate buffer (pH 7.5) containing 0.1% SDS, 10 mM 2-mercaptoethanol and 5 mM EDTA. After cooling, 3 U of N-glycanase from Flavobacterium meningosepticum EC 3.5.1.52 (Genzyme Diagnostics, MA, USA) was added, and incubation was performed for 20 h at 37°C in the presence of 1% Nonidet P40 (NP40) and 0.3 mM PMSF. For digestion of sialic acids, 30 μg of the purified G form of rCD59#77 was treated with 1 U of neuraminidase from Arthrobacter ureafaciens EC 3.2.1.18 (Sigma Chemical Co., MO, USA) at 37°C for 20 h in 80 mM sodium acetate buffer (pH 5.0) containing 1.6 mM $CaCl_2$ and 0.3 mM PMSF. The G form thus treated was purified again by affinity chromatography with the monoclonal antibody against CD59.

2.6. Other procedures

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with a 15–25% gradient gel under reducing conditions, and proteins were detected by silver staining. To calculate the isoelectric point (pI) values, rCD59 molecules were separated by electrophoresis in polyacrylamide gel containing ampholine (Pharmacia Biotech, Uppsala, Sweden). The separated proteins were electroblotted to a polyvinylidine difluoride (PVDF) membrane. The membrane with proteins was incubated with 1 μg/ml of biotinylated rabbit antibodies against human CD59 in 20 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 0.1% Tween 20 and 1% BSA, and then with 1 μg/ml horse radish peroxidase-conjugated avidin (Amersham International plc, Buckinghamshire, UK). The blot was developed by a chemiluminescence method using an ECL kit (Amersham). The protein concentrations in the rCD59 preparations were measured with a Micro BCA protein assay kit (Pierce Chemical Co., Rockford, IL).

3. Results and discussion

3.1. Complement inhibitory activities of G and NG forms of rCD59#77

Soluble rCD59#77 was prepared using a gene expression system in CHO cells and purified by affinity chromatography

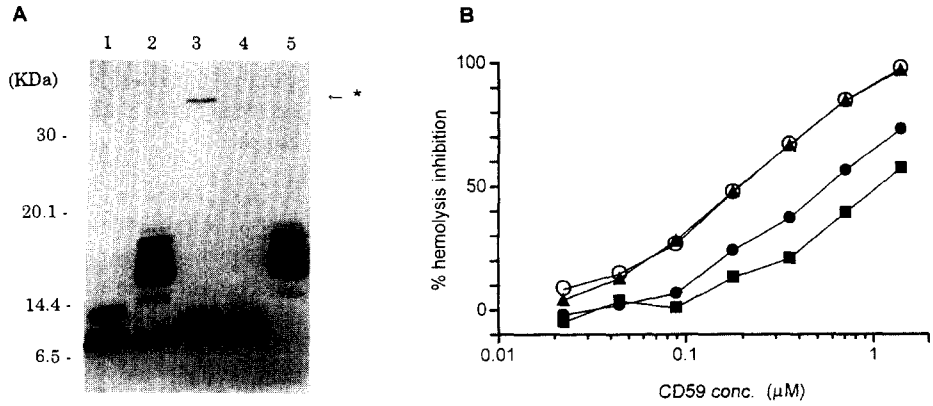

Fig. 1. Separation of G and NG forms of rCD59#77 and their complement inhibitory activities. (A) rCD59#77s which were expressed in insect (lane 1) and CHO (lane 2) cells were affinity-purified with an anti-CD59 antibody. The purified rCD59#77 (CHO) was either treated with N-glycanase (lane 3) or loaded on a DEAE-5PW column to separate the NG form (lane 4) and G form (lane 5). They were analyzed by SDS-PAGE and silver staining. The asterisk indicates N-glycanase (34.6 kDa). (B) The complement-inhibitory activities of these rCD59#77s were assessed in a reactive hemolysis system. The rCD59#77 expressed in insect (○) or CHO (●) cells, NG (▲) and G (■) forms.

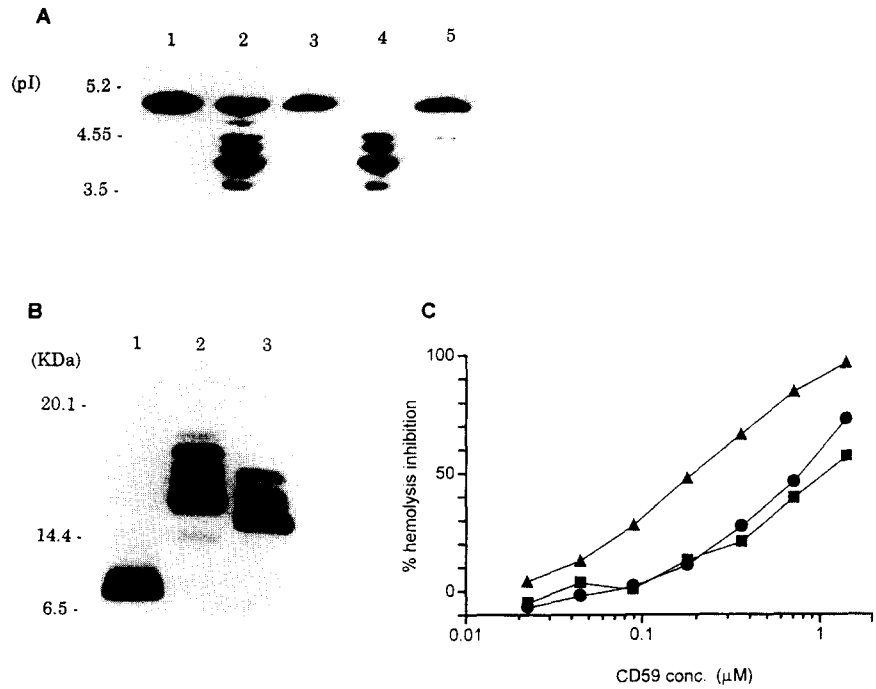

Fig. 2. Treatment of G form with neuraminidase. (A) Isoelectric points. The purified G form of rCD59#77 was treated with neuraminidase and then analyzed by isoelectrofocusing. The proteins were transferred to a PVDF membrane and visualized by immunoblotting with rabbit anti-CD59 antibodies. rCD59#77 produced in insect cells (lane 1), CHO cells (lane 2), NG form (lane 3), G form (lane 4) and G form treated with neuraminidase (lane 5). (B) Molecular weights determined by SDS-PAGE. NG form (lane 1), G form (lane 2) and G form treated with neuraminidase (lane 3). (C) Complement-inhibitory activities of NG form (▲), G form (■) and neuraminidase-treated G form (●) in reactive hemolysis.

with an antibody against CD59. The rCD59#77 preparation was analyzed by SDS-PAGE (Fig. 1A). At least seven protein bands were observed, ranging from 7 to 18 kDa. The smallest protein migrated at the position of 7 kDa, like the smallest protein expressed in the baculovirus expression system [21]. Some proteins produced in CHO cells were larger than the proteins produced by insect cells. Treatment of rCD59#77 with N-glycanase resulted in disappearance of the molecules from 14 to 18 kDa and an increase in the amount of the smallest molecule (Fig. 1A, lane 3). In addition, these protein bands, except the 7 kDa one, were detected as glycoproteins by the periodate oxidation method (data not shown). These results imply that the molecules from 14 to 18 kDa are N-glycosylated proteins, while the 7 kDa molecule is a non-glycosylated one. The differences in the molecular weights seem to be due to heterogeneity in their N-linked sugar moiety. The relative amounts of the G and NG forms of rCD59#77 produced by the CHO cells were estimated to be 4:1 from the densities of the protein bands on SDS-PAGE. It has been shown that CD59 purified from human erythrocytes was all N-glycosylated [17].

The G and NG forms of rCD59#77 were separated by anion exchange chromatography to determine their complement inhibitory activities. These two forms were clearly separated from each other (Fig. 1A, lanes 4 and 5). The complement inhibitory activities were assessed in reactive hemolysis of guinea pig erythrocytes with human C5b6 complexes and human serum as the source of C7, C8 and C9 (Fig. 1B). The concentrations required to inhibit hemolysis by 50% (IC_{50}) were 0.17 and 1.2 μM for the NG and G forms, respectively. Thus, rCD59#77 lacking the sugar chain was 7 times more potent than the major constituent, rCD59#77 having the sugar chain, in inhibiting reactive hemolysis. In addition, another rCD59#77 preparation produced by the baculovirus expression system was as potent as the NG form produced by the CHO cells.

Ninomiya et al. [17] reported that the complement-inhibitory activity of CD59 purified from human erythrocyte membranes decreased by 90% after N-glycanase treatment and that the CD59-transfected CHO cells reduced the resistance to complement attack after growth in the presence of the 1-deoxymannojirimycin, which inhibits the conversion of high mannose-type to complex-type glycoproteins. Their findings were thus inconsistent with ours. In their experiments, CD59 was treated with N-glycanase by incubation for 24 h at pH 8.5 in the presence of 0.1% NP40. There is a possibility that CD59 lacking the sugar moiety is unstable under these conditions, thus decreasing its activity. In addition, 1-deoxymannojirimycin affects not only CD59 but also all glycosylated proteins, and this broad effect may make the cells less resistant to complement attack.

3.2. Effect of sialic acids on the activity of G form

Since baculovirus rCD59#77 was as active as the NG form and was assumed to have a high mannose-type sugar chain, which does not contain sialic acids, we postulated that the sialic acids hinder the access of CD59 molecules to nascent MAC on the cell surface, which is abundant in sialic acids because of their electric repulsion. In fact, erythropoietin showed 3 times higher activity after removal of sialic acids from the sugar moiety [22]. Thus, the sialic acids on the sugar moiety of rCD59#77 were removed with neuraminidase to test our hypothesis. In isoelectric focusing, NG form and baculovirus rCD59#77 showed an pI of 5.0, which is consistent with

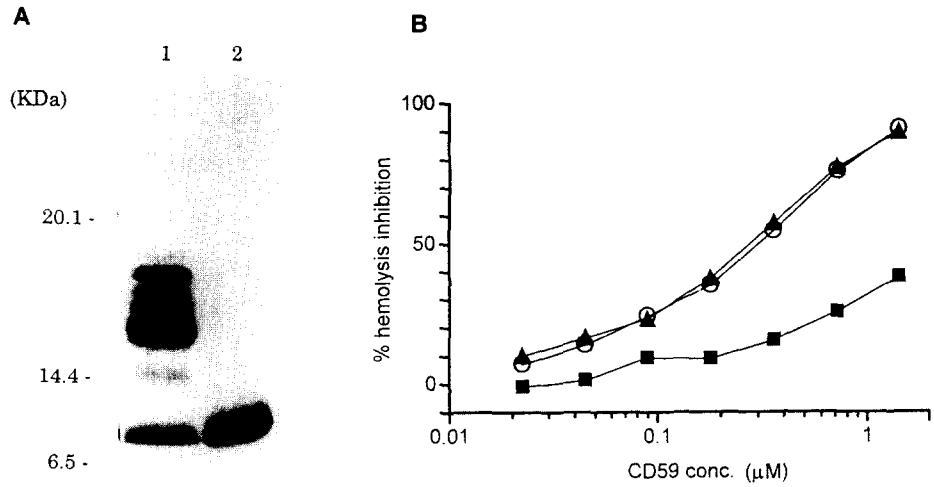

Fig. 3. A mutant molecule, rCD59#77(N/Q), and its complement-inhibitory activity. Mutated rCD59#77(N/Q), in which Gln was substituted for Asn[18], was expressed in CHO cells and purified by affinity column chromatography. (A) Molecular weights. The wild-type rCD59#77 (lane 1) and rCD59#77(N/Q) (lane 2). (B) Complement-inhibitory activities in reactive hemolysis. rCD59#77(N/Q) (○), NG form (▲) and G form (■) of rCD59#77.

the value calculated from the primary a.a. sequence (Fig. 2A). The pI values of rCD59#77 produced in CHO cells were more acidic, ranging from 4.7 to 3.8. The desialylated G form of rCD59#77 showed a single band at pI 5.0, implying the complete removal of sialic acids from the rCD59#77 molecules. The complement inhibitory activity of the desialylated G form was not increased and remained less potent than that of the NG form (Fig. 2C). Therefore, the decreased activity of the G form is not attributable to the presence of sialic acids.

The molecular weights of desialylated G form became only a little smaller than the original G form, ranging from 14 to 17 kDa (Fig. 2B). The molecular weights of the sugar moiety were still as large as 7 to 10 kDa, while the peptide is as small as 7 kDa. These data suggest that the bulky sugar chains sterically hinder the interaction of rCD59#77 with C8 and C9.

3.3. *A mutant molecule, rCD59#77(N/Q,) which lacks the N-linked sugar*

To confirm that the sugar moiety of rCD59#77 decreases the complement inhibitory activity, a plasmid expressing rCD59#77(N/Q), in which Gln was substituted for Asn[18], was constructed. The rCD59#77(N/Q) protein was expressed by transfecting the plasmid into CHO cells. rCD59#77(N/Q) showed a single protein band of 7 kDa in SDS-PAGE (Fig. 3A), indicating that the protein is not glycosylated at all. The complement inhibitory activity of rCD59#77(N/Q) was as high as that of the NG form, and its IC_{50} was 0.20 μM (Fig. 3B). Furthermore, other non-glycosylated mutants, i.e., CD59#77(S/G) and rCD59#77(S/A), in which Ser[20] was changed to Gly or Ala, were also as potent as the NG form (data not shown). These results indicate that the N-linked sugar moiety at Asn[18] decreases the complement inhibitory activity of rCD59#77. The fact that the N-linked sugar is not necessary for the complement inhibitory activity and actually hampers the access to nascent MAC on the cell surface is not restricted to soluble CD59#77: the same case seems to apply to membrane-bound CD59, as shown in a short communication [18]

The results shown here were unexpected because in general sugar moieties are necessary for the full biological activitiy of glycoproteins. For instance, N-glycanase digestion resulted in loss of the biological activities of glucose transporter [23] and complement component C9 [24]. Urokinase plasminogen activator receptor [25], which has homology with CD59 in the a.a. sequence, showed a decreased affinity for the ligand after the N-glycosylation sites were removed by mutation [25].

Recent studies on the three-dimensional structure of recombinant truncated CD59 (Leu[1] to Asn[70]) and CD59 purified from urine have revealed that CD59 is disc-shaped with a diameter of 30 Å and a height of 15 Å and closely packed with five disulfide bonds [26,27]. On the basis of this structure, Fletcher et al. [26] suggested that its inhibitory activity involves two loops composed of the 7th to 14th and the 20th to 24th amino acid. In addition, a synthetic peptide consisting of Cys[19] to Cys[39] was as active as urinary CD59 in its inhibition of reactive hemolysis [28]. These findings suggest that the 20th to 24th amino acid loop seems to be important for complement-inhibitory activity. Since Asn[18] is close to the active site, it is highly likely that the bulky sugar moiety linked to Asn[18] hampers the interaction with C8 and C9.

MAC formation plays an important role in the pathogenesis of inflammatory diseases such as ischemia and autoimmune diseases [29,30]. CD59 prevents cells from complement attack by interacting with activated C8 and C9 on nascent MAC [1]. In human myocardial infarction, CD59 disappeared and MAC was concomitantly deposited at the infarcted lesions [12]. In these pathogenic conditions, administration of soluble CD59 is anticipated to be effective for suppressing the MAC formation which accompanies inflammation. Our present findings suggest that non-glycosylated CD59#77 is more effective than the glycosylated protein.

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