Immunoinhibitory effect of soluble HLA-G1 on the cytotoxicity of NK92 cells*

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Abstract HLA-G (human leukocyte antigen-G) is a non-classical HLA class I molecule, playing an important immuno-modulatory role in maintaining maternal immune tolerance of the semiallogenic fetus and organ transplantation. In this study, the cDNA sequence of extracellular domain of HLA-G1 was subcloned into the pET28a vector and a soluble 35 kD fusion protein (His-sHLA-G1) with six histine residues was obtained. In the 4 h ^{51}Cr-release assay the fusion protein obviously inhibited the cytotoxicity of NK92 cells in a dose-dependent manner. These results indicated that sHLA-G1, as an activated immunoinhibitor, may provide an effective approach to overcoming the immune rejection of transplantation.

Keywords: sHLA-G1, NK92, cytotoxicity, immunoinhibition.

Ever since the role of the immune system in transplantation rejection was discovered in the middle of the 20th century, biologists have been trying to find out a variety of strategies to make the graft survive in the host. The hyperacute rejection can be avoided or inhibited by clearing natural antibody and blocking the activation of complement in xeno-transplantation. The presence of delayed xenograft rejection caused by the infiltration of macrophages and NK (natural killer) cells is the biggest obstacle in transplantation.

The pregnancy of mammalian species provides a natural successful immunotolerance model of semiallograft. Though a large number of NK cells are found in the uterine deciduas, semi-allogenic fetus is not attacked and subsequently rejected. Different mammalian species seem to use a variety of strategies to avoid maternal immune attack, but one common of them is the altered expression of major histocompatibility complex (MHC) class I molecules on trophoblast[1]. In humans, the classical class I molecules HLA-A, -B are not expressed on trophoblast, but low-level HLA-C and high-level non-classical class I molecules HLA-G and -E are expressed[1,2].

The primary mRNA of HLA-G is alternatively spliced into seven transcripts, which might encode four membrane-bound isoforms (G1 ~ G4) and three soluble isoforms[3~5]. Soluble HLA-G has been reported to be secreted by trophoblast at the maternal-fetal interface and also been detected in the amniotic fluid and serum of pregnant women. As an effective immunomodulator, it has been demonstrated to induce apoptosis of activated CD8$^+$ T cells through the interaction with CD8 molecules[6], and to modulate the NK and allo-cytotoxic T lymphocyte responses[7]. Furthermore, the expression of soluble HLA-G has been found to be associated with a decrease incidence of acute rejection episodes and an absence of chronic rejection in patients undergoing heart transplantation[8].

However, the physiological resources of sHLA-G are absent and the purification of activated sHLA-G molecules is very expensive. In our present study, a large quantity of sHLA-G1 have been yielded by gene engineering and its activity has been examined by a 4 h ^{51}Cr-release assay, which has a potential in clinical applications.

1 Materials and methods

1.1 Construction of pET28a/sHLA-G1 vector

The encoding sequence of extracellular domain of HLA-G1 was amplified by PCR using a set of primers containing *Bam*H I and *Hin*d III restriction sites, with pGEM-T Easy/HLA-G1 vector as the source.

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The PCR product was digested with *Bam*H I and *Hin*d III and ligated into pET28a expression vector using T4 DNA ligase. The upstream primer containing *Bam*H I restriction site was 5′-TTAGGAT-CCG-GCTCCCACTCCATG-3′, and the downstream primer containing *Hin*d III restriction site was 5′-CCTAAGCTTCCATCTCAGCATGAGGG-3′. PCR reactions were conducted under the following conditions: 94 ℃ 3 min, 1 cycle; 94 ℃ 1 min, 62 ℃ 1 min, 72 ℃ 1 min, 30 cycles; 72 ℃ 10 min. The kanamycin-resistant transformants were confirmed by cloning PCR, restriction mapping and subsequent sequencing.

1.2 Induction of His-sHLA-G1 fusion protein

E. Coli BL21(DE3) competent cells were transformed with pET28a/sHLA-G1 vector. The transformants were selected on LB plates with 30 μg/mL kanamycin and cultured in LB medium at 37 ℃ with shaking to an OD_{600} value of 0.6～1. Isopropyl-β-D-thiogalactopyranoside (IPTG, Promega) was added to a final concentration of 1 mmol/L to induce the expression of the protein. After 3～5 h, the whole cell lysates were collected by centrifugation.

1.3 Purification of His-sHLA-G1 fusion protein

The bacterial pellet was resuspended in sonication buffer (20 mmol/L Na_3PO_4, 500 mmol/L NaCl, pH7.8) with 100 μg/mL lysozyme (Promega) and incubated for 15 min on ice. Cells were then lysed by sonication with five 15-second bursts, at the interval of 10 seconds. The inclusion bodies were collected by centrifugation of the lysate at 3000 g for 15 min and were solubilized in denaturing solubilization buffer A (6 mol/L guanidine-HCl, 20 mmol/L Na_3PO_4, 500 mmol/L NaCl, pH7.8). After being agitated for 15 min, the solution was centrifuged for 15 min at 3000 g and the supernatants were collected to bind with ProBond™ resin (Invitrogen) in a column for 15 min. The ProBond™ resin was then transferred into denaturing solubilization buffer B (8 mol/L urea, 20 mmol/L Na_3PO_4, 500 mmol/L NaCl, pH7.8) and the mixture was stirred for 15 min for equilibrating. The ProBond™ resin was then transferred into denaturing washing buffer (8 mol/L urea, 20 mmol/L Na_3PO_4, 500 mmol/L NaCl, pH6.0) and the mixture was stirred for 15 min for equilibrating. These steps were repeated until the A_{280} value of supernatant was less than 0.1. The column was eluted with linear imidazole gradient elution buffers (8 mol/L urea, 20 mmol/L Na_3PO_4, 500 mmol/L NaCl, pH6.0, with increased imidazole of 37.5 mmol/L, 75 mmol/L, 150 mmol/L and 300 mmol/L), with being stirred for 15 min each time. The fusion protein was eluted by 300 mmol/L imidazole elution buffer finally. The samples of each stage were analyzed by SDS-PAGE analysis to detect the fusion protein (except the sample containing guanidine-HCl).

1.4 Renaturing of the fusion protein

The protein eluted was dialyzed against urea-containing PBS buffers at 4 ℃ (the concentration of urea descended from 8, 6, 4, 2, 1, 0.5, to 0 mol/L). The fusion protein was solubilized in PBS or dried for a long-term storage.

1.5 Western blot

The monoclonal antibody 87G, against HLA-G1 and G5, was provided by D. Geraghty, Fred Hutchinson Cancer Research Institute, USA.

The whole cell lysates were separated onto a 12% SDS-polyacryamide gel and transferred onto nitrocellulose membranes. The membranes were reacted with the 87G antibody overnight at 4 ℃, followed by an incubation for 1～2 h at room temperature with peroxidase-conjugated goat anti-mouse IgG. After washing, the color was developed by a LumiGLO™ kit.

1.6 Cytotoxicity assay

1.6.1 Cell lines The human erythroleukemia K562 cell line (HLA-A, -B, -C and -G-negative) was maintained in RPMI1640 medium (GIBCO) supplemented with 10% fetal calf serum (FCS).

NK92 cells were cultured in a α-MEM (GIBCO)-based medium supplemented with 2 mmol/L L-glutamine, 1.5 g/L sodium hydrogen carbonate, 12.5% horse serum, 12.5% FCS, 0.2 mmol/L inositol, 0.1 mmol/L α-mercaptoethanol, 0.02 mmol/L folic acid and 100 U/mL recombinant IL-2.

1.6.2 4-h ^{51}Cr release assays Nk92 cells, used as effector cells (E), were preincubated for 30 min at 4 ℃ with fusion His-sHLA-G1 protein at the concentration of 10 μg/mL and 20 μg/mL. Killing of the target cells (T) K562 mediated by NK92 cells was tested in a 4-h ^{51}Cr release assay as described previously[9].

2 Results

2.1 Analysis of restriction mapping and sequencing

The fragment of PCR product, amplified with a pair of primers containing *Bam* H I and *Hin* d III restriction site respectively, was 837 bp. The recombinant plasmid was analyzed by the restriction mapping (Fig. 1).

The extra-cellular domain of HLA-G1 was confirmed by sequencing and GenBank sequence alignment. It had 274 amino acids and contained α1, α2, and α3 chains, lacking the trans-membrane and cytoplasmic domains. The total molecular weight of this fusion protein (including six histidine residues at its N-terminus and some other residues encoded by sequence of the vector) was approximately 35 kD. The protein sequence translated by Vector NTI Suite 6 is shown in Fig. 2.

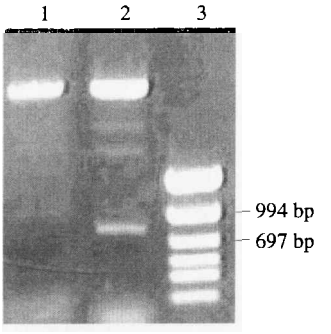

Fig. 1. Restriction analysis of recombinant pET28a/sHLA-G1. 1, *Bam* H I and *Hin* d III restriction fragments of pET28a vector; 2, *Bam* H I and *Hin* d III restriction fragments of recombinant pET28a/sHLA-G1 vector; 3, DNA marker.

GGC TCC CAC TCC ATG AGG TAT TTC AGC GCC GCC GTG TCC CGG CCC GGC CGC
Gly Ser His Ser Met Arg Tyr Phe Ser Ala Ala Val Ser Arg Pro Gly Arg

GGG GAG CCC CGC TTC ATC GCC ATG GGC TAC GTG GAC GAC ACG CAG TTC GTG
Gly Glu Pro Arg Phe Ile Ala Met Gly Tyr Val Asp Asp Thr Gln Phe Val

CGG TTC GAC AGC GAC TCG GCG TGT CCG AGG ATG GAG CCG CGG GCG CCG TGG
Arg Phe Asp Ser Asp Ser Ala Cys Pro Arg Met Glu Pro Arg Ala Pro Trp

GTG GAG CAG GAG GGG CCA GAG TAT TGG GAA GAG GAG ACA CGG AAC ACC AAG
Val Glu Gln Glu Gly Pro Glu Tyr Trp Glu Glu Glu Thr Arg Asn Thr Lys

GCC CAC GCA CAG ACT GAC AGA ATG AAC CTG CAG ACC CTG CGC GGC TAC TAC
Ala His Ala Gln Thr Asp Arg Met Asn Leu Gln Thr Leu Arg Gly Tyr Tyr

AAC CAG AGC GAG GCC AGT TCT CAC ACC CTC CAG TGG ATG ATT GGC TGC GAC
Asn Gln Ser Glu Ala Ser Ser His Thr Leu Gln Trp Met Ile Gly Cys Asp

CTG GGG TCC GAC GGA CGC CTC ATC CGC GGG TAT GAA CGG TAT GCC TAC GAT
Leu Gly Ser Asp Gly Arg Leu Ile Arg Gly Tyr Glu Arg Tyr Ala Tyr Asp

GGC AAG GAT TAC CTC GCC CTG AAC GAG GAC CTG CGC TCC TGG ACC GCA GCG
Gly Lys Asp Tyr Leu Ala Leu Asn Glu Asp Leu Arg Ser Trp Thr Ala Ala

GAC ACT GCG GCT CAG ATC TCC AAG CGC AAG TGT GAG GCG GCC AAT GTG GCT
Asp Thr Ala Ala Gln Ile Ser Lys Arg Lys Cys Glu Ala Ala Asn Val Ala

GAA CAA AGG AGA GCC TAC CTG GAG GGC ACG TGC GTG GAG TGG CTC CAC AGA
Glu Gln Arg Arg Ala Tyr Leu Glu Gly Thr Cys Val Glu Trp Leu His Arg

TAC CTG GAG AAC GGG AAG GAG ATG CTG CAG CGC GCG GAC CCC CCC AAG ACA
Tyr Leu Glu Asn Gly Lys Glu Met Leu Gln Arg Ala Asp Pro Pro Lys Thr

CAC GTG ACC CAC CAC CCT GTC TTT GAC TAT GAG GCC ACC CTG AGG TGC TGG
His Val Thr His His Pro Val Phe Asp Tyr Glu Ala Thr Leu Arg Cys Trp

GCC CTG GGC TTC TAC CCT GCG GAG ATC ATA CTG ACC TGG CAG CGG GAT GGG
Ala Leu Gly Phe Tyr Pro Ala Glu Ile Ile Leu Thr Trp Gln Arg Asp Gly

GAG GAC CAG ACC CAG GAC GTG GAG CTC GTG GAG ACC AGG CCT GCA GGG GAT
Glu Asp Gln Thr Gln Asp Val Glu Leu Val Glu Thr Arg Pro Ala Gly Asp

GGA ACC TTC CAG AAG TGG GCA GCT GTG GTG GTG CCT TCT GGA GAG GAG CAG
Gly Thr Phe Gln Lys Trp Ala Ala Val Val Val Pro Ser Gly Glu Glu Gln

AGA TAC ACG TGC CAT GTG CAG CAT GAG GGG CTG CCG GAG CCC CTC ATG CTG
Arg Tyr Thr Cys His Val Gln His Glu Gly Leu Pro Glu Pro Leu Met Leu

AGA TGG
Arg Trp

Fig. 2. Nucleotide and amino acid sequences of sHLA-G1.

2.2 Expression and purification of fusion HLA-G1 protein

The pET28a/sHLA-G1 recombinant was transferred into BL21 (DE3) *E. Coli* and induced by IPTG for protein expression. As shown in Fig. 3, sHLA-G1 (T5-p35) occupies 30% of total protein of the cells. After purification by ion-chromatography and renaturing, this protein has the purity of 95%.

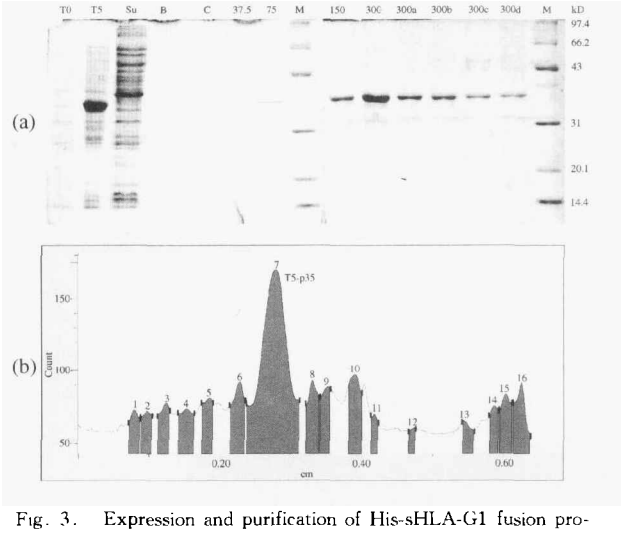

Fig. 3. Expression and purification of His-sHLA-G1 fusion protein. (a) PAGE result. T0, total cell protein before induction; T5, total cell protein after induction for 5 h; Su, the protein in supernatant after sonication; B and C, fractions eluted by denaturing solubilization buffer B and C, respectively; 37.5, 75, 150, 300 are proteins eluted by different concentrations of imidazole buffer (mmol/L); 300 a~d indicate 5 times of elution; M, protein standards. (b) His-sHLA-G1 (T5-p35) occupies 30% of total cell protein through profile analysis.

2.3 Western blot analysis

The expressed His-sHLA-G1 was confirmed by its reaction with 87G antibody in Western blot analysis (Fig. 4). There was no expression of sHLA-G1 in the total cell protein before induction by IPTG.

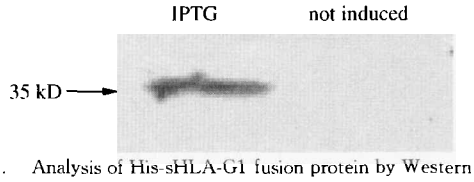

Fig. 4. Analysis of His-sHLA-G1 fusion protein by Western blot.

2.4 Inhibition of cytolysis by His-sHLA-G1 in NK92 cells

The 4h-^{51}Cr release assay was carried out with the HLA-class I negative human cell line K562 as the target cells. His-sHLA-G1, at the concentrations of 10 μg/mL and 20 μg/mL, did not block the cytolysis of NK92 at a E:T ratio of 5:1. However, when the number of effector cells reduced, e.g. at E:T ratios of 2.5:1 and 1.25:1, His-sHLA-G1 inhibited cytolytic activity of NK92 in a dose-dependent manner (Fig. 5). The highest inhibition was observed when the E:T ratio was 1.25:1 and fusion protein was at concentration of 20 μg/mL.

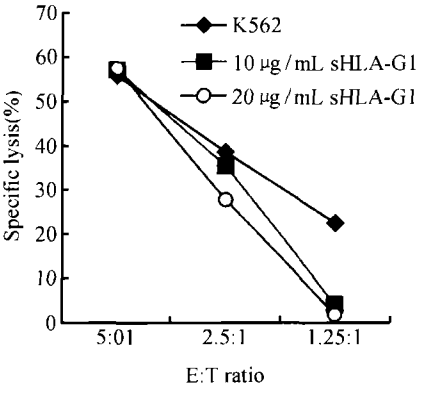

Fig. 5. Effect of His-sHLA-G1 on NK92 mediated lysis of K562.

3 Discussion

HLA-G is a non-classical MHC class I molecule. Its alternatively high level expression has been found on extravillous cytotrophoblasts and it has also been detected in the endothelial cells of placenta villous blood vessel in the first-trimester of pregnancy, amnion epithelial cells, thymic epithelial cells and activated macrophages and dendritic cells during viral infections and some carcinoma cells or tissues[3]. The full-length HLA-G1 molecule is encoded by six exons, with exon 1 encoding leader peptide and exon 5 and exon 6 encoding transmembrane region and intracellular regions respectively[2]. The presence of a stop codon within intron 4 of the alternatively spliced product prevents the translation of both transmembrane and cytoplasmic domains, resulting in a soluble HLA-G1 (also termed as HLA-G5) molecule. His-sHLA-G1 we produced in this study is not associated with β_2-microglobulin, but the native HLA-G1 does.

In order to study the function of His-sHLA-G1, we carried out the 4-h ^{51}Cr release assay. The soluble His-sHLA-G1 was shown to obviously inhibit the cytolytic activity of NK92 cells in a dose-dependent manner. With the result showed by Marchal-Bras-Goncalves et al.[10], we speculate that this kind of unglycosylated soluble fusion protein obtained through the prokaryotic expression system has an active im-

munoinhibitory function.

The inhibition of NK92 cytotoxicity induced by His-sHLA-G1 may result from the interaction of sHLA-G1 with NK receptors. NK92 is an active, interleukin-2(IL-2)-dependent human natural killer cell line. It expresses a relatively large number of activating (NKp30, NKp46, 2B4, NKGD, E, CD28) receptors and a few inhibitory receptors (NKGA/B, low levels of KIR2DL4, ILT-2), while lacking most of the killer inhibitory receptors (KIRs) clonally expressed on normal NK cells[11]. The ILT2 and KIR2DL4 (p49) receptors expressed on the uterine decidual were identified to directly recognize the membrane-bound HLA-G[4]. Because His-sHLA-G1 contains most functional domain of full-length HLA-G, it might take the same recognization pathway as the latter one.

In mixed lymphocyte cultures, Lila et al. observed that the soluble HLA-G5 protein but not the membrane-bound HLA-G isoform was secreted by allo-specific $CD4^+$ T cells, which suppressed the allogeneic proliferative T cell response[7]. In addition to allo-specific $CD4^+$ T cells, the soluble HLA-G5 molecules were also detected in the cytotrophoblast, activated placental macrophages, and the serum and amniotic fluid of pregnant women[6,7]. In this regard, the soluble HLA-G5 was speculated to be responsible for the allo-geneic reaction against paternal antigens during pregnancy[7]. And what is more important is that HLA-G has been identified to be closely related to the rejection of organ transplantation, especially to heart transplantation. The membrane-bound and soluble HLA-G have been found to be associated with a decreased incidence of acute rejection episodes and an absence of chronic rejection in patients undergoing heart transplantation[7,9].

GST-HLA-G fusion protein obtained by Marchal-Bras-Goncalves et al. could inhibit the cytotoxic activity of freshly obtained NK cells from peripheral blood and YT2C2-PR NK cells[10]. The His-sHLA-G1 fusion protein we obtained also inhibited activated NK92 to kill K562 cells. All of the evidence suggested that the soluble HLA-G1, very similar to the native HLA-G5, can not only suppress allogeneic proliferative T cell response, but also functions as immunomodulators by inhibiting the innate cytotoxicity of NK cells. In the maternal-fetal immunotolerance, a large quantity of soluble HLA-G secreted into amniotic fluid and then into the peripheral blood might produce immunotolerant effect of system. So when NK cells become the major lymphoid cells that are involved in rejection, this inhibitory capacity would be invaluable during immune response following transplantation. As soluble immunoinhibitors, sHLA-G1 produced by genetic engineering could become a new alternative immunotherapy aimed at inducing immune tolerance during allograft, xenograft, and autoimmune diseases.

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