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Identification of Amino Acid Positions Involved in HLA-E Expression

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The cell surface expression of HLA-E molecules by transfection is faint in xenogeneic cells. Therefore, this study was done for the aim of better expression of HLA-E molecules on the surface of pig cells in order to overcome xenograft rejection mediated by human natural killer (NK) cells. The importance of the loading peptide sequence for HLA-E expression has been studied extensively, but much less information is available concerning the HLA-E heavy chain sequence. In our previous study, we developed the S147C substitution of HLA-E as a useful gene tool for xenotransplantation. In this study, a more extensive substitution analysis throughout the entire region led to the identification of nine amino acid positions, positions-9, 11, 25, 40, 66, 67, 74, 99 and 174, that are significantly involved in the cell surface expression of HLA-E molecules. In view of xenotransplantation usage, double and triple point substitutions, HLA-Ev(11,147) and HLA-Ev(11,66,147), were constructed. These constructs led to a high expression on the xenogeneic cell surface and possessed inhibitory functions against human NK cell-mediated cytolysis in an *in vitro* pig to human xenotransplantation model system.

Key words: acute rejection, HLA-E, NK, porcine endothelial cell, xenotransplantation.

Abbreviations: NK, natural killer cell; PEC, porcine endothelial cell; HLA, human leucocyte antigen; SLA, swine leucocyte antigen; CHO, Chinese hamster ovary tumor; DMEM, Dulbecco's modified eagle's medium; FBS, fetal bovine serum; mAb, monoclonal antibody; TM, transmembrane; β_2m, β_2 microglobulin.

Xenotransplantation is one of the most attractive solutions for solving the worldwide organ shortage. However, at least three types of xenograft rejections, hyperacute, acute and chronic rejections need to be overcome (*1*). The first hurdle, hyperacute rejection, has been overcome by the genetic manipulation of pig organs to express human complement regulatory proteins and/or to be knocked out the α-1,3-galactosyltransferase gene (α-1,3-GT) (*2–5*). As a subsequent hurdle, so-called acute vascular rejection, characterized by tissue infiltration by macrophages and natural killer (NK) cells, has now become a subject of great interest.

NK cells express various receptors that play roles in decisions concerning subsequent actions. The activation of NK cells is controlled by a balance of signals between inhibitory and activating receptors. In particular, several types of NK receptors that recognize HLA class I molecules, such as NKG2A/CD94 hetero dimeric receptor and KIR2DL4, are able to transmit a major turn-off signal to the cell (*6*, *7*). On the other hand, pig cells are highly susceptible to human NK cell-mediated cytotoxicity, presumably due to the mismatch between the swine leucocyte antigen (SLA), counterpart genes of HLA, and human NK inhibitory receptors (*8*).

HLA-G and HLA-E, which are classified into a special group known as non-classical class I molecules, also designated as HLA-class Ib, have recently attracted attention as immune tolerant molecules for fetus–maternal immunity and tumour immunity (*9*, *10*). Both molecules are quite effective in down-regulation of NK cell activity via various NK inhibitory receptors. Therefore, in our previous studies, we attempted to apply these non-classical HLA genes in xenotransplantation (*11*, *12*). In the case of HLA-G1, the cell surface expression of HLA-G1 could be easily observed on pig cells by the introduction of the HLA-G1 gene. On the other hand, HLA-E was expressed little on the pig cell surface despite the high expression of mRNA (*11*, *12*). HLA-G1 and HLA-E have a high homology (78.6% of amino acid in their extra cellular domain), which indicated that the remaining amino acid positions defined the difference of expression profile of these molecules. In our recent work, we reported the substitution of 147-Ser to Cys resulted in the enhanced expression of HLA-E on xenogeneic cell and the increased NK-inhibitory activity. In this study, a more extensive substitution analysis was performed to define the other positions related to the expression. We also discussed the newly constructed gene tools, HLA-Ev(11,147) and HLA-Ev(11,66,147), as

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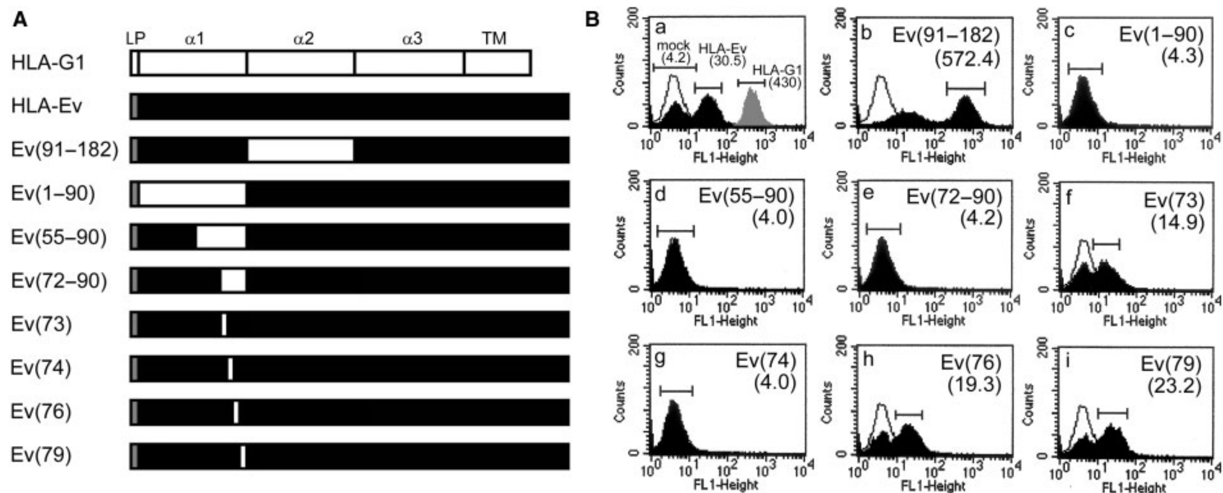

Fig. 1. **Schematic presentation of gene construction and the expression profiles in transfection experiments.** CHO-β cells, CHO cells transfected with the human β_2m (β_2 microglobulin) gene, were transfected with the constructed genes (*11*). In this figure, only a part of all constructs is shown for avoiding a complication. Cell surface expressions were analysed by flow cytometry using an anti pan-MHC class I antibody, B9.12.1. (A) Schematic presentation of the constructed chimeric genes. LP, α1, α2, α3 and TM: leader peptide, α1 domain, α2 domain, α3 domain and transmembrane domain, respectively. White and black boxes indicate HLA-G1 and HLA-E sequences, respectively. The leader peptide indicated as gray boxes was previously described (*17*) and used for the following construction. This leader peptide possessed V instead of F at 10th position of the HLA-G1 leader peptide sequence, *i.e.* MVVMAPRTL**V**LLLSGALT LTETWA (*17*). (B) Flow cytometric profiles of the transfected genes on CHO-β cells. Open histogram indicates the mock transfectant. Closed histogram indicates the transfectants with constructed genes. The expression of stable transfectant of HLA-G1 is also indicated in the first panel as gray colour. Parenthesis indicates the mean fluorescence intensity ($n = 3$). The horizontal bar shows the region calculated.

candidates for regulators of human NK-cell mediated xenogeneic cell rejection.

MATERIALS AND METHODS

Cell Culture—Chinese hamster ovary tumour (CHO) cells were obtained from the American Type Culture Collection (VA, USA) and were cultured in Ham's F-12 medium. The porcine endothelial cell (PEC) line, MYP30, was established and cultured in Dulbecco's modified Eagle's medium (DMEM) (*13*). These culture media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) and kanamycin/amphotericin B. The cultures were maintained in 5% CO_2/95% air atmosphere at 37°C. Transfectants of CHO cells and PEC with the human β_2 microglobulin (β_2m) gene, CHO-β and PEC-β have been established in our previous work (*11*).

Preparation of Gene Constructs—The cDNA cloning of HLA-G1 and HLA-E (HLA-E*01032) genes has been described previously (*11*). The gene constructions were performed by the overlap extension PCR method using four specific primers corresponding to the substitution points. The reaction products were flanked by Sma I and Xho I sites and cloned into the pCXN$_2$L expression vector (CMV enhancer, chicken beta actin promoter, neomycin resistant gene) (*14*). The DNA sequences were confirmed by means of an ABI-310 genetic analyzer (Applied Biosystems, CA, USA) (Fig. 1A).

Establishment of Stable Transfectants—The plasmid constructs (20 μg) were introduced into CHO-β and PEC-β cells by electroporation, as described previously (*12*). The transfected cells were maintained in complete medium for several days, and then transferred to selection medium containing 0.7 mg/ml G418 (Calbiochem, CA, USA) for PEC, 1.0 mg/ml for CHO cell. More than 200 of stable colonies on several culture dishes were combined and used as a bulk mixture. Single stable clones were also isolated by the limiting dilution method.

Flow Cytometry Analysis—The stable clones were pre-cultured at three different temperature conditions depending on the experiments, *i.e.* overnight at 37°C, overnight at 26°C, and 1 h at 37°C following overnight culture at 26°C. The stable clones (1×10^6 cells) were incubated with 4 μg/ml of a mouse monoclonal antibody (mAb) B9.12.1 (anti-human MHC class I molecule), that is able to react with both HLA-G1 and E (Cosmo Bio, Japan), for 1 h at 4°C, followed by incubation with a 40 μg/ml solution of fluorescein isothiocyanate (FITC)-labelled rabbit anti-mouse IgG Ab (MP biomedicals, CA, USA) as a secondary Ab for 1 h at 4°C. The stained cells were analysed by means of FACS Calibur flow cytometer (BD Biosciences, NJ, USA). Isotypic irrelevant control IgG2a (MP biomedicals, CA, USA) was also used as the first Ab at 4 μg/ml.

Peptide Binding Assay—The oligo peptides, RIIPRHLQL, KIPAQFYIL, MAPRTLLLL, VMAPRTLVL and VMAPRTLFL, were synthesized using an ABI-432A peptide synthesizer (Applied Biosystems, CA, USA). RIIPRHLQL and KIPAQFYIL are the peptides which can be bound to HLA-G1. MAPRTLLLL is the leader sequence of the wild-type HLA-E which cannot be bound to HLA-E as a negative control. VMAPRTLVL and VMAPRTLFL are the leader sequences of HLA-A2 and HLA-G, respectively, which can be reportedly bound to HLA-E, the former are recognized exclusively by

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the CD94/NKG2A inhibitory receptor and the latter the CD94/NKG2C activating receptor (*15*). Various concentrations of the synthetic peptides were added to the culture medium and cultured overnight at 26°C prior to the flow cytometry analysis according to a previously described method (*16*).

NK Cell-Mediated Cytotoxicity Assay—Target cells (1.5×10^4) were seeded in each well of a 96-well micro plate and cultured for 16 h prior to assay. The cells were then incubated in complete medium supplemented with 10 μCi/ml $Na_2{}^{51}CrO_4$ at 37°C for 2 h. Peripheral blood was obtained from a healthy volunteer, and NK cells were purified by negative selection, $CD3^-$, $CD4^-$, $CD14^-$, $CD19^-$, $CD66b^-$, glycophorin A^-, with a magnetic cell sorter using the StemSep^{TM} Human NK Cell Enrichment Cocktail (StemCell Technologies, Canada) following Ficoll-Hypaque method. After washing the micro titer plate, purified NK cells were added to each well ($E/T = 10:1$), followed by incubation for 4 h at 37°C. The released ^{51}Cr was measured with a γ-counter. The spontaneous release of ^{51}Cr from the target cells was <20% of the maximum release, as determined by a complete target cell lysis by treatment with 10% Triton-X100 (*12*).

Northern Blotting—Total RNA was isolated from transfectant and naive cells with the TRIZOL reagent (Invitrogen, CA, USA). Aliquots containing 25 μg of total RNA were separated by electrophoresis and transferred to a nylon membrane. The hybridization signals on the northern blots were developed using an ECL detection system (Amersham, UK) with specific probes that were prepared from the cDNA inserts of the HLA-E gene (*11*).

Western Blotting—Total cell lysate was prepared from transfectants with lysis buffer. Aliquots containing 50 μg of total cell lysate were separated by denature condition of SDS-PAGE and transferred to a nitrocellulose membrane, Hybond ECL (Amersham, UK). The signals on the western blot were developed using an ECL detection system (Amersham, UK) following incubation with 20 μg/ml of MEM-E/02 as first Ab (anti-HLA-E, MBL, Japan) and HRP labelled rabbit anti-mouse IgG Ab (MP biomedicals, CA, USA).

RESULTS AND DISCUSSION

Identification of Important Amino Acid Positions, 9, 25, 40, 67, 74 and 99, for HLA-E Expression—In our previous studies, we reported on the expression and NK cell inhibitory function of HLA-G1 and HLA-E in a pig cell system (*11*, *12*). However, the cell surface expression amounts of these two class Ib molecules were quite different. That is, HLA-G1 can be expressed but HLA-E cannot be expressed sufficiently on the pig cell surface by introduction of the corresponding expression vector constructs. Therefore, it would be helpful for the development of gene modified pigs in clinical xenotransplantation to analyse the expression profiles by substitution analysis between these two molecules.

In this study, the HLA-Ev sequence constructed previously was used as a parent sequence for the following gene constructions. HLA-Ev is designed to possess the typical binding sequence, VMAPRTLVL, in its leader peptide region (*17*). In our previous study, the chimeric gene of HLA-Ev with the α3 and transmembrane (TM) sequence of HLA-G1 did not change the expression profile significantly, suggesting that the difference between HLA-E and HLA-G1 in the amino acid sequences of this region, *i.e*. position-183, 194, 195, 197, 214, 219, 223, 224, 228, 270 and 271, were not significantly important for the expression feature of HLA-E (*17*). This result was confirmed by the construction of each single point mutants that have been replaced with single amino acid corresponding to the amino acid located in the same amino acid residue of HLA-G1 (Fig. 2). We next investigated the α1 and α2 domain, which comprises the peptide-binding groove of HLA-E. In our previous work, we also constructed a chimeric gene replaced with the α1 domain of HLA-G1, designated as HLA-Ev(1–90). HLA-Ev(1–90) completely lost cell surface expression (*17*) (Fig. 1). We therefore focused on the α1 domain and narrowed down the region that is relevant to the complete loss of expression of HLA-E. As a result, we found that position-74 is critically involved in the expression of HLA-E (Fig. 1B). In addition, in a continuation of the same procedure and the construction of single point substitutions through the entire region, positions-9, 25, 40, 67, 74 and 99 were successfully identified as critical positions for HLA-E expression (Fig. 2). On the other hand, during the above substitution study, three positions, 11, 66 and 174, were also identified as the important positions for the up-regulation of the HLA-E expression (Fig. 2).

In X-ray crystallographic analyses of HLA-E, O'Callaghan *et al*. (*18*) reported that the pocket accommodating P2 (peptide position 2) is composed of residues 9, 22, 24, 45 and 67, the pocket accommodating P3: 97, 99, 156 and 159, the pocket accommodating P6: 70, 73, 74, 97 and 116, the pocket accommodating P7: 114, 116, 124, 133 and 147, the pocket accommodating P9: 81, 95, 116, 123 and 124. Thus many amino acid positions on the HLA-E heavy chain appear to be involved in leader peptide binding. However, the degree of contribution of each amino acid positions in the HLA-E heavy chain sequence for cell surface expression has not been clarified. Our results indicated the particular importance of these nine amino acid positions in the expression of the HLA-E molecule at least in CHO cell system.

Reactivity to the Leader Peptide—Stable single clones were established by the transfection of constructed genes to CHO-β cells, which possessed a single amino acid substitution at each 9, 25, 40, 67, 74 and 99. The cell surface expression of the HLA protein was evaluated by flow cytometry analysis (Fig. 3A). The mRNA and protein of transgenes were well expressed (Fig. 3B), whereas the cell surface expression of HLA-E protein was not in the cases of point mutants (Fig. 3A c–h). HLA molecule have been known to be well expressed on cell surface at low temperature such as 26°C. The low temperature stabilize the 'empty' HLA and allow to capture exogenously added peptide, which stabilize the HLA–peptide complex on cell surface at 37°C incubation for 1 h. However, even in the case of wild type HLA-E, the 1 h incubation at 37°C drastically reduced the expression (Fig. 3C b), which makes it difficult to compare the expression profile of

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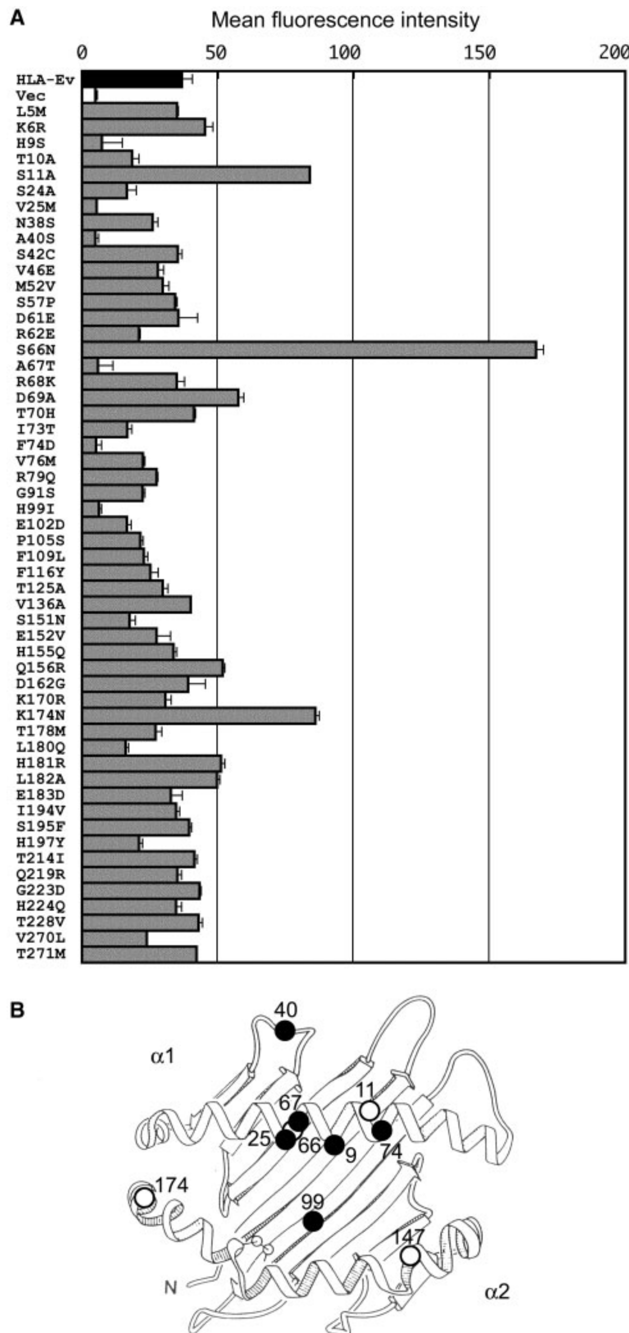

Fig. 2. **Comparison of the mean fluorescence intensities of constructed genes by flow cytometry analysis.** The cell surface expression on CHO-β cell was evaluated by flow cytometer. (A) Mean fluorescence intensities are indicated with the SEM. The substituted sequences are identical to the corresponding amino acid residues of the HLA-G1 molecule. (B) Schematic representation of the important amino acid positions on α1 and α2 domains. Closed and open circles indicate the positions of down-regulation and up-regulation, respectively. The amino acid residue 147 is also indicated for reference, which have been described in our previous work (*17*).

these point substitutions. Therefore, we next performed a peptide stabilizing assay without 1 h incubation at 37°C. The EC_{50} values were determined for each substitutions along with the concentration of the peptide required to achieve 50% of the expression of HLA-Ev at 50 μM. In the case of HLA-Ev, the expression intensity was elevated as the increased concentration of VMAPRTLVL. However, all six substitutions, at position-9, 25, 40, 67, 74 and 99, showed reduced responses to this peptide (Fig. 3C). In particular, Ev(67) and Ev(74) almost completely lost the response. The same phenomena were also observed when the leader peptide of HLA-G1, VMAPRTLFL, was used (data not shown). In addition, these substitutions failed to react with the HLA-G1 binding sequences, RIIPRHLQL and KIPAQFYIL, even though these constructs contain a portion of the HLA-G1 sequence (data not shown). In the case of alanine substitution method, there is a possibility that the translocation machinery could not recognize the mutant HLA-E by 'nonsense' alanine substitution. However, we substituted by HLA-G1 sequence of compatible position, which might not affect the translocation machinery. In addition, considering the position of these substitution, *i.e.* in the peptide binding domain α1 and α2, these results indicate that these six amino acid might be most critical positions for binding to the leader peptide.

Application of Double and Triple Substitutions to Xenotransplantation—In the above substitution experiments, we identified three other positions, position-11, 66 and 174, which moderately enhanced HLA-E expression. In our recent study, the S147C substitution, designated as HLA-Ev(147), showed a high expression and NK inhibitory function (*17*). Therefore, double and triple substitutions with an S147C substitution, HLA-Ev(11,147), HLA-Ev(66,147) and HLA-Ev(11,66,147), were constructed to evaluate their possible use in xenotransplantation. As a result of the flow cytometric analysis, the transfectants with HLA-Ev(11,147) and HLA-Ev(11,66,147) exceeded the expression intensity of HLA-Ev(147) (Fig. 4A). It is noteworthy that the expression of HLA-Ev(11,66,147) was nearly equal to that of HLA-G1 in the case of CHO cells (Fig. 4A upper panel). These three positions seemed completely to relax the peptide specificity of HLA-E in CHO cell system.

We next analysed the inhibitory function of these molecules, HLA-Ev(11,147) and HLA-Ev(11,66,147), against purified human NK cells using an *in vitro* xenotransplantation model system. We established the single isolated stable PEC transfectants with these constructs (Fig. 4B). Bulk mixtures of stable clones were also used to confirm the functional analysis. The stable bulk mixtures were assumed to represent the average features of the transfected genes, irrespective of the position effect of the integrated site. As a result, both transfectants with the HLA-Ev(11,147) and HLA-Ev(11,66,147) gene showed a moderate inhibition as HLA-Ev for human NK cells, but less than HLA-Ev(147).

In conclusion, in this and our previous studies, we demonstrated the particular importance of positions 9, 11, 25, 40, 66, 67, 74, 99, 147 and 174 for HLA-E expression and leader peptide binding. Positions-9, 25, 40, 67, 74 and 99 are important for the leader peptide binding, since the substitution of these position resulted in the significant loss of the cell surface expression of HLA-E. On the other hand, point substitutions at position-11, 66, 147 and 174 resulted in an enhancement

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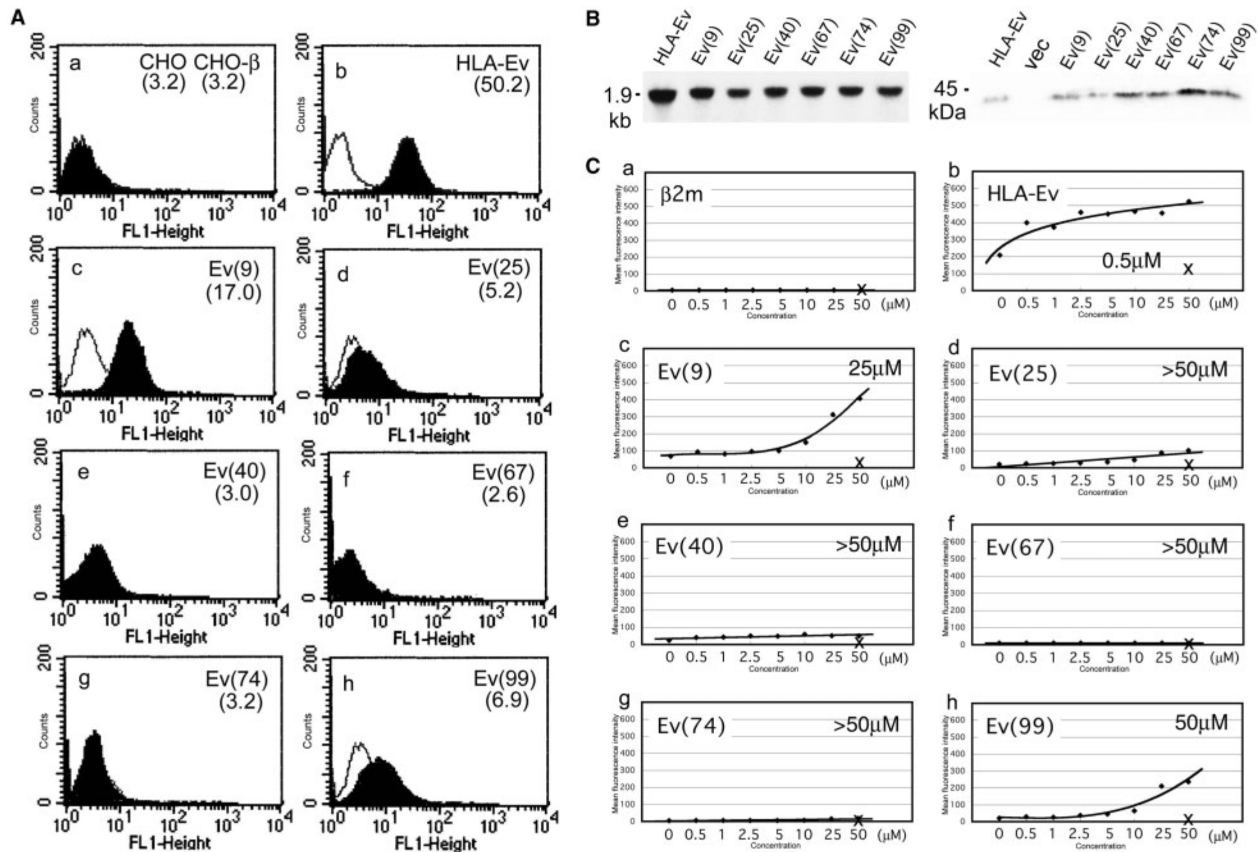

Fig. 3. **Establishment of stable transfectants on CHO-β cells and a peptide-stabilizing assay.** (A) Flow cytometric analysis of stable clones. Parentheses indicate the mean fluorescence intensity. The cells were cultured at 37°C (B) Northern and Western blot analysis. The cells were cultured at 37°C. MEM-E/02 (anti-HLA-E) was used as first Ab for Western blot analysis. (C) Peptide-stabilizing assay. The cells were precultured overnight at 26°C with synthetic peptide prior to FACS analysis. X also indicate the fluorescence intensity of the cells that were incubated for 1 h at 37°C prior to FACS analysis following overnight preculture at 26°C with 50 μM of VMAPRTLVL peptide. The mean fluorescence intensity was plotted versus various concentrations of the VMAPRTLVL peptide. The approximate EC_{50} values compared with the expression intensity of HLA-Ev at 50 μM are indicated in each panel.

of cell surface expression. In particular, position-147 is a key position, showing a drastic enhancement of HLA-E expression (*17*). In addition, we evaluated the expression and inhibitory function of double and triple substitutions, HLA-Ev(11,147), HLA-Ev(66,147) and HLA-Ev(11,66,147). Concerning the issue of cell surface expression, HLA-Ev(11,147) and HLA-Ev(11,66,147) were both expressed higher than HLA-Ev(147). However, concerning the inhibitory function, these multiple substitutions failed to show any improvement compared with HLA-Ev(147) in this system. The CD94/NKG2A inhibitory receptor recognizes, not only the HLA-E heavy chain sequence alone, but also the peptide sequence that is located in the binding groove. In addition, in Chinese hamster to mouse xenotransplantation system, the Ly-49D activation receptor on C57BL/6 mouse NK cells is known to recognize Chinese hamster MHC class I and trigger natural killing. Thus, the multiple substitutions of amino acid sequences might change the magnitude of inhibitory signal and/or might trigger NK activation through activation receptor such as Ly-49D. Therefore a smaller alteration is preferable for inhibitory function.

Other strategies for the regulation of NK cell-mediated xenogeneic cell lysis have been reported, including the transfection of DAF(CD55) and antibody blocking of the activating receptor, NKp44 and NKG2D, on NK cells (*19*, *20*). A combination of these strategies would be quite beneficial for the complete down-regulation of NK-mediated cell lysis. HLA-E provides inhibitory signal to NK cell through inhibitory receptor CD94/NKG2A expressed on NK cell. The CD94/NKG2A inhibitory receptor is also found on a subset of CD8+ T cells. Therefore, HLA-E have the potential to down-regulate the T cell activation. We next have to investigate the inhibitory function of HLA-E and mutant HLA-E to T cell activation for the success of clinical xenotransplantation.

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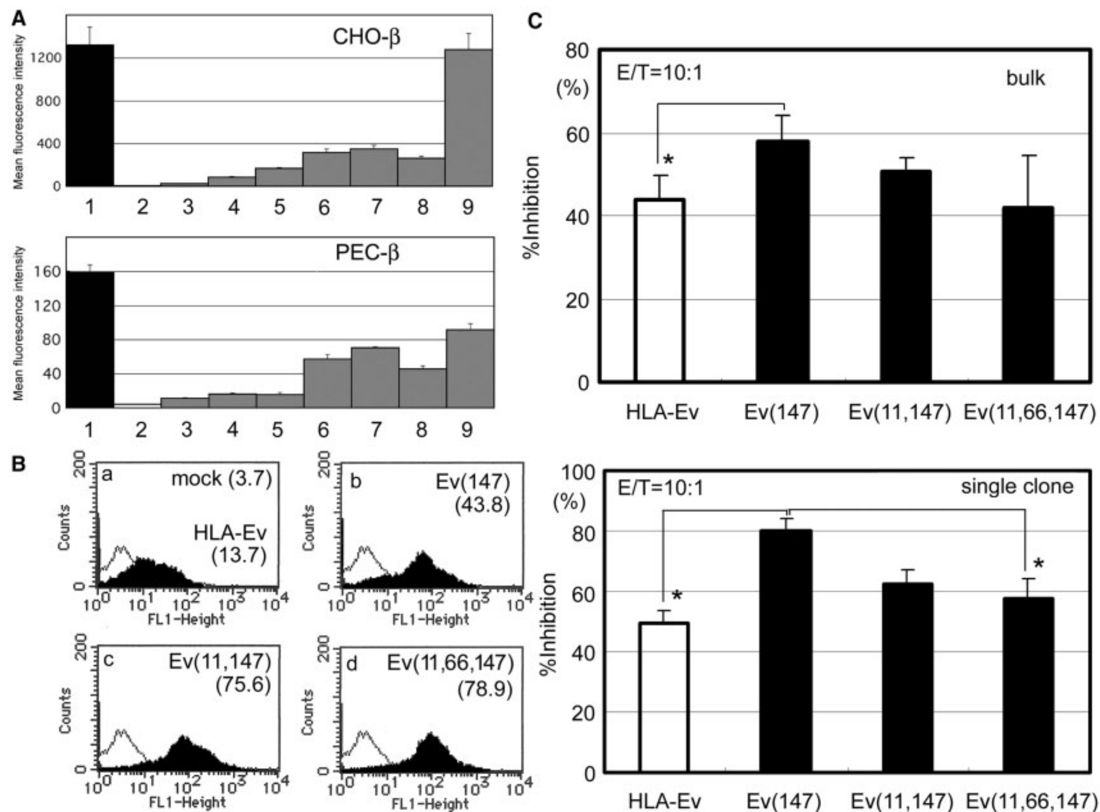

Fig. 4. **Expression profiles and an NK cell-mediated cytolysis assay using PEC-β transfectants with the double and triple substitutions.** (A) Mean fluorescence intensity with SEM of these bulk mixtures of stable transfectants evaluated by flow cytometer were summarized ($n = 3$). Lane 1: HLA-G1, lane 2: wild type HLA-E, lane 3: HLA-Ev, lane 4: HLA-Ev(11), lane 5: HLA-Ev(66), lane 6: HLA-Ev(147), lane 7: HLA-Ev(11,147), lane 8: HLA-Ev(66,147), lane 9: HLA-Ev(11,66,147). Upper panel: transfectants on CHO-β cells, lower panel: on PEC-β. PEC-β cell has been established by the transfection with human β_2 microglobulin gene (*17*). (B) Establishment of PEC-β transfectants. The results of the flow cytometry analysis are shown. The mean fluorescence intensity is indicated in the each panel. Open histogram shows the mock transfectant. (C) A ^{51}Cr release assay to determine the inhibitory function of the double and triple substitutions of the modified HLA-E molecules on PEC-β transfectants. Freshly prepared human NK cell were used as effecter cells. The percentage inhibition is shown at $E/T = 10:1$, calculated by the percentage lysis of the mock transfectant. The average percentage lysis of the mock transfectant was about 20% under these conditions. Upper panel shows the results using stable bulk mixtures, and the lower panel the single isolated stable clones. Asterisk indicates $P < 0.05$, compared with HLA-Ev(147). Error bar indicates the SEM ($n = 4$).

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