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学 位 論 文 内 容 の 要 旨

博士の専攻分野の名称　博士（薬科学）　氏　名　　髙橋 愛実

学 位 論 文 題 名

The functional analysis of domain-deleted isoform of human leukocyte antigen (HLA)-G
(ドメイン欠損型HLA-Gアイソフォームの機能解析)

[Introduction] Human leukocyte antigen (HLA)-G is an important immunomodulatory molecule belonging to the non-classical HLA class Is. There are several splicing isoforms of HLA-G, and HLA-G1 is a typical one which has been well-studied. HLA-G1 induces immunosuppression or immune tolerance by binding to leukocyte immunoglobulin-like receptor (LILR) B1 and LILRB2, human immunoreceptor tyrosine-based inhibition motif (ITIM)-bearing receptors, and correlates with pregnancy, autoimmune diseases and cancers. On the other hand, the existence of HLA-G null allele, *HLA-G*0105N*, suggests functional importance of the domain-deleted HLA-G2 isoform In other words, some fetuses who are homozygous for the HLA-G null allele could survive even in absence of a HLA-G1 isoform, indicating that the other isoforms such as HLA-G2 might be functionally sufficient for survival during prenatal period. However, few researches focusing on HLA-G2 have been reported.

Our laboratory has already established a refolding method for preparation of recombinant HLA-G2 protein. HLA-G2 protein forms the nondisulfide-bonded β2m-free homodimer, and binds to LILRB2, but not to LILRB1. These characteristics are equivalent to mammalian cell-derived HLA-G2.

In this thesis, I focused on the function of HLA-G2. First, I evaluated the effect of recombinant HLA-G2 in collagen-induced arthritis (CIA) mice. Surprisingly, single administration of the HLA-G2 showed significant immunosuppressive effects over the one month period. Then, I tried to elucidate the immunosuppressive mechanism of HLA-G2 using human peripheral blood monocytes and monocyte-derived dendritic cells (DCs). HLA-G2 induced cytokine productions and down-regulation of co-stimulatory molecules in monocytes. HLA-G2 could induce suppressive antigen presenting cells (APCs).

[Experimental Methods] *Preparation of recombinant HLA-G2 protein.* The extracellular domain (α1 and α3) of HLA-G2 polypeptides were expressed as inclusion bodies in *Escherichia coli* strain ClearColi BL21(DE3) competent cells. The solubilized protein was refolded by dilution method for three days and purified by size exclusion chromatography (SEC). Then, buffer was exchanged by dialysis with PBS. Just before using, the protein solution was concentrated and 0.22 μm-filtered.

Animal experiment with CIA mice. Fifty of DBA/1J mice, male, were used for each experiment and randomly divided into three groups. They were immunized twice by bovine type II collagen solution containing Freund's adjuvant. One week after the last immunization (= Day 0), they got subcutaneous injection of HLA-G2 solution or PBS (as a control) at a hind foot pad and were evaluated by rheumatoid arthritis (RA) score five times a week. These injection and evaluation were performed double-blinded.

Human primary cell experiment. The healthy donor was defined as a person who has never suffered blood or immune problem. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation, followed by MACS $CD14^+$ cell separation. RPMI-1640 including 10% fetal bovine serum (FBS) and antibiotics was used as cell culture medium. Under 37 ^oC and 5% CO_2 condition, $CD14^+$ monocytes were incubated with 1000 U/mL of GM-CSF and 500 U/mL of IL-4 for 6 days to generate IL-4-DCs. The HLA-G2 solution or PBS (as a control) were added on the first day of incubation, and the medium was exchanged to new one every 3 days. To generate IFN-DCs, monocytes were cultured with 1000 U/mL of GM-CSF and IFN-α for 2 days, then, HLA-G2 or PBS were added for 2 days.

[Results] *HLA-G2 has immunosuppressive effects in CIA mice (A. Takahashi et al., 2016). (i) The binding of human HLA-G2 with mouse paired immunoglobulin receptor (PIR)-B.* Firstly, the binding of human HLA-G2 with mouse PIR-B was verified. PIR-B is murine orthologue of human LILRB2. I established the preparation method of the extracellular domains of PIR-B as a C-terminal biotinylated protein. The kinetic analysis of HLA-G2 binding to immobilized PIR-B using surface plasmon resonance (SPR) analysis fitted reasonably well to the bivalent analyte model and showed slow dissociation. The apparent K_D, calculated by 1:1 Langmuir binding model fitting was 130 nM. On the other hand, the HLA-G2-LILRB2 binding in other orientation is much lower (~μM). These indicate that HLA-G2 homodimer can utilize two receptor sites to strongly bind to PIR-B on the cell surface. Therefore, HLA-G2 has the potential to transmit effective signals through PIR-B receptor in mice.

(ii) RA score after HLA-G2 administration. Because HLA-G1 proteins exhibited efficient anti-inflammatory effects on CIA in mice, I used the same model to evaluate the effect of HLA-G2 *in vivo*. One week after the second immunization, mice were administered HLA-G2 solution (high dose; 1.4 μg/mouse, low dose; 0.14 μg/mouse) or PBS as a control on day 0, because a clear effect was not observed by HLA-G2 injection during early arthritis phase, and observed RA score five times a week. RA score indicate the clinical severity of arthritis, and its maximum score per mouse was 40. The HLA-G2 high dose group showed consistently lower RA scores than that of the PBS control group, especially during the last three days, when it showed the statistically significant differences. The immunosuppressive effects showed in dose-dependent manner, and low dose (0.14 μg) might be non-effective in mice.

(iii) Production of cytokines in lymph node and joint after HLA-G2 administration. To find a clue to understand the immunosuppressive effect of HLA-G2, mRNA expression levels of cytokines in lymph nodes and a limb joint on Day 7 and Day 41 were measured by real-time PCR. Anti-inflammatory cytokines including IL-10 and TGF-β tended to be up-regulated in lymph nodes, and IL-17, expressed by Th17 cells, tended to be down-regulated locally in limb joint. However, it did not show any statistical significance due to large standard deviation.

HLA-G2 induces immunosuppressive phenotype in human monocytes and human monocyte-derived DCs. (i) The expression of cell surface molecules on HLA-G2-stimulated monocytes. To elucidate the immunosuppressive mechanism of HLA-G2 in human cells, I studied using LILRB2-expressing human primary cells. In this study, I focused on monocytes and monocyte-derived DCs. $CD14^+$ cells from human PBMCs were isolated by positive selection and confirmed the LILRB2 expression (over 90%). After a two-day incubation period with 2.3 μM (0.05 mg/mL) of HLA-G2, the cell surface phenotype was analyzed by flow cytometry (FCM). HLA-G2 tended to down-regulated HLA-DR and CD86 expression in the monocytes. The immature IL-4-DCs which incubated with 2.3 μM HLA-G2 during differentiation also showed a tendency for down-regulation of HLA-DR and CD86.

Horuzsko's group showed that HLA-G1 stimulation to LILRB2 on human monocyte-derived DCs and murine DCs from LILRB2 transgenic mice leads to diminished expression of HLA class II molecules and co-stimulatory molecules and prolongation of skin allograft survival. Therefore, HLA-G2 could induce immunosuppressive phenotype on monocytes and DCs, similarly to HLA-G1.

(ii) Cytokine productions from HLA-G2-stimulated human primary $CD14^+$ monocytes, monocyte-derived IFN-DCs and IL-4-DCs. After HLA-G2-incubation, the monocytes, immature IL-4-DCs and IFN-DC significantly produced some kinds of cytokines.

[Conclusion] In this study, the following things were suggested: the recombinant soluble HLA-G2 bind to mice PIR-B receptor, has immunosuppressive effects in CIA mice, and induces immunosuppressive phenotypes in monocytes and monocyte-derived DCs. I hope that this study will contribute further understanding of function of HLA-G2 and new clinical application for problems related to pregnancy and poor prognostic cancer correlating with HLA-G.