論文の内容の要旨

論文題目　Molecular mechanism of HLA class II association with Type I diabetes
Establishment of a plate-based HLA peptide binding assay and its application to HLA-DR9 and ZnT8 interaction
(HLA クラスII と一型糖尿病の関連の分子機序に関する研究:
HLA-ペプチド結合測定法の確立と HLA-DR9-ZnT8 ペプチド相互作用解析への応用)

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Study Purpose

Type 1 diabetes (T1D) is an autoimmune disorder that mediates destruction of insulin-producing pancreatic cells. Previous genetics association studies reported HLA II to be the major factor for increased susceptibility to T1D. Yet, knowledge remained limited on disease mechanism especially in the Japanese. Even though ELISA based peptide binding assays are frequently performed to determine the binding affinity between HLA and peptides, limitations such as the requirements of excess quantity of purified HLA proteins and monoclonal antibodies are found to be not only time consuming but also inefficient when dealing with a large number of alleles. Thus, current study was conducted with the two major purposes: first, to establish a plate-based peptide binding assay system where peptide binding to HLA II protein can be tested using cell-lysates of HLA-DR or -DQ -expressing cells. Second, to screen the synthetic peptide ligands derived from autogenic peptide ZnT8 in order to identify the regions that are able to be presented by the T1D risk or protective HLA-DR and -DQ T1D alleles in Japanese and might act as pathogenic self-epitopes.

Study Methods

Japanese susceptible HLA – *DR8* (*DRA*01:01- DRB1*08:02*), *DR4* (*DRA*01:01-DRB1*04:05*), *DR9* (*DRA*01:01-DRB1*09:01*), HLA- *DQA*03 - DQB1*03:02, DQA*03 - DQB1*03:03, DQA*03 - DQB1*04:01*, protective HLA - *DRB1*15:01, DQA*01:02 - DQB1*06:02* were sub-cloned into expression vectors and expressed in mouse embryonic fibroblast NIH3T3 cell lines.

A plate based peptide binding assay system was established in utilization of Ni-NTA coated 96-well ELISA plates and HLA-*DR9* containing cell lysates. The assay system was developed in three steps with distinct experimental purposes: 1) lysate binding assay to ensure the immobilization of HLA II protein on the Ni-NTA coated plates. 2) single peptide binding assay to examine the potential binding of HLA II and indicator peptide. 3) peptide competition assay to determine the binding affinity of synthetic peptides. Moreover, the assay system has optimized with modified reaction mixture, incubation condition and time where chemiluminescence and chemifluorescence substrate were used respectively to determine the HLA II/peptide binding affinity.

Lastly, peptide competition assay was conducted following the protocol established in prior experiments using 20-mer ZnT8 peptide library designed to cover the entire sequence of ZnT8.

Results

Eight stable cell lines were established in order to be representative of T1D risk and protective alleles in Japanese. Flow cytometry analysis confirmed the expression of HLA II molecules at varied expression levels. Sufficient quantities of HLA II recombinant proteins were generated for peptide competition assays.

A peptide binding assay system was successfully developed using Ni-NTA coated plates and HLA II cell lysates. First, a success immobilization of HLA II proteins was confirmed by lysate binding assay with anti-HLA II primary antibody and HRP labeled secondary antibody. Second, a suitable indicator peptide was selected and used for both of the single peptide binding assay and peptide competition assay to determine its peptide binding affinity to HLA-*DR9*. The binding of HLA-DR9 and the indicator peptide was determined to be dose dependent by the chemiluminiscence and chemifluorescent signals, whereas, inhibition of the peptide binding was also confirmed using the biotinylated and non-biotinylated indicator peptides.

In summary, the peptide binding assay with DR9 are to be performed with 3 hours pre-immobilization of HLA II protein to the plate, followed by the washing of the plates to eliminate the un-immobilized proteins, and incubation with peptide for 24 hours at 37 ºC where both lysate immobilization and peptide reaction conditions are to be performed at pH 7.0.

Peptide competition assay was conducted using 24 ZnT8 peptides from the ZnT8 peptide library and 3 potential binding peptides to HLA-*DR9* were determined.

Discussion

In distinct to previously reported peptide binding assays, current assay system is developed to immobilize the protein onto the plates relying on the strong binding of nickel ion and the His tag instead of anti-MHC II mAb or streptavidin coated plates. Such method has improved from the previous methods not only to allow the study of binding characteristics of a large variety of HLA molecules as long as His tag is present, but also enable the HLA II protein to be localized on the plate surface in a uniform orientation which maximize the interaction with the peptide. Furthermore, current assay system also permits the use of cell lysate instead of purified MHC molecule where the process of protein purification could be exempted. The limitation in the current assay system is that the use of pH is restricted between 7.0 and 8.0 for the immobilization of MHC II protein to the plate and the insolubility of synthetic peptides. Peptide insolubility is expected to be improved by the use of organic solvents such as DMF instead of DMSO.

Three ZnT8 peptides were determined to be potentially bounded to the Japanese T1D susceptible allele of HLA-*DR9*. Such results has not only proven the success of the establishment of peptide binding assay but also, provide an inventory for mapping the epitopes for ZnT8 peptides, one of the newest established auto-antigens in T1D and new insights underlying the mechanism of T1D especially in the Japanese and the Asian populations.