Altered collagen II peptides inhibited T-cell activation in rheumatoid arthritis

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Abstract

It has been reported that collagen II (CII)-derived peptide induced T-cell activation via its amino acids responsible for T-cell receptor (TCR) recognition. In this study, three altered CII263–272 peptide ligands (APL) containing multiple substitutions of TCR contact residues were synthesized. Their roles in inhibition of T-cell activation were evaluated in peripheral blood lymphocytes (PBL) of rheumatoid arthritis (RA) in vitro. It was shown that 41% (25/61) of RA patients were responsive to the wild-type antigenic CII263–272. In contrast, marginal or silent T-cell responses to the three APLs were found, accompanied by inhibitory effects on secretion of Th1 type cytokines and expression of cell surface markers, CD69 and CD25. In addition, T-cell activation induced by the wild-type antigenic CII263–272 was inhibited by all the three APLs in a dose-dependent manner. It is demonstrated that APLs with substitutions of TCR contact residues are capable of down-regulating T-cell responses in PBLs of RA, suggesting that the CII-derived APLs are potentially therapeutic in RA.

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Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that primarily affects peripheral joints with cartilage destruction and subsequent bone erosion. Although the etiologic agent of RA remains unclear, there is convincing evidence that antigenic peptides presented by disease-associated HLA (predominately HLA–DRB1*0101, *0401, *0404 and *0405) are involved in the pathogenesis of RA [1–3]. They might initially activate auto-reactive T cells and mediate autoimmune responses in the disease [4,5]. A number of auto-antigens in RA have been identified, such as collagen II (CII), HCgp-39 and BiP [6–8]. Among these, CII has been extensively studied in the last several years. It is suggested that the CII263–272 peptide is one of the predominant antigenic peptides in RA [9–13]. Crystallographic analysis and alanine scanning showed that 263F and 264K of CII263–272 were mainly responsible for binding with HLA–DR, while 267Q and 270K were the TCR contact residues [9,11,12,14]. However, inconsistent results were shown in different studies on residues 266E, 269P and 272E [9,11,12].

T-cell activation requires the interaction of a TCR with its cognate ligand of peptide bound to class II MHC molecules on the surface of antigen presenting cells (APC). In the TCR–antigen–HLA–DRB1 complex, some side chains of a few residues of the peptide contact with TCR, while other side chains are partially buried in the antigen-binding pockets of the HLA–DRB1 molecules [15]. This interaction is highly sensitive, whereby subtle changes in the peptide can invoke drastic variations in T-cell effector function. As a result, peptides with amino acid substitution, termed as altered peptide ligands (APLs), have been used to modulate the activation of T cell in experimental autoimmune encephalomyelitis (EAE) and have also been suggested to be a mean of immunotherapy of multiple sclerosis (MS) and myasthenia gravis (MG) patients [16–19].

Recently, Myers et al. reported that an analog peptide containing HLA–DR binding amino acid substitutions was a potent suppressor of the HLA–DR-mediated immune response and collagen-induced arthritis [14]. Studies from our group demonstrated that the altered CII peptides with individually or consecutively substitution of the TCR contact amino acids in CII263–272 inhibited T-cell activation induced by an antigenic CII peptide and suppressed arthritis of the CIA model [11,20,21]. We had also shown that the peptide with consecutive substitution of three residues was more efficient
than those with single or double substitutions of residues in inhibition of T-cell responses [13]. Therefore, it was hypothesized that a potential HLA–DRB1-binding and non-T-cell-stimulating APL could be selected by comparing different peptides with multiple substitutions of the TCR contact residues of CII236–272.

In this study, a panel of CII-derived APLs were designed and analyzed to select a potential inhibitory APL in CII-mediated T-cell response. Firstly, 267Q and 270K, the main TCR contact residues in CII263–272, were replaced by alanine to abort T-cell-stimulating effect of the peptide. Secondly, 265G or 271G was substituted, respectively, with alanine to increase their binding ability to HLA–DRB1*0401/*0101, which was demonstrated by Rosloniec and Andersson et al. [9,11]. To evaluate the role of these peptides in a condition more closely to clinic, peripheral blood lymphocytes (PBL) from RA patients rather than transfection cells were used in T-cell activation and inhibition assay. This is the first study so far to examine the effect of APLs in down-regulation of T-cell response in a cohort of HLA–DRB1*04/*01 typed rheumatoid arthritis patients in vitro.

Materials and methods

Peptides synthesis

Wild-type CII263–272 peptide, three APLs with substitutions (underlined) of 265G, 267Q or 271G were synthesized using solid-phase techniques on an Applied Biosystems Peptide Synthesizer (Genomed Synthesis, Inc. San Francisco, CA, USA). Irrelevent peptide with reversed sequence of CII263–272 was synthesized as negative control. Peptide sequences were FKGEQGPKGE (wild-type CII263–272), FKGEAQPGAE (APL1), FKAEQPAGAE (APL2), FKGEAQPGAE (APL3) and EGKPGQEGKF (irrelevant peptide). A myristic acid group was added to the N-terminal of each peptide to facilitate their intracellular delivery. The peptides were purified by reversed phase high pressure liquid chromatography with a purity of more than 95%.

Molecular modeling

Interaction of the APLs with HLA–DR4 molecules was analyzed using Red Hat Linux 9 operating system on the CPU 2.4 GHz Xeon CPU×2 Workstation with Sybyl 6.9 + SURM software [22]. Crystal structures of HLA–DR4–CII1168–1179 (PDB: 2SEB) were used as template. The affinity of CII263–272 and the APLs to HLA–DR4 molecule was calculated by using SCORE software. The method, SCORE, has an empirical scoring function to describe the binding free energy which includes van der Waals contact, metal–ligand bonding, hydrogen bonding, desolvation effect and deformation penalty upon the binding process. The coefficients of each term are obtained by multivariate regresional analysis of a diverse training set of 170 protein–ligand complexes. The final scoring function reproduces the binding free energies of the whole training set with a cross-validated deviation of 6.3 kJ/mol. The scoring function takes the following form:

$$pK_d = K_o + K_{vdw} + K_{metal} + K_{hbond} + K_{desolv} + K_{deformation}.$$ 

Here, $K_{vdw}$ represents the contribution of van der Waals interaction between the protein and its ligand, $K_{metal}$ the contribution of metal–ligand bonding, $K_{hbond}$ the contribution of hydrogen bonding, $K_{desolv}$ the contribution of desolvation effect and $K_{deformation}$ the contribution of deformation. $K_o$ is the regression constant which may contain the translational and rotational entropy loss upon the binding process. Increase of $pK_d$ value indicates higher affinity.

Patients

Informed consent was obtained from 61 patients (6 men and 55 women) with RA who fulfilled the 1987 revised criteria of the American College of Rheumatology. The mean age of the RA patients was 50.6 ± 13.0 years (20–74 years). The mean disease duration was 9.5 ± 5.2 years (8 months–32 years). All patients had clinically active synovitis. The majority of patients were being treated with nonsteroid anti-inflammatory drugs, prednisolone (>10 mg/day) and slow-acting anti-rheumatic drugs (methotrexate, sulfasalazine, hydroxychloroquine or leflunomide).

PBL isolation and T-cell proliferation assay

Heparinized peripheral blood was collected under sterile conditions from RA patients and diluted 1:1 with PBS. PBL were isolated by Ficoll–Paque (Pharmacia, USA) density gradient centrifugation and washed twice with PBS and resuspended in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum (Gibco, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. Cell viability was >95% by trypan blue exclusion assay.

Cells were then cultured at 1 × 10⁷ cells per well in triplicate in 96-well plates (Costar, USA) and stimulated with CII263–272 or the altered peptides at a final concentration of 10 μg/ml (optimal dose in T-cell proliferation assay as shown previously). PHA (20 μg/ml) was used as positive control. Cells were incubated at 37°C in 5% CO₂ for 5 days. Before the last 8 h of culture, 1 μCi of [³H]-thymidine was added to each well. The cells were then harvested, and the incorporated radioactivity was counted in a β-scintillation counter. Data are presented as stimulation index (SI), which was calculated as the ratio of the counts per minute (cpm) obtained with peptide stimulation divided by the cpm in cultures of cells with medium alone. T-cell proliferative responses were considered positive if the SI was ≥2 and Δcpm > 1000.

Assessment of cytokine production

The supernatant (150 μl) was taken at 24 h from wells for the detection of IL-2 and 72 h for IFN-γ and IL-4 production. The highly sensitive enzyme-linked immunosorbent assay kit (R&D, USA) was used according to instruction from the manufacturer. Each sample was tested with duplicated wells. The detection limit was 7.8 pg/ml for each cytokine.
**Flow cytometry**

To examine CD25 and CD69 expression on CD4+ T cell, isolated PBL from patient responsive to CII263–272 were cultured at 2 × 10^5 cells/well in 96-well plates with 10 μg/ml CII263–272 or APLs at the same concentration at 37°C in 5% CO₂. For detection of CD69 expression, cells were harvested at 18 h. After washing with PBS once, anti-CD4-FITC and anti-CD69-PE were added to the cells and incubated 30 min at 4°C. Cells were fixed with 4% paraformaldehyde/PBS solution after washing with PBS twice. Flow cytometry was performed using a Beckton Dickinson FACScan instrument. For detection of CD25 expression, cells were harvested at 72 h, and dual flow cytometry stained with anti-CD4-FITC and anti-CD25-PE was performed as indicated above.

**Inhibition of CII263–272-induced T-cell response in vitro**

To detect whether APLs could inhibit T-cell proliferation induced by CII263–272, PBL (2.0 × 10^5 cell/well) from 10 of CII263–272-responsive patients were incubated with various concentrations of APLs (0, 2, 10 and 50 μg/ml, respectively) as indicated in the presence of 10 μg/ml antigenic peptide CII263–272 and incubated at 37°C in 5% CO₂ for 5 days. Irrelevant peptide with reversed sequence of CII263–272 was synthesized as a specific control. 3H-thymidine (1 μCi/well) was added to the cultures 8 h before the cells were harvested. The cells were then harvested, and the incorporated radioactivity was counted in a β-scintillation counter. Counts per minutes (cpm), stimulation index (SI) and cytokine concentration are indicated as mean ± SD. Student’s t test was used to determine differences between groups. Pearson Chi-square test was used to analyze the correlation between HLA–DRB1*04/01 subtypes and the T-cell responses of CII peptide in RA patients. Spearman’s rank correlation test was used to analyze the correlation between the increases in cytokine production by CII stimulation and stimulation index. A P value < 0.05 was considered statistically significant.

**Results**

**HLA–DR4 and APLs interaction**

As shown by computer modeling, APL1 containing substitutions of 267Q and 270K with alanine bind to HLA–DR4 molecules (Fig. 1). Very similar binding was also found from APL2 and APL3 with multiple substitutions as indicated above (data not shown). The binding ability indicated by pKₐ values of APL1 (5.84), APL2 (6.13) and APL3 (6.16) was higher than the wild-type CII263–272 (5.30), suggesting that removal of 265G, 267Q, 270K or 271G might increase the affinity CII263–272 to HLA–DR4. These results are consistent with previous studies [9,11]. It is suggested that the APLs derived from CII263–272 can bind to HLA–DR4 molecules, and replacement of 265G or 271G yields better binders to HLA–DR4.

**Impact of substitution of TCR contact residues of CII on T-cell proliferation**

To investigate T-cell responsiveness to the wild-type CII263–272 and the altered CII peptides, PBL from 61 RA
patients were cultured in the presence of these peptides in the indicated concentrations. It was found that 41% (25/61) of RA patients were responsive to the wild-type antigenic CII263–272 with a stimulation index (SI) of 2.40 ± 0.39. In contrast, there were significant low stimulatory responses to the altered CII263–272 peptides, with 1.6% (1/61) to APL1, 6.6% (4/61) to APL2 and 11.5% (7/61) to APL3. As shown in Table 1, the stimulation indices were also much lower in the altered CII263–272 peptides, with SI 1.32 ± 0.37* to APL1, 1.26 ± 0.31* to APL2 and 1.20 ± 0.43* to APL3, compared to SI of the wild-type CII263–272 (2.40 ± 0.39). There was no correlation in sex, age and disease duration between CII263–272-responsive and non-responsive RA patients (data not shown).

In addition, the responsiveness of T cells to CII263–272 stimulation was 48% (12/25) in RA-associated HLA–DRB1*04/*01 positive group which was higher than in HLA–DRB1*04/01 negative group (36%, 13/36), though there was no statistically significant.

**Low cytokine production by T cells with APL stimulation**

To further examine the responses of T cells to CII263–272 and the altered peptides, we detected IL-2 and IFN-γ secretion by the PBL from 10 of the CII263–272-responsive RA patients. It was found that production of IL-2 (30.6 ± 16.0 pg/ml) and IFN-γ (92.8 ± 60.0 pg/ml) by PBL induced by wild-type CII263–272 was significantly higher than those of medium control (11.8 ± 5.3 and 43.3 ± 29.0 pg/ml, respectively, P < 0.01). These results suggested that the wild-type antigenic CII263–272 might be involved in the initiation of RA by promoting secretion of the Th1 type cytokines. In addition, it was shown that the levels of IL-2 and IFN-γ production from these 10 patients correlated with the SI value (r = 0.793, P < 0.01; r = 0.936, P < 0.01) (Fig. 2).

In comparison with high level of IFN-γ induced by wild-type CII263–272 (92.8 ± 60.0 pg/ml), the altered CII263–272 yielded a significant lower level of IFN-γ by APL1 (56.7 ± 39.0 pg/ml, P = 0.127), APL2 (46.5 ± 33.0 pg/ml, P < 0.05) and APL3 (32.3 ± 17.0 pg/ml, P < 0.01). As indicated in Fig. 3, IL-2 production by PBL in these RA patients induced by the altered CII263–272 was also much lower with the levels of 13.6 pg/ml (APL1: 6.8 pg/ml and 21.7 ± 13.6 pg/ml (APL3), when compared to that of the wild-type CII263–272 (30.6 ± 16.0 pg/ml). These results suggested that the altered CII263–272 peptides might have no stimulatory effect on Th1 cells in RA patients. We also measured IL-4 production by PBL in the supernatant in this study. Unfortunately, the results were unable to be included since the level of IL-4 was below the detection limit of the kit (data not shown).

**Low expression of CD25 and CD69 induced by APLs on CD4+ T cell**

To further evaluate the effect of the altered CII peptides on T-cell activation in RA, the early T-cell activation marker CD69 and the late marker CD25 were analyzed, respectively. As shown in Fig. 4, levels of CD69 expression on CD4+ T cells induced by the altered CII263–272 peptides were much lower (APL1: 56.7 ± 39.0%; APL2: 5.1 ± 3.5%; APL3: 4.7 ± 3.4%) in comparison with wild-type CII263–272 (12.2 ± 3.6%). Similarly, compared to the high level of CD25 expression on CD4+ T cells induced by wild-type CII263–272, lower levels of CD25 expression mediated by the altered CII263–272 peptides were found (APL1: 10.6 ± 2.1%; APL2: 9.0 ± 1.6%; APL3: 8.0 ± 1.7%). These results further suggested that the
The wild-type CII263–272 peptide is antigenic, while the altered CII263–272 bound to HLA–DR4/DR1 had no significant effect in T-cell activation. They might be potentially competitive to the antigenic CII263–272 in inhibition of autoimmune T-cell responses in RA.

**Inhibition of PBL activation in RA by APLs**

Based on the above results, it is likely that the altered CII263–272 peptides can be competitive blocker of the antigenic CII263–272 in down-regulation of T-cell activation in RA. To test this possibility, the inhibitory effects of the APLs in CII263–272-induced T-cell responses were studied using PBL from 10 RA patients responsive to CII263–272. It was shown that CII263–272-induced response of PBL was inhibited by the APLs in a dose response manner in all these patients (Fig. 5). When the concentration of APLs was increased to 50 μg/ml (5-fold of wild peptide CII263–272), the PBL response induced by CII263–272 was efficiently inhibited with the mean cpm values 2331.8 ± 905.3 (APL1), 1993.9 ± 825.3 (APL2) and 1916.5 ± 883.2 (APL3), which were significantly lower than that of CII263–272 peptide control (5363.1 ± 2536.0). The inhibitory rates of APL1, APL2 and APL3 to CII263–272 peptide reach to 74.2 ± 18.5%, 90.3 ± 11.6% and 93.8 ± 10.8%, respectively. As a control, the irrelevant peptide with reversed sequence of CII263–272 did not inhibit the PBL response to CII263–272 antigenic peptide (Fig. 5). The results suggested that the APLs could inhibit T-cell activation induced by the antigenic peptide CII263–272 in RA.

**Discussion**

We have shown in this study that enhanced T-cell responses to CII263–272 peptide can be detected in 41% of RA patients. This is consistent with the results by Kim et al. in which 54% of PBMC and 61.9% of SFMC from RA patients were CII responsive.[23] Moreover, there was an increased production of Th1 type cytokines IFN-γ and IL-2 by CII263–272-stimulated PBL from RA patients responsive to CII263–272. The elevation of IFN-γ and IL-2 was closely correlated with the T-cell responsiveness to the antigenic peptide CII263–272. These findings suggested that CII263–272-reactive T cells play a role in the pathogenesis of RA, and immunotherapy towards this CII fragment might be a useful approach in treatment of RA.

It was shown by Andersson et al. that Ala substitutions of 267Q or 270K of CII263–272 only marginally changed the apparent binding affinity to HLA–DRB1 molecule, whereas 265G or 271G substitution resulting analogs have approximately 5–10 times higher affinity than the parent peptide.[11] Analogs with substitution of 267Q or 270K disrupted CII-specific T-cell stimulation. These results were in agreement with the studies by Rosloniec et al.[9]. In our molecular modeling analysis, CII263–272 peptide fitted well into the binding cleft of HLA–DR4 molecule, while the long side chain of 267Q and 270K stretched out of the binding groove of HLA–DR4. The APLs with substitution of 265G, 267Q, 270K or 271G did not change the binding affinity to HLA–DR4...
molecule. In contrast, the stimulatory effect of CII263–272 peptide on T cells was eliminated by the APLs. These results suggested that 267Q and 270K are involved in TCR contact, as indicated by the previous studies from our group and others [9,11,13,21].

It is suggested in the present study that the APLs down-regulated the secretion of Th1 type cytokine in PBL from RA patients and barely induced the expression of the T-cell activation markers, CD69 and CD25. The APLs inhibited the CII263–272-induced T-cell proliferation in a dose-dependent manner. The inhibition was specific since a control peptide, namely, the irrelevant peptide with reversed sequence of CII263–272, had no inhibitory effect on CII263–272-induced T-cell response.

It has been proposed that APLs might inhibit T-cell response to antigenic peptide by antagonism or partial agonism [24–27]. For example, Chen et al. had demonstrated that altered peptide ligands carrying certain residue substitutions of wild-type antigenic peptide exhibited TCR antagonism by inhibiting T-cell activation. Furthermore, some of these TCR antagonistic peptides might partially activated the T cells to induce the expression of T-cell surface markers such as CD28 [26]. These results suggested that the altered peptide ligands of an antigenic peptide stimulated T cell via the substituted residue of the peptide to exhibit antagonism and partial agonism.

It was also suggested that the altered peptides might prime and expand a novel population of APL-specific T cells with immunoregulatory properties. These APL-specific T cells are able to cross-recognize native antigenic peptide and down-modulate inflammatory response. In the inflammatory site, these APL-specific T cells are activated upon cross-stimulation with the native antigen released during tissue destruction and produce regulatory cytokines such as IL-4, IL-10, IL-13 and TGF-β. The release of these anti-inflammatory cytokines subsequently inhibits the ongoing inflammatory process in an antigen-nonspecific manner [28,29]. Such regulatory mechanism termed as bystander suppression has been showed in some animal experiments treated with APLs, such as in EAE models [16,28]. In the present study, we have identified that the APLs were capable of antagonizing CII263–272 specific T-cell activation by ameliorating cell proliferation and cytokine secretion. It is still unknown whether these APLs can mediate partial agonism or bystander suppression.

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References


