

Therapeutic effect of two altered peptide ligands derived from the human heat shock protein 60 in experimental models of rheumatoid arthritis

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ABSTRACT

Induction of immune tolerance as therapeutic approach for autoimmune diseases constitutes a current research focal point. In this sense, two Altered Peptide Ligands (APLs) were evaluated for the induction of peripheral tolerance in patients with Rheumatoid Arthritis (RA). Two novel T cell epitopes from human heat-shock protein 60 (hHsp60), an autoantigen involved in the pathogenesis of RA, were identified by bioinformatics tools and two APLs were designed from these epitopes (APL-1 and APL-2). APL-1 increases the proportions of the CD4+CD25^{high}FoxP3+ regulatory T cells in *ex vivo* assays using PBMCs isolated from RA patients. While, APL-2 increased the IL-10 level and suppressed IL-17 secretion, and induces the activation of T cells through his ability to modify cell cycle phase's distribution of CD4+ T cells from RA patients. Additionally, the therapeutic effect of these APLs in two animal models was evaluated: adjuvant induced arthritis (AA) in Lewis rat and collagen induced arthritis (CIA) in DBA/1 mice. Our approach was compared to metotrexate (MTX), the treatment of reference for RA, in CIA model. Clinical score, TNF- α levels and histopathology were monitored. Both APLs efficiently inhibited the course of AA and CIA, with significant reduction of the clinical and histopathology scores. The therapeutic effect induced by APLs is mediated by different molecular mechanisms, associated with immunologic tolerance. These results indicate a therapeutic potentiality of these APLs and support further investigation for treatment of RA. This study won the Annual Award of the Academy of Sciences of Cuba in 2012.

Keywords: rheumatoid arthritis, altered peptide ligand, hHsp60, immune tolerance, regulatory T cells, collagen induced arthritis, adjuvant induced arthritis

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RESUMEN

Demostración del efecto terapéutico de dos péptidos modificados derivados de la proteína de estrés celular de 60 kDa, en modelos experimentales de artritis reumatoide. La inducción de tolerancia periférica mediante el uso de autoantígenos involucrados en la patogénesis de las enfermedades autoinmunes, constituye una alternativa muy atractiva para el tratamiento de estas afecciones. Se evaluaron dos ligandos peptídicos alterados (LPA-1 y LPA-2) para la inducción de tolerancia periférica en pacientes con artritis reumatoide (AR). Estos se derivaron de dos nuevos epitopos de células T humanas, identificados en la proteína de estrés celular de 60 kDa (Hsp60), autoantígeno involucrado en la patogénesis de la AR. El LPA-1 aumentó las proporciones de células T reguladoras CD4+CD25^{high}FoxP3+ en ensayos *ex vivo* en células mononucleares de sangre periférica aisladas de pacientes con AR. El LPA-2 incrementó los niveles de IL-10, suprimió la secreción de IL-17 e indujo la activación de células T mediante la modificación de la distribución de fases del ciclo celular en linfocitos T CD4+ de pacientes con AR. Se comprobó el efecto terapéutico de los LPA en dos modelos animales: artritis adyuvante (AA) en ratas Lewis, y artritis inducida por colágeno (AIC) en ratones DBA/1. Este se comparó con el tratamiento con metotrexato (MTX), medicamento de referencia para la AR. Se monitorearon los niveles del factor de necrosis tumoral alfa, la clasificación clínica y la histopatología. Ambos LPA inhibieron eficazmente el curso de la AA y de la AIC, y redujeron significativamente los dos últimos parámetros. Su efecto terapéutico estuvo mediado por mecanismos moleculares relacionados con la tolerancia inmune. Estos resultados indicaron el potencial terapéutico de estos dos LPA en la AR, y las posibilidades investigativas en torno a ellos como candidatos. Este estudio mereció el Premio Anual de la Academia de Ciencias de Cuba, en el año 2012.

Palabras clave: artritis reumatoide, ligando peptídico alterado, proteína de choque térmico 60 humana, tolerancia inmune, células T reguladoras, artritis inducida por colágeno, artritis inducida por adyuvante

Introduction

Recently, relevant progresses have been achieved in the knowledge of the immunological and molecular mechanisms of autoimmune diseases. They translated into a generation of biological therapeutic agents that target pro-inflammatory cytokines, with the aim of

interfering with their mechanisms of action [1]. These agents are expected to progressively complement or replace currently used immunosuppressive and anti-inflammatory therapies. Available anticytokine approaches remain vulnerable by limitations associated

eminently with generalized immunosuppression and subsequent increased of the occurrence of malignancies and infectious diseases [2].

The main challenge to treat autoimmune diseases is the development of therapeutic strategies that could eliminate pathogenic T cells with specificity, without affecting other non-related T cells. For this purpose, the induction of peripheral tolerance using autoantigens involved in the autoimmune disease pathogenesis constitutes an alternative which facilitates the restoration of the tolerance lost in the course of autoimmune diseases [3]. It can be mediated by mechanisms of bystander suppression or anergy, depending on the therapeutic doses, and the route and frequency for antigen administration [4].

An antigen that can be used to induce tolerance is Hsp60, a protein belonging to the family of the heat shock proteins (Hsp), which are immunogenic and show exceptional evolutionary conservation. Hsps are considered as candidate antigens to restore tolerance based on the possibilities of triggering the activation of regulatory T cells [5, 6]. In this sense, Hsp60 epitopes involved in the mechanisms of regulation have been identified in animal models of autoimmune arthritis (AA) [7] and epitopes from the human Hsp60 (hHsp60) have been selected to induce tolerance in patients with autoimmune diseases [8].

On the other hand, these epitopes can be modified to modulate their immunological properties, being called altered peptide ligands (APLs). They are similar to native immunogenic peptides but with one or several substitutions in the essential contact positions with the T cell receptor or with the major histocompatibility complex (MHC), and, therefore, interfering the cascade of necessary events for the complete activation of T cells [9].

In this work, we have predicted two novel T cell epitopes from hHsp60 with the aid of bioinformatic tools. These epitopes were used to design two APLs, which therapeutic effects were evaluated in two animal models: AA in Lewis rats and collagen induced arthritis (CIA) in DBA/1 mice; and in *ex vivo* assays using peripheral blood mononuclear cells (PBMCs) isolated from rheumatoid arthritis (RA) patients. Therapeutic effects of both APLs were similar to methotrexate (MTX), the standard treatment for RA, suggesting that these peptides are potential therapeutic treatments to control RA.

Results

APLs design

In contrast, with previous reports, attention was put on the N-terminal region of human Hsp60. In this region, the computer algorithm Propred [10] predicted two epitopes, corresponding to amino acid 90-109 and 55-75, directly involved in the interaction with MHC class II molecule. These peptides were called E18-3 and E18-12 and the sequences are shown in Figure 1. An amino acid residue involved in the interaction with the MHC II was changed in each peptide to increase the affinity for this molecule. MHC II molecules frequently expressed by RA patients were considered. The affinity improved new peptides were called APL-1 and APL-2 and their sequences are shown in figure 1.

E18-3 (wild-type peptide)
SIDLKDKYKNIGAKLVQDVANNTNEEA
SIDLKDKYKNIGAKLVQLVANNTNEEA APL-1

E18-12 (wild-type peptide)
MGPKGRTVIIIEQSWGSPKVTK
MGPKGRTVIIIEQSLGSPKVTK APL-2

Figure 1. Amino acid sequences of wild-type peptides and the derived altered peptide ligands (APLs). The amino acid modified in each APL is represented in bold.

Evaluation of regulatory T cells induced by APL-1 in PBMC from RA patients

The induction of regulatory T cells (Tregs) by the APL-1 peptide in *ex vivo* assays using PBMCs or synovial fluid mononuclear cells (SFMCs) from RA patients was analyzed. In total, 13 patients were included in this study. PBMCs were stimulated with 40 µg/mL of APL-1 or wild-type peptide for 5 days. The samples were screened for frequency of FoxP3+ cells among CD25^{high}CD4+ T cells by flow cytometry. For this purpose, T lymphocytes were first gated for CD4+, and further gates were applied on that subset to select those coexpressing low or high levels of CD25. FoxP3 was performed on CD25^{high}CD4+ gated T cells. A relative increase in FoxP3+ CD25^{high}CD4+ T cells was found in PBMCs treated with APL-1 in comparison to PBMCs stimulated either with the wild-type peptide or without stimulation (Figure 2).

Evaluation of IL-17, IL-10 and TNF-α levels induced by APL-2 in PBMCs from RA patients

Once demonstrated that APL-2 induced proliferation of CD4+ T cells, the effect of this peptide on cytokine secretion was further evaluated. PBMCs without peptide stimulation were used as negative control. APL-2 suppressed the interleukin 17 (IL-17) levels *in vitro* by more than 50 % and remarkably increased IL-10 levels more than 3 times compared to negative controls. Tumor necrosis factor alpha (TNF-α) levels remained steady regardless the treatment.

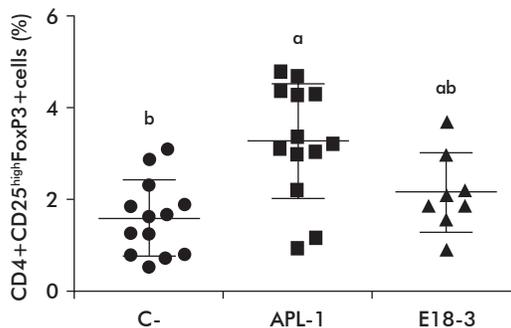


Figure 2. The altered peptide ligand APL-1 increases frequency of CD4+CD25^{high}Foxp3+ regulatory T cells in *ex vivo* assays with peripheral blood mononuclear cells (PBMCs) from rheumatic patients. PBMCs were stimulated with APL-1 or the native E18-3 peptide for 5 days. PBMCs in the absence of peptide were used as control of basal levels for each patient (C-). Samples were screened for CD4+CD25^{high}Foxp3+ T cells frequencies by flow cytometry. Different letters mean statistically significant differences (p < 0.05).

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Therapeutic evaluation of APLs on AA model

Therapeutic effects of APLs on arthritis was evaluated and compared with wild-type peptides in the AA model. The signs associated with the development of arthritis began gradually in all animals inoculated with *Mycobacterium tuberculosis*. These signs were evident on day 10, characterized by a slight redness and swelling of the posterior joints. On day 11, the rats were randomly divided in several groups (n = 12). The mean arthritis score on day 21 (the day of maximum arthritis severity) was the main parameter used in this study to measure clinical outcomes and evaluation of the effects of the peptides. A significant reduction of AA means arthritis score on day 21 was observed with APL-1 and APL-2 compared to rats treated with phosphate buffer saline (PBS). The improvement induced by both APLs was comparable to healthy animals. Clinical improvement of AA with the APLs is corresponded with a decrease of joint destruction by the arthritic process. Four animals were sacrificed per group and ankle joints were collected on day 21 after induction of AA and scored for severity. The histological examination of the joints showed severe erosion of cartilage and bone as well as massive inflammatory cell infiltration and obvious pannus formation in all rats inoculated with PBS. In contrast, subcutaneous administration of APL-1 or APL-2 resulted in a suppression of these histological signs characteristic of AA. However, in all rats treated with wild-type peptides presented a histological score of 3, with massive cellular infiltration, synovitis and moderate erosions of bone and cartilage. These results indicate that both APLs suppressed AA efficiently (Figure 3A-C).

In addition, the production of TNF- α was investigated in the spleen from sacrificed rats at day 21 after disease induction. Administration of APLs significantly reduced TNF- α levels.

On the other hand, the capacity of APL-1 to induce regulatory T cells during the therapy with this peptide in rats was confirmed by a significant increase in the percentages of T cells with phenotype CD4+CD25^{high}FoxP3+ in rats treated with APL-1, compared to rats either without treatment or treated with the corresponding wild-type peptide.

Therapeutic evaluation of APL-2 on CIA model

Therapeutic effect of APLs were evaluated and compared to MTX, in another animal model where arthritis is induced by a different autoantigen. CIA was elicited in DBA/1 mice by two subcutaneous immunizations with collagen type II. Thirty-two out of thirty-six mice (88 %) developed arthritis between days 23 and 26, showing evidences of clinical inflammation in one or two hind paws. CIA mice were randomly allocated in several groups (n = 12 per group), and showed no differences in the arthritis score among them. Then, the CIA mice were treated with APL-1, APL-2 or MTX. PBS was injected as negative control.

Treatment with APLs reduced the severity of CIA compared to PBS treated mice from day 37 after the first immunization until the end of protocol. Similar results were observed in MTX treated animals. On day 46, 4 mice per group were sacrificed for histological examination. A close correlation between the clinical signs and the histopathological findings was

found. All untreated mice developed a histological score of 3. In contrast, mice inoculated with APLs only showed slight histological damages in the joints (Figure 3D-F).

To investigate the mechanism of action of both APLs in suppressing CIA, the levels of TNF- α , IL-17 and IL-10 were measured in serum of mice sacrificed on day 46. The treatment with APL-2 significantly reduced TNF- α and IL-17 secretion compared to PBS inoculated mice. Levels of both cytokines in this group were similar to those obtained in healthy animals. The administration of APL-1 or MTX significantly reduced TNF- α secretion, but did not modify IL-17. Otherwise, only the combination of APL-1 with MTX increased the percentages of T cells with CD4+CD25^{high}FoxP3+ phenotype in mice.

Discussion

Conceptually, the therapeutic intervention presented here is based on modulating the T cell function and therefore, higher specificity and lower toxicity is expected [11]. In this study, two novel T cell epitopes from the hHsp60 were defined. According to our prediction, these peptides represent strong epitopes directly involved in the interaction with human MHC II

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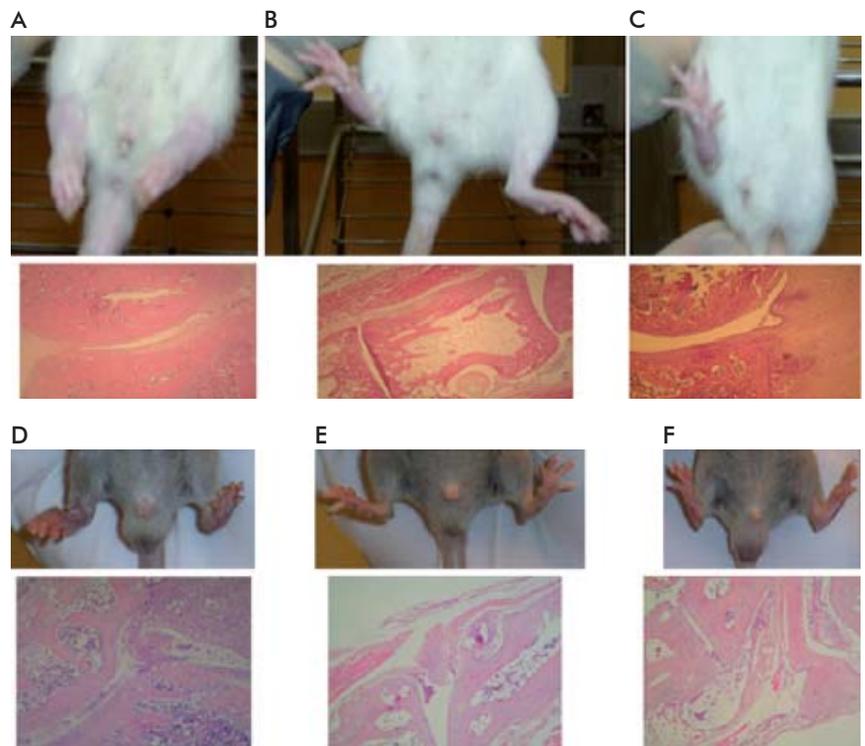


Figure 3. Treatment with APL-1 and APL-2 peptides caused reduction of clinical score and histological damages in ankle joints after the induction of autoimmune arthritis in Lewis rats (A-C) and collagen induced arthritis in DBA-1 mice (D-F), respectively (4 animals per group). Upper panels show the clinical status of animals without (A and D; PBS-treated control animals) or after treatment with APLs (B, C, E, and F) on day 21. Upper panels A and D evidence arthritic inflammation, which is absent in APL-treated animals. For microscopy evaluations (lower panels), joints were harvested after the induction autoimmune arthritis in rats and collagen induced arthritis in mice (4 animals per group), and were stained with haematoxylin and eosin (original magnification 10 \times). Paw sections were examined by two independent observers who were blinded to the experimental group. The histopathological damage score in ankle joints was assigned with grades 0 to 3. A and D) Pannus infiltration, diminished synovial space and cartilage damage (grade 3); B and E) APL-1 treated animals (grade 0). C and F) APL-2 treated animals (grade 0). APL treatments preserved the synovial spaces.

molecules, particularly those related to RA. We evaluated the potential of these wild-type peptides to modify proinflammatory and immunoregulatory cytokine levels in *ex vivo* assays using PBMCs from patients with RA. In these assays, an increment of TNF- α and INF- γ levels was confirmed.

Two APLs were designed from these wild-type peptides, retaining the binding properties to MHC II molecules, but carrying single mutations of an essential contact position for its binding. All these modifications were aimed at transforming the T cell response generated by the wild-type peptides towards the tolerance induction in RA patients.

Recent studies have reported defects in the number and/or activity of Tregs in humans with RA, similar to those observed in mouse models of arthritis [12]. Failures in the function of the Tregs can therefore be responsible for the development of autoimmune diseases, and enhancing their functions may represent a feasible treatment strategy. We suggested that the modification in APL-1 could induce Tregs in patients that may attenuate the pathogenic T cells with specificity, without affecting other non-related T cells. To test this hypothesis, the induction of Tregs by APL-1 was analyzed in *ex vivo* assays using PBMCs or SFMCs from RA patients, and it was confirmed that APL-1 induced an increment of Tregs with a CD4+CD25^{high}FoxP3+ phenotype in all the analyzed patients.

On the other hand, APL-2 preferentially increased IL-10 and decreased IL-17 but do not have any effect on TNF- α level in *ex vivo* assays with PBMCs isolated from RA patients, suggesting a deviation from inflammatory to regulatory cytokine profile. This peptide increased IL-10 levels in all patients' PBMCs irrespective of their HLA background; therefore APL-2 could bind to several allelic variants of the human MHC II (DQ and DR).

To explore the therapeutic effect of the APLs, AA and CIA animal models were chosen. The treatment with the APL-1 induced excellent clinical in both models. This effect was correlated with improvement of the histological score of the joints induced by the peptide, and it was comparable to healthy animals. In contrast, the wild-type peptide (E18-3) did not induce any clinical or histological improvement in the animals. According to these results, we can confirm that the modification carried out in E18-3 was very effective through attenuation of the pathogenic inflammation in these animal models. The clinical efficacy achieved by the treatment with APL-1 was associated with an increment in Tregs in the spleen. The Tregs induced by APL-1 in the periphery apparently could migrate to the joints and induce suppression of the local self-reactive response. In addition, we found that the therapy

with the APL-1 reduces significantly the TNF- α level in spleen. Given these facts, we think that probably the potent therapeutic effect of the APL-1 in the reduction of AA and CIA would be due to the processing and presentation of the peptide by the antigen presenting cells to the autoreactive T lymphocytes in periphery. The recognition of this altered ligand may induce the expansion of T cells with immunoregulatory phenotype like CD4+CD25^{high}FoxP3+ Tregs. The activated cells migrate to the inflammation site and they could cross-recognize the native epitope from the hHsp60, where it is highly expressed due to the inflammation process.

This new contact with the autoantigen may induce potent immunoregulatory effect, attenuating the autoreactive T cells responsible for arthritis pathogenesis and inhibiting the TNF- α expression. The pivotal role of TNF- α in the induction and progression of rheumatoid synovitis is well established [13]. Consequently, the result demonstrating that APL-1 inhibited the expression of TNF- α represents a beneficial effect for the control of the inflammatory process.

Similarly, APL-2 was effective for down-regulating the inflammatory response in AA model with a reduction of TNF- α level. Also, APL-2 reduced the TNF- α level in sera of CIA mice to concentrations comparable to healthy animals. Additionally, pannus formation and joint damage were not observed. Similar results were obtained in MTX-treated mice.

Many evidences support the role of IL-17 in the pathogenesis of human RA and its animal models such as CIA [14, 15]. The decrease of IL-17 and TNF- α secretion induced by APL-2 could indicate that the therapeutic effect of this peptide is mediated by downregulation of inflammatory cytokines rather than induction of Tregs. Consistent with this, APL-2 treatment does not induce Tregs with a Foxp3+ CD4+ phenotype at day 46 in spleen of CIA mice.

Conclusions

The therapeutic effect induced by APLs in both animal models is mediated by different molecular mechanisms, associated with immunologic tolerance. These results indicate a therapeutic potential for these APLs and support further investigation of these candidate drugs for RA treatment.

Intervention on T cell function in a specific manner as shown here would bring the possibility of focusing on one or more antigens involved in the autoimmune pathogenesis, thus avoiding the general immune suppression in patients as happens with anti-TNF- α treatment. This study contributes to the knowledge of mechanisms and tools needed for the induction of tolerance in humans using autoantigens or their variants.

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