Treatment of lupus patients with a tolerogenic peptide, hCDR1 (Edratide): Immunomodulation of gene expression

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Abstract

Systemic lupus erythematosus (SLE) is an autoimmune disorder characterized by dysregulation of cytokines, apoptosis, and B- and T-cell functions. The tolerogenic peptide, hCDR1 (Edratide), ameliorated the clinical manifestations of murine lupus via down-regulation of pro-inflammatory cytokines and apoptosis, up-regulation of the immunosuppressive cytokine TGF-β, and the induction of regulatory T-cells. In the present study, gene expression was determined in peripheral blood mononuclear cells of 9 lupus patients that were treated for 26 weeks with either hCDR1 (five patients), or placebo (four patients). Disease activity was assessed by SLEDAI-2K and the BILAG scores. Treatment with hCDR1 significantly down-regulated the mRNA expression of the pathogenic cytokines IL-1β, TNF-α, IFN-γ, and IL-10, of BlyS (B-lymphocyte stimulator) and of the pro-apoptotic molecules caspase-3 and caspase-8. In contrast, the treatment up-regulated in vivo gene expression of both TGF-β and FoxP3. Furthermore, hCDR1 treatment resulted in a significant decrease in SLEDAI-2K (from 8.0 ± 2.45 to 4.4 ± 1.67; P = 0.02) and BILAG (from 8.2 ± 2.7 to 3.6 ± 2.9; P = 0.03) scores. Thus, the tolerogenic peptide hCDR1, immunomodulates, in vivo, the expression of genes that play a role in SLE, consequently restoring the global immune dysregulation of lupus patients. Hence, hCDR1 has a potential role as a novel disease-specific treatment for lupus patients.

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of autoantibodies and by impaired functions of B- and T-cells, accompanied by systemic clinical manifestations [1,2]. Previous studies demonstrated the role of cytokines [3,4], apoptosis [5,6], and dysfunction of regulatory T-cells [7,8] in the pathogenesis of murine and human SLE. For a specific treatment of SLE, our laboratory designed a peptide, designated hCDR1 [9], which is based on the sequence of the complementarity-determining region (CDR)1 of a human anti-DNA monoclonal antibody that bears the major idiotype designated 16/6Id [10,11]. hCDR1 was shown to ameliorate the serological and clinical manifestations of induced or spontaneously developed lupus in mice [12]. The beneficial effects of hCDR1 were associated with the down-regulation of the pathogenic cytokines IL-1β, IFN-γ, and IL-10 [12], and the up-regulation of the immunosuppressive cytokine TGF-β [13]. Treatment with hCDR1 also diminished significantly the production of the B-cell stimulator (BlyS, BAFF) in lupus-prone mice [14]. Furthermore, treatment of SLE-afflicted mice with hCDR1 resulted in reduced apoptosis rates of T-cells [15–17], and in specific induction of CD4+CD25+Foxp3+ regulatory cells [13,18].

We have previously shown that hCDR1 down-regulated, in vitro, autoreactive T-cell responses of peripheral blood mononuclear cells (PBMC) of SLE patients in association with an increased production of TGF-β [9]. Moreover, we established a human model of SLE by transferring PBMC of lupus patients into severe combined immunodeficient mice [19]. Treatment with hCDR1 ameliorated the serological (human anti-DNA antibodies) and clinical lupus-related manifestations in the latter model of human SLE [20]. Furthermore, we have recently shown that in vitro incubation of PBMC of lupus patients with hCDR1, but not with a control peptide, down-regulated gene expression of IL-1β, TNF-α, IFN-γ, IL-10, and caspase-3 and up-regulated the expression of TGF-β, FoxP3, the anti-apoptotic molecule Bcl-xL and of the negative regulators Foxj1 and...
Foxo3a [21]. In addition, the in vitro incubation with hCDR1 resulted in an increase of CD4+CD25+FoxP3+ functional regulatory T-cells [21]. It has been of great interest to determine whether hCDR1 functions via similar mechanisms of actions when administered to SLE patients. The present study was therefore aimed at determining the in vivo effects of hCDR1 on human SLE. To this end, blood samples were taken from 9 lupus patients that were treated with hCDR1 or with the vehicle for a period of 26 weeks. Gene expression of the pathogenic cytokines (IL-1β, TNF-α, IFN-γ, and IL-10), pro-apoptotic molecules (caspase-3, caspase-8), BlyS, and immunoregulatory TGF-β and FoxP3 molecules was determined by real-time RT-PCR. The results indicate that hCDR1 immunomodulated in vivo the expression of the above genes in SLE patients in a manner similar to that demonstrated for SLE-afflicted mice.

2. Materials and methods

2.1. Patients

The data presented here are of nine SLE patients from two Israeli Medical Centers (Kaplan, Rehovot; Rabin, Petah-Tikva). These patients participated in a large double-blinded clinical trial. Included are all patients from the two medical centers who completed the study and from whom blood samples were taken at least twice (before treatment initiation and at week 24) for mRNA preparation. We present here the effect of treatment with hCDR1 on cytokine, apoptosis, BlyS, and regulatory gene expression and the clinical outcomes of these patients. All patients were diagnosed with SLE according to 4 or more of the ACR-revised diagnostic criteria [22]. All patients signed an informed consent form prior to the initiation of the study. The patients had a mild to moderate active SLE with an SLE disease activity index 2000 (SLEDAI-2K) [23] of 6–12 (inclusive) and a stable (for at least 4 weeks) dosage of all lupus-related medications (changes in prednisone ≤ 10 mg/day were allowed). This study was approved by the Kaplan and Rabin Medical Centers ethic committees and was conducted according to all good clinical practice (GCP) rules.

2.2. Study medication and design

hCDR1 (Edratide) is a peptide (GYYWSWIRQPPGGEWIG) based on the CDR1 of an autoantibody [9,10]. hCDR1 (synthesized by Polypeptide Laboratories, Torrance, CA), dissolved in Captisol (Sulfoethyl ether cycloexdrin sodium, CyDex, Lenexa, KS), was subcutaneously injected weekly for 26 consecutive weeks. The patients were screened 7–30 days prior to their randomization into four treatment groups: those receiving placebo (Captisol, four patients), and those receiving three doses of hCDR1 0.5 mg (two patients), 1.0 mg (two patients), and 2.5 mg (one patient). Patients were then evaluated clinically (lupus-related manifestations, medications, adverse effects, study drug adherence) and serologically at weeks 4, 8, 12, 16, 20, 24 and 26. SLE disease activity was determined by SLEDAI-2K [23], and by the British Isles Lupus Assessment Group (BILAG) scores [24].

2.3. Real-time RT-PCR

Blood samples were collected in PAXgene (PreanalytiX, Switzerland) tubes and frozen at −70 °C until mRNA isolation. The mRNA levels of IL-1β, IFN-γ, TNF-α, IL-10, BlyS, Caspase-3, Caspase-8, TGF-β, and FoxP3 were determined by real-time RT-PCR using LightCycler (Roche Mannheim, Germany). Complementary DNA was prepared from the mRNA and subjected to real-time RT-PCR, according to the manufacturer’s instructions. Because there are no available assays to determine protein levels for all nine molecules investigated in the sera of patients, and since a correlation was previously shown between mRNA and protein expression in murine models of SLE [12,17,21], we chose to assess gene expression levels in the present study. Real-time RT-PCR was performed at the same time on blood samples taken at different visits from the same patient to allow accurate comparison of gene expression at baseline and following in vivo treatment with hCDR1. The gene expression levels were determined by examiners who were blinded to the treatment groups of the blood donors. The following primer sequences (forward and reversed, respectively) were used: IL-1β (5′-cagaaagtccggtct-3′, 5′-gcactatccaaagagcag-3′), IFN-γ (5′-tcaggtcgagagcagagaa-3′, 5′-gacagtaagctgtctgatcagc-3′), TNF-α (5′-gcagccggttcattacct-3′, 5′-aggccgattacacagaca-3′), IL-10 (5′-aagccatgtaggtttg-3′, 5′-acatgtgttgcacctc-3′), BlyS (5′-cttgcctgatcctca-3′, 5′-gaacgcacgctattct-3′), Caspase-3 (5′-gcaggaagccctaca-3′, 5′-ggtgtagctgccgtta-3′), Caspase-8 (5′-cattgctgtatcgct-3′, 5′-atagccatgcgcacag-3′), TGF-β (5′-gcagactatccagacag-3′, 5′-actgtctagactctgttg-3′), FoxP3 (5′-ccacacagtaacctc-3′, 5′-cttcttctgtccaatc-3′), and GAPDH (5′-ctgcacagtctgct-3′, 5′-gttaggggaactccca-3′). The levels of GAPDH were used for normalizing the expression levels of all other genes. Results for all genes are expressed as percent expression at week 24 of treatment compared to day 0, which was defined as 100%.

2.4. Statistical analysis

The non-parametric Mann–Whitney and unpaired Student’s t-tests were used for statistical analyses. Values of p ≤ 0.05 were considered statistically significant.

3. Results

3.1. Patients

The mean (±SD) age of our nine patients (one male, eight females) at the time of the study was 46.4 ± 10.5 (range 29–61) years. All had antinuclear antibodies and anti-dsDNA autoantibodies in their sera at study entry. Hypocomplementemia (either C3 or C4) was observed in five patients. The main SLE-related clinical manifestations at study entry were skin involvement (6/9), arthritis (5/9), alopecia (4/9), thrombocytopenia and lymphopenia (2/9), mucosal ulcers (2/9), and pleuritis (1/9). The mean SLEDAI-2K and BILAG scores, determined for all patients at entry to the study (week 0), were 8.2 ± 2.5 (range 6–12) and 7.8 ± 2.4 (range 4–13), respectively, indicating mild to moderate SLE. Seven patients were treated with Hydroxychloroquine (400 mg/day) and seven with oral corticosteroids (mean daily dose of prednisone 18.2 ± 11.3 mg; range 5–40). There were no significant differences in serological and clinical manifestations or in treatment regimes among the five patients who were treated with the study drug (hCDR1) and the four patients in the placebo group at entry to the study.

3.2. In vivo effects of hCDR1 treatment on gene expression of inflammatory cytokines and BlyS

The in vivo effects of weekly administrations of hCDR1 to the SLE patients on IL-1β, TNF-α, IFN-γ, and IL-10 gene expression were determined. Table 1 shows the expression of the genes studied for the individual patients and Fig. 1 presents the mean percent of gene expression at week 24 compared to the levels at week 0 (defined as 100%; dotted line). Table 1 and Fig. 1 demonstrate that the expression of the four inflammatory cytokines decreased significantly (p < 0.01) in five patients (with the three treatment doses) following 24 weeks of hCDR1 treatment, as compared with their levels in the same patients prior to the initiation of treatment.
3.3. In vivo effects of hCDR1 treatment on apoptotic gene expression

Since hCDR1 treatment of SLE-afflicted mice down-regulated the pro-apoptotic genes, caspase-3, and caspase-8 [16,17], we studied the in vivo effects of hCDR1 on these genes in lupus patients. As can be seen in Table 1 and Fig. 2, significant ($p < 0.01$) down-regulation of the gene expression of both caspase-3 and caspase-8 was observed in all five lupus patients following 24 weeks of hCDR1 treatment, regardless of the treatment dose. In the placebo-treated patients, however, BLYS expression was insignificantly increased. In spite of the small number of patients, the difference between the hCDR1- and the placebo-treated patients was statistically significant ($p = 0.01$; Fig. 1).

3.4. In vivo effects of hCDR1 treatment on regulatory gene expression

Our laboratory previously reported that treatment of SLE-afflicted mice with hCDR1 resulted in the up-regulation of the immunosuppressive cytokine TGF-$\beta$ and the regulatory master gene Foxp3 [13,17,18]. As shown in Table 1, both genes TGF-$\beta$ and Foxp3 were up-regulated in the four patients that were treated with either 0.5 mg or 1 mg of hCDR1. These genes were rather down-regulated in PBMC of the patient treated with 2.5 mg of hCDR1. Foxp3 gene expression was elevated in only one patient from the placebo group (Table 1). TGF-$\beta$ gene expression was significantly higher (compared with baseline at week 0) in the hCDR1-treated lupus patients than in the control group of patients (Fig. 3). Although 24 weeks of hCDR1 treatment resulted in a prominent increase in Foxp3 gene expression (216 ± 94.6%), the difference did not reach statistical significance (most probably due to the low number of patients) compared with the placebo-treated patients (Fig. 3).

3.5. Kinetics of hCDR1-affected gene expression

To further investigate the in vivo effects of hCDR1 treatment on gene expression of lupus patients, we studied the changes in the expression of various genes throughout the study period (weeks 0, 4, 8, 16, 24). Fig. 4 shows the kinetics of gene expression in two representative patients, one treated with 1 mg (Fig. 4A) and the other with 0.5 mg of hCDR1 (Fig. 4B). Fig. 4A demonstrates that whereas no effect was noted regarding the expression of IFN-$\gamma$, IL-10, and TGF-$\beta$ genes after four weeks of hCDR1 treatment, the expression of BLYS diminished whereas that of FoxP3 already

![Graph 1](image1.png)

Fig. 1. hCDR1 down-regulates in vivo IL-1$\beta$, TNF-$\alpha$, IFN-$\gamma$, IL-10 and BLYS gene expression in SLE patients. SLE patients with mild to moderate lupus were treated (subcutaneously, once a week) with either hCDR1 or placebo. Gene expression in PBMC obtained from lupus patients treated with hCDR1 or the placebo was determined by real-time RT-PCR. Results are presented as mean percentage of gene expression (+SE) at week 24 compared to the levels at week 0 (defined as 100%; dotted line).

![Graph 2](image2.png)

Fig. 2. hCDR1 down-regulates in vivo gene expression of the pro-apoptotic molecules caspase-3 and caspase-8 in SLE patients. Results are presented as mean percentage of gene expression (+SE) in PBMC obtained from lupus patients treated with either hCDR1 or placebo at week 24 compared to the levels at week 0 (defined as 100%; dotted line).

Table 1

The effect of in vivo treatment with hCDR1 on gene expression in PBMC of SLE patients.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Treatment</th>
<th>Dose (mg)</th>
<th>% Expression relative to baseline</th>
</tr>
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<td></td>
<td></td>
<td></td>
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<td>IL-1$\beta$</td>
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<td>70103</td>
<td>hCDR1</td>
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<td>23</td>
</tr>
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<td>34</td>
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</table>

SLE patients with mild to moderate lupus were treated (subcutaneously, once a week) with either hCDR1 or placebo. Gene expression in PBMC was determined by real-time RT-PCR. Results are presented as the percentage of gene expression at week 24 compared to that at week 0 (before the study was initiated), defined as 100%.
increased. Note that after four weeks of treatment, no clinical improvement was observed in this patient. At all time points thereafter, hCDR1 treatment resulted in the down-regulation of IFN-γ, IL-10, and BlyS and in the up-regulation of TGF-β and Foxp3 gene expression (Fig. 4A) concomitantly with clinical improvement (decrease in BILAG score from 6 at week 0 to 0 at week 24).

The patient presented in Fig. 4B, a 29-year-old female, was treated with 0.5 mg hCDR1. The treatment resulted in clinical improvement (decrease in BILAG score from 13 to 8 along with the discontinuation of her prednisone treatment) concomitantly with improvement (decrease in BILAG score from 13 to 8 along with the discontinuation of her prednisone treatment) concomitantly with worsening of her alopecia, requiring the initiation of prednisone (week 36), the patient had a lupus flare with fever, pleuritis, and a new inflammatory skin rash along with worsening of her alopecia, requiring the initiation of prednisone (30 mg/day). At that time, an up-regulation of IFN-γ, IL-10, and BlyS and a down-regulation of TGF-β and Foxp3 gene expression were observed (Fig. 4B).

As was demonstrated at week 24 (Figs. 1–3 and Table 1), no significant effects on the expression of the above genes were observed throughout the study (weeks 4–24) in the placebo-treated patients (data not shown).

3.6. The effects of hCDR1 treatment on the clinical score

The main finding of the present study is that the tolerogenic peptide hCDR1 is capable of suppressing, in vivo, gene expression of pro-inflammatory cytokines, apoptosis, and of B-cell stimulation. Furthermore, in vivo treatment with hCDR1 up-regulated immunosuppressive and regulatory molecules in PBMC of SLE patients.

4. Discussion

The clinical trial lasted for 26 weeks. However, since gene expression was determined on blood samples obtained at week 24, we present here the clinical and laboratory evaluation for this week. Nevertheless, the clinical, laboratory, and disease activity scores (BILAG, SLEDAI-2K) were similar at the end of the study (week 26).

Treatment of the five SLE patients with hCDR1 resulted in clinical improvement. Thus, as shown in Fig. 5, the mean BILAG score of the hCDR1-treated patients decreased significantly (from 8.2 ± 2.7, range 6–13 at week 0 to 3.6 ± 2.9, range 0–8 at week 24; p = 0.03), whereas the change in the BILAG score in the placebo-treated group was not significant (from 7.2 ± 2.2, range 4–9 at week 0 to 6.25 ± 1.7, range 4–8 at week 24; p = 0.50). Similarly, the mean SLEDAI-2K score decreased significantly in the hCDR1-treated patients (from 8 ± 2.45, range 6–12 at week 0 to 4.4 ± 1.67, range 2–6 at week 24; p = 0.02), compared with an insignificant change in the placebo-treated patients (from 7.0 ± 0.8, range 6–8 at week 0 to 6.0 ± 2.45, range 4–9 at week 24; p = 0.50).

All nine patients presented here completed the entire study. Neither site injection discomfort nor rash or any significant clinical or biochemical disturbances were observed during the 26 weeks of injections of either hCDR1 (at all three doses) or the placebo.
mice or spontaneously developed in (NZBxNZW)F1 mice [12]. Similarly, synthetic peptides were used successfully in several laboratories for the treatment of various murine models of SLE [28–30].

The beneficial effects of hCDR1 were due to the generation of CD4⁺CD25⁺FoxP3⁺ functional regulatory T-cells [13,17,18], to the up-regulation of the immunosuppressive cytokine TGF-β, and to the suppression of lupus-associated pathogenic cytokines [12]. Suppression of apoptosis via down-regulation of the pro-apoptotic molecules caspase-3 and caspase-8 was also shown to play a role in the mechanism of action of hCDR1 [16,17]. Furthermore, in vitro incubation of PBMC of lupus patients with hCDR1 resulted in similar immunomodulatory effects on the above gene expression and on the induction of functional regulatory T-cells [21]. In the present study, treatment of lupus patients with hCDR1 resulted in significant in vivo effects on these SLE-related genes (Figs. 1–3), suggesting that, as was previously shown in mice [12–14,17,31], hCDR1 has potential beneficial effects in human lupus.

The underlying mechanisms involved in the development of SLE are not precisely understood, though global T-cell dysregulation, an increased rate of apoptosis, and B-cell stimulation play major roles in the pathogenesis of the disease [1]. The elevated levels of the pro-inflammatory cytokines IL-1β, IFN-γ, IL-10, and TNF-α, and the down-regulated production of the immunosuppressive cytokine, TGF-β, were shown to be involved in the pathogenesis of murine and human lupus [4,13,17], and human [3] SLE. In addition, high rates of apoptosis of immune cells were reported in active lupus [6,32]. We showed here that, as previously demonstrated following treatment of SLE-afflicted mice [12,13,16,17], in vivo treatment of lupus patients with hCDR1 down-regulated the lupus-related pro-inflammatory cytokines (Fig. 1), and the pro-apoptotic molecules caspase-3 and caspase-8 (Fig. 2), and up-regulated TGF-β (Fig. 3).

The B-cell stimulator, BlyS, which promotes the differentiation and maturation of B-cells, also was shown to play a role in the pathogenesis of murine and human lupus [25–27]. Nevertheless, clinical trials of lupus patients with BlyS antagonists resulted in only very modest beneficial effects [33,34], suggesting that targeting BlyS alone is an insufficient therapeutic approach. In vivo treatment of SLE patients with hCDR1 resulted in down-regulated expression of the BlyS gene (Fig. 1), in addition to the immunomodulation of the above-discussed genes that play a role in this autoimmune disease, suggesting that this peptide has a potential role in the treatment of human lupus.

CD4⁺CD25⁺FoxP3⁺ regulatory T-cells play a major role in maintaining immunological homeostasis [7,8]. Lower levels and dysfunction of regulatory T-cells were demonstrated in various autoimmune diseases [35]. Indeed, low levels of functional CD4⁺CD25⁺FoxP3⁺ regulatory T-cells were reported in the peripheral blood of active SLE patients [36,37]. Moreover, adoptive transfer of hCDR1-induced specific CD4⁺CD25⁺FoxP3⁺ regulatory T-cells led to the amelioration of disease manifestations in lupus-afflicted mice [13]. Recently, we demonstrated an up-regulation of FoxP3 gene expression associated with an increased number and suppressive function of CD4⁺CD25⁺FoxP3⁺ regulatory T-cells in PBMC obtained from lupus patients following in vitro incubation with hCDR1 [21]. In agreement, Hahn et al. demonstrated in vitro restoration of CD4⁺CD25⁺FoxP3⁺ regulatory T-cells (increased numbers as well as suppressive function) in PBMC of lupus patients by an anti-DNA-based peptide [38]. The fact that hCDR1 up-regulated, in vivo, gene expression of the suppressive master gene FoxP3 in PBMC of lupus patients (Fig. 3) further suggests its ability to up-regulate functional regulatory T-cells, as demonstrated in murine models [13,17,18], and in vitro in human studies [21].

We present here only nine lupus patients (five treated with hCDR1 and four with placebo) from two Israeli centers that completed the entire study and for whom mRNA samples were available at least at two time points (week 0 and week 24). Thus, our clinical results should be interpreted with caution. Nevertheless, 24 weeks of treatment with hCDR1 was safe and led in this limited number of patients to significant clinical improvement as indicated by both SLEDAI-2K and BILAG scores (Fig. 5). The kinetics of the hCDR1-afllected gene expression (Fig. 4) was associated with lupus disease activity (clinical improvement was observed 6–8 weeks following the initiation of hCDR1 treatment and disease exacerbation 10 weeks after the end of the study). The latter further supports the significance and relevance of the in vivo hCDR1-induced effects on gene expression in lupus patients.

All patients that participated in this study had mild to moderate lupus without renal or central nervous system involvement. Thus, the beneficial effects of hCDR1 on the latter manifestations could not be tested. Nevertheless, it should be noted that in the lupus models in mice, hCDR1 had significant beneficial effects on both...
the renal [12,13,20], and central nervous system (Lapert S. et al., unpublished observations) lupus-related manifestations.

To conclude, in the limited group of patients that participated in this study, hCDR1, a peptide with significant beneficial therapeutic effects in murine SLE, down-regulated in vivo gene expression of pathogenic cytokines, apoptosis and BLyS and up-regulated immunosuppressive molecules, thus restoring the global immune dysregulation of lupus patients. The latter was associated with clinical amelioration. Taking into consideration the safety of treatment with hCDR1 and the fact that its effects are specific to SLE-associated responses, our results suggest a potential role for hCDR1 in the treatment of lupus patients.

Acknowledgement

The clinical trial was conducted by Teva Pharmaceutical Industries, Ltd., and the blood samples used in our study were of patients that participated in this trial. However, the study reported here was performed independently at the Weizmann Institute of Science.

References


