Induction of hippocampal neurogenesis by a tolerogenic peptide that ameliorates lupus manifestations

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To determine the effect of the tolerogenic peptide hCDR1 on hippocampal neurogenesis, we treated SLE-affected (NZBxNZW)F1 mice with hCDR1 (once a week for 10 weeks). The treatment resulted in the up-regulation of neurogenesis in the dentate gyrus and restored the NeuN immunoreactivity in brain hippocampi of the mice in association with increased gene expression of IGF-1, NGF and BDNF. Furthermore, hCDR1 treatment significantly up-regulated p-ERK and p-Akt that are suggested to be key components in mediating growth factor-induced neurogenesis. The observed effects of hCDR1 on hippocampal-neurogenesis and on associated signaling pathways suggest a potential role for hCDR1 in CNS lupus.

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1. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease affecting organ systems including the kidney, central nervous system (CNS), joints, and skin (Hahn, 2006). The disease is characterized by dysregulated T cell and B cell immune responses (Nagy et al., 2005). Our laboratory designed a peptide, designated as hCDR1, that is based on the sequence of the complementarity-determining region (CDR) 1 of a human anti-DNA monoclonal antibody (Waisman et al., 1995). Our previous studies demonstrated that hCDR1 ameliorated the serological and clinical manifestations of murine lupus that was either induced or spontaneously developed in mice (Luger et al., 2004). Thus, hCDR1 down-regulated anti-dsDNA autoantibody levels, proteinuria, and the formation of immune complex deposits in the kidney resulting in better survival rates of the treated mice (Luger et al., 2004). The beneficial effects of hCDR1 were associated with a reduced production and expression of the pathogenic cytokines (e.g. IL-1β, IFN-γ, TNF-α, and IL-10), whereas the immunosuppressive cytokine TGF-β was up-regulated (Luger et al., 2004). hCDR1 was shown to inhibit T cell receptor signaling following its binding to class II major histocompatibility complex (Sela et al., 2006). The induction of CD4 and CD8 regulatory T cells plays a key role in the mechanism of action of hCDR1 (Sharabi et al., 2006; Sharabi and Mozes, 2008). Further, hCDR1 was shown to decrease the rates of T cell apoptosis (Rapoport et al., 2005; Sharabi et al., 2007). Treatment with hCDR1 affected the B cell compartment as well. Thus, it diminished the rate of maturation and differentiation of B cells by reducing the levels of B-cell-activating factor (BAFF), and increasing B cell apoptosis (Parameswaran et al., 2009). Moreover, treatment with hCDR1 inhibited the maturation of dendritic cells (Sela et al., 2009).

CNS involvement in SLE leading to a neuropsychiatric disease (NPSLE) is a debilitating consequence of this disease associated with substantial morbidity and occasional mortality (van Dam, 1991). Volume loss and structural abnormalities of the hippocampus were observed in the different mouse models of lupus and correlated with behavioral changes, similar to those observed in humans (Tomita et al., 2004). Recently, we demonstrated cell infiltration, immune complex deposits, gliosis, loss of neuronal nuclei (NeuN) immunoreactivity, and an altered cytokine profile in the hippocampi of (NZBxNZW)F1 mice with lupus (Lapter et al., 2009). Treatment with hCDR1 ameliorated the CNS manifestations and improved the behavioral performance of the mice with lupus (Lapter et al., 2009).

It has been suggested that brain inflammatory processes associated with autoimmune diseases can interfere with neurogenesis and contribute to impaired cognitive function (Monje et al., 2003). As the hippocampus is the core center for learning and memory, it has been hypothesized that there is an association between hippocampal neurogenesis and hippocampal function related to specific aspects of learning and memory (Leuner et al., 2004). A number of reports suggest that SLE progression is associated with a widespread neuronal loss (Appenzeller et al., 2006). The observed pathological changes including hindered neuronal differentiation and increased neuronal death suggest that the development of lupus induces neurodegenerative changes...
that involve impaired proliferative capacity of the brain (Stanojcic et al., 2009).

Several mechanisms may explain these brain alterations. Neuroinflammation is reported to disrupt neurogenesis via increased pro-inflammatory cytokine expression and increased microglia activation in the hippocampus. Stress hormones, chronically elevated in lupus-diseased mice, have also been shown to inhibit neurogenesis, and may additionally account for impaired brain growth and regeneration along the progression of autoimmune disease (Ballok, 2007). Elevated levels of brain-reactive antibodies were detected in the serum or cerebrospinal fluid (CSF) of patients with NPSLE (Zandman-Godder et al., 2007; Tin et al., 2005). CSF from diseased MRL-Lpr mice and from a deceased SLE patient, with a history of psychosis, memory loss and seizures, was reported to reduce viability of the C17.2 neural stem cell line (Sakic et al., 2005). Further, dsDNA-specific antibodies that cross-react with NR2, a subunit of the N-methyl-d-aspartate receptor on neuronal cells, were shown to cause neuronal death in vivo and in vitro (DeGiorgio et al., 2001). An additional subpopulation of CNS-associate autoantibodies consists of the anti-ribosomal-phosphoproteins (anti-Ribosomal-P). Indeed, intracerebroventricular injection of affinity purified anti-Ribosomal-P antibodies from a patient with NPSLE into naïve mice resulted in a depression like behavior in the injected mice (Katzav et al., 2007).

Various neurotrophins, such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), and growth factors such as insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF) were reported to be involved in the mechanisms regulating hippocampal neurogenesis. Thus, BDNF has been intensively studied and shown to increase hippocampal neurogenesis, and also to be involved in learning and memory. NGF is involved in a multitude of biological functions such as immune processes and peripheral tissue regeneration. IGF-1 plays an important role in cell growth and development, promotes the generation of newborn cells, and up-regulates the neurogenesis in adult hippocampus. Finally, VEGF, in addition to regulating vasculogenesis and angiogenesis, also stimulates hippocampal cell proliferation, survival and neurogenesis (Balu and Lucki, 2009). The action of these growth factors is associated with the activation of several signal transduction pathways including extracellular-regulated kinase (ERK) and protein kinase B (Akt) pathways (Johnson-Farley et al., 2007). These kinases were reported to be required for mediating growth factor-induced neurogenesis and neuroprotection (Brunet et al., 1999; Hetman et al., 1999; Datta et al., 1999).

The objective of the present study has been to determine the status of hippocampal neurogenesis in SLE-affected mice and to evaluate the effect of treatment with hCDR1 on the latter. We report here that treatment with hCDR1 up-regulated significantly the reduced levels of neurogenesis observed in mice with full-blown SLE. The latter was associated with increased expression of IGF-1, NGF, and BDNF whereas the expression of VEGF was not affected by hCDR1. Furthermore, a significant up-regulation in activated ERK and Akt, suggested to be key components in mediating growth factor-induced neurogenesis, was observed following treatment of SLE-affected mice with hCDR1.

2. Materials and methods

2.1. Mice

Female (NZBxNZW)F1 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were handled according to the protocols approved by the Weizmann Institute Animal Care and Use Committee, using international guidelines.

2.2. Synthetic peptides and treatments

The synthetic peptide hCDR1 (Stoeger et al., 2003), with a sequence GYYWSWIRQPPKGGEWIG that is based on the CDR1 of a human monoclonal autoantibody (Waisman et al., 1995), was synthesized at Polypeptide Laboratories (Torrance, CA). A peptide containing the same amino acids as hCDR1, with a scrambled order (SKGIPOYGWPGWEGWRYE), designated scrambled peptide, was used as a control. PBS (Dulbecco’s phosphate buffer; Sigma-Aldrich, UK) was used as a vehicle. Eight-month-old (NZBxNZW)F1 female mice (n = 8–10/group) were treated subcutaneously with injections of hCDR1, scrambled peptide (both at a dose of 50 μg per mouse), or with the vehicle alone (PBS), once a week for 10 weeks.

2.3. Treatment with 5-Bromo-2-deoxyuridine (BrdU) and immunochemistry

BrdU (Sigma), a thymidine analog that incorporates into the DNA of dividing cells, was injected intraperitoneally (50 mg/kg body weight) for the last 8 days (once a day) of treatment with hCDR1. One day after the last injection of BrdU, the mice from all treatment groups were deeply anesthetized and sacrificed by transcardial perfusion with PBS. Brains were then postfixed in 4% paraformaldehyde, and cryoprotected with 15% sucrose solution in PBS. Serial sagittal sections from the lateral 1.08–1.68 mm area were prepared. Frozen cryostat sections (20 μm thick) were used (Leica CM 1850; Leica, Nussloch, Germany).

For BrdU and NeuN immunocchemistry, the earlier sections were incubated in 2 M HCl for 30 min at 37 °C and rinsed in 0.1 M boric acid pH 8.5 for 10 min. Sections were then incubated with the primary antibodies, mouse anti-NeuN (1:200 Chemicon, Temecula, CA) and rat anti-BrdU (1:200, Serotec, Düsseldorf, Germany) diluted in PBS containing 2% BSA and 0.1% Triton X-100. After washing the secondary antibodies, anti-mouse FITC (1:200, Jackson ImmunoResearch, West Grove, PA) and biotinylated anti-rat (1:250, Chemicon) were used. Cy3-conjugated streptavidin (1:1000, Jackson ImmunoResearch) was added thereafter. Sections were analyzed with a fluorescence microscope (Nikon Eclipse E800) using Nikon ACT-1 software. Cell counts were performed on 1–4 series of sagittal sections of the dentate gyrus area in the hippocampi, by two examiners who were blinded to the treatment groups.

2.4. RNA isolation and polymerase chain reaction (PCR)

Brain hippocampi were isolated from mice in the different groups. Total RNA was extracted and pooled from the hippocampi of each group. The RNA was prepared using PerfectPure RNA Cell Kit-50 (Zotal, Biologicals & Instrumentation). Complementary DNA was prepared and subjected to PCR, to detect changes in gene expression. Primer sequences (forward and reversed, respectively) were used as follows: for IGF-1, 5'-atactcccttacagc-3', 5'-atacctgcgggttta-3'; for NGF, 5'-aaggacgcagctttct-3', 5'-gtctgataggggatc3'; for BDNF, 5'-ggtctgcgctctttacc-3', 5'-cgatgtaaggggaactc-3'; for VEGF, 5'-tcctcccagctggcagc-3', 5'-ccgatataagagactc3'; and for β-actin, 5'-ggtagctggcatactcc-3', 5'-cagtagaaggctcgc-3'. Each sample that was amplified contained 1 μl cDNA, 12.5 μl Ready Mix for PCR (Bio-Lab Ltd., Jerusalem, Israel), 9.5 μl DEPC water, and specific primer pairs.
The PCR was performed using Biometra® system (Tamar, Laboratory Supplies, Ltd., Jerusalem, Israel) for 35 cycles. The PCR products were separated by agarose electrophoresis to ensure the correct size.

2.5. Quantitative real-time reverse transcription-PCR (RT-PCR)

Real-time RT-PCR was performed using the LightCycler system (Roche, Basel, Switzerland), according to the manufacturer’s instructions. The primer sequences used for IGF-1, NGF, BDNF, and β-actin were as shown earlier. β-Actin levels were used to normalize samples for calculation of the relative expression levels of the genes. The results are expressed as percent relative to the vehicle treated group (considered as 100%).

2.6. Western blot analysis

Lysates were extracted and pooled from the hippocampi of mice of the different groups and protein concentrations of the lysates were determined using the Bradford Protein Assay Reagent (Bio-Rad Laboratories GmbH, Munich, Germany). The lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as described previously (Sharabi et al., 2007). The membranes were incubated with antibodies against total ERK and its phosphorylated form (Santa Cruz Biotechnology, Santa Cruz, CA), and against total Akt (Cell Signaling Technology, Inc., Danvers, MA) and phosphorylated Akt (Biosource, Camarillo, CA). The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Detection was performed using the enhanced chemiluminescence method. Densitometric units were determined using the NIH Image program.

2.7. Statistical analysis

Statistical analysis was performed using the paired two-tailed Student’s t-test and the Mann–Whitney U test using InStat software. p values of less than 0.05 were considered significant.

3. Results

3.1. Treatment with hCDR1 up-regulates BrdU positive cells and restores the immunoreactivity for NeuN

In order to study whether treatment with hCDR1 results in enhanced neurogenesis we injected BrdU to mice from all treatment groups, as described in the Materials and methods. Fig. 1A shows the representative images of sections of the hippocampi of individual mice that were treated with the vehicle, hCDR1, or with the control peptide (for 10 weeks starting at the age of 8 months). Fig. 1B presents the mean of BrdU positive cell counts in the dentate gyrus per section. Only rare BrdU positive cells could be detected in the dentate gyrus region of vehicle and control peptide treated mice (1.9±0.4 and 2.2±0.4 BrdU cell count/section, respectively). However, in mice treated with hCDR1 a significant up-regulation (t21 =2.9, p =0.008 and t20 =2.5, p =0.001, respectively) of two-fold in BrdU positive cells (4.2±0.7) was observed in the dentate gyrus region. Fig. 1C shows, for comparison, the mean dentate gyrus length/section of mice of the three treatment groups. As can be seen no significant differences were observed in the mean length.
between groups. Fig. 1D demonstrates representative images of sections showing the dentate gyrus region in an aged matched healthy BALB/c mouse and in a 2 month old (NZBxNZW)F1 mouse. In accordance with the results shown in Fig. 1D, a mean of 13.5 ± 1.8 BrdU positive cells was counted in the dentate gyrus region of the sections of 2 month old, free of disease, (NZBxNZW)F1 mice (n = 12). On the other hand, in healthy age matched BALB/c mice (n = 6) that were used as a control we detected a mean of 5.7 ± 2.1 BrdU positive cells in the dentate gyrus region, similar to the counts determined for the hCDR1 treated, SLE-affected (NZBxNZW)F1 mice. It is shown in Fig. 1A that in brain sections of vehicle and control peptide treated mice the immunoreactivity for NeuN was lost as reported by us previously (Lapter et al., 2009). It can be seen in the figure that treatment with hCDR1 restored the immunoreactivity for NeuN and that the BrdU positive cells in the hippocampi of mice treated with hCDR1 were stained positively for NeuN, indicating that those BrdU positive cells were neurons.

3.2. Effect of hCDR1 on the expression of IGF-1, NGF, BDNF, and VEGF in brain hippocampi of SLE-affected mice

Because neurogenesis was reported to be regulated by internal growth factors including IGF-1, NGF, BDNF, and VEGF (Balu and Lucki, 2009) it was of importance to determine the effect of treatment with hCDR1 on the expression of the latter factors. To this end we first examined gene expression of each of the factors by PCR in mRNA prepared from brain hippocampi of mice that were treated with hCDR1 as compared to those treated with vehicle alone or with the control peptide. As can be seen in Fig. 2, the expression of IGF-1 and NGF was markedly enhanced in the hippocampi of SLE-affected mice, which were treated with hCDR1 in comparison to the very low levels observed in brain hippocampi of mice that were treated with the vehicle. Treatment with the scrambled control peptide did not up-regulate the expression levels of these factors. Furthermore, the expression of BDNF was higher in mice treated with hCDR1 in comparison to both control groups, the differences in this case were less prominent than in the case of IGF-1 and NGF (Fig. 2). It can be seen in Fig. 2 that the gene expression of VEGF was similar in all three examined groups.

Fig. 3 demonstrates the results of a quantitative analysis performed by real-time RT-PCR. Because no difference could be determined in the expression of VEGF measured by PCR for the 3 different treatment groups, suggesting that the latter factor is not involved in the mechanism of action of hCDR1, real time RT-PCR was performed only for IGF-1, NGF, and BDNF. The results shown in Fig. 3 that represents 4 experiments, confirmed the observation by PCR that treatment with hCDR1 significantly increased the expression of IGF-1, NGF, and BDNF in comparison to hippocampi of vehicle-treated mice and of mice treated with the control peptide.

3.3. Treatment with hCDR1 stimulates activation of ERK and Akt kinases in the hippocampi of SLE-affected mice

Since, ERK and Akt kinases were suggested to be involved in the development of central nervous system diseases and to play an important role in regulation of neurogenesis, mediated by growth factors (Johnson-Farley et al., 2007), we determined whether up-regulation of the three above mentioned factors, following treatment with hCDR1, is associated with an increased activation of ERK and Akt kinases in the hippocampi of the treated mice. Protein levels of phosphorylated and total forms of ERK and Akt were measured in brain hippocampi of hCDR1-treated mice and were compared to those in the vehicle-treated group. Representative western blots that are shown in Fig. 4A indicate the prominent up-regulation in both phosphorylated ERK and Akt. Fig. 4B demonstrates the mean results of 3 experiments measured by densitometry and expressed relatively to the control group of vehicle-treated mice (considered as 100%). The results confirm the significant increase in the levels of activated pERK and pAkt in the hippocampi of hCDR1-treated mice.

4. Discussion

The main findings of the present study are that treatment of SLE-affected (NZBxNZW)F1 mice with the tolerogenic peptide, hCDR1, resulted in the up-regulation of neurogenesis in the dentate gyrus, and restored the NeuN immunoreactivity in brain hippocampi of the treated mice. The tolerogenic peptide up-regulated the gene expression of three factors that control neurogenesis, namely, IGF-1, NGF, and BDNF whereas treatment with a control peptide had no such effect. Also, protein levels of pERK and pAKT kinases were up-regulated in brain hippocampi of the hCDR1-treated mice. These results suggest that IGF-1, NGF, and BDNF-induced ERK and AKT

Fig. 4. Treatment with hCDR1 increases protein expression of pERK and pAkt kinases in brain hippocampi of (NZBxNZW)F1, SLE-affected mice. (A) Representative western blots of ERK and Akt from vehicle-treated mice and hCDR1-treated mice. Results are representatives of 3 experiments performed. (B) Results based on densitometric units, indicating the ratio of pERK to tERK and pAkt to tAkt. Data are expressed as mean percent ± SEM for hCDR1 (n = 17) relative to vehicle-treated mice (n = 16), considered as 100%.
activation plays a role in the observed hCDR1 stimulated hippocampal neurogenesis in the (NZBxNZW)F1 mouse model of lupus.

Minute numbers of BrdU-positive cells could be detected in the dentate gyrus region of SLE-affected mice, however, the number of the latter cells increased significantly following treatment with hCDR1 (Fig. 1). Further, the BrdU-positive cells in the treated mice were neurons because these cells expressed NeuN as well (Fig. 1). We showed that treatment with hCDR1 up-regulated the expression of mainly IGF-1 and NGF, but also that of BDNF (Fig. 2). Neurotrophic and growth factors are considered extracellular signals that are essential for hippocampal adult neurogenesis (Calof, 1995; Kuhn et al., 1997; Poo, 2001; Frielingsdorf et al., 2007). BDNF was shown to be a regulator of neuronal survival and neurogenesis in the adult brain (Lesmann et al., 2003), and it was reported to play a role in multiple sclerosis (Aharoni et al., 2005b). Likewise, IGF-1 elicits neurogenesis in the adult hippocampus (Aberg et al., 2000). In addition, NGF was described to be neuroprotective in autoimmune conditions (Molnár and Bokk, 2006). Moreover, previous studies indicated that NGF led to increased survival of neurons (Frielingsdorf et al., 2007). Thus, the ability of hCDR1 to increase the production of the neurotrophins and growth factors is likely to contribute to the neurogenesis process.

In the present study we showed that treatment of SLE-affected mice with the tolerogenic peptide resulted in up-regulated phosphorylation of ERK and AKT in the hippocampi of the treated mice (Fig. 4). ERK and Akt kinases were reported to control neuron formation and proliferation (Sung et al., 2007). Indeed, the results of our study may suggest that ERK phosphorylation in the hippocampi of hCDR1-treated mice could contribute to the neurogenesis associated with up-regulation of IGF-1, NGF, and BDNF, as the latter three factors were reported to promote ERK and AKT pro-survival pathways (Johnson-Farley et al., 2007). Previous studies suggested that ERK, that was expressed within the hippocampal region, could participate in the regulation of behavior, synaptic plasticity and memory (Daw et al., 2002; Duman et al., 2007). In agreement, our group reported that the tolerogenic treatment of mice with SLE restored behavioral dysfunction (Lapier et al., 2009). Thus, it is possible that hCDR1-induced phosphorylation of ERK could play a role in the improved behavioral performance either in association with neurogenesis or via other mechanisms.

The significance of ERK and AKT in neurogenesis can also be exemplified following the administration of different kinds of drugs. For instance, it was shown that statins, which control the formation of cholesterol, could induce neurogenesis through the phosphorylation of ERK and AKT (Chen et al., 2003). Further, a mood stabilizer such as valproate was demonstrated to promote neurogenesis via ERK pathway-regulated Bcl-2 gene (Hao et al., 2004). Hence, the role of hCDR1 in controlling the ERK signaling pathway in the hippocampi of the diseased mice appears to be essential for the accomplishment of neurogenesis.

Another pathway by which hCDR1 might affect a better survival of neurons is due to the induction of Bcl-xL. We recently demonstrated that gene expression and protein levels of the survival molecule Bcl-xL were up-regulated in the hippocampi of SLE-affected mice in response to treatment with hCDR1 (Lapier et al., 2009). In addition, inflammatory mediators were also suggested to affect neurogenesis. For example, the pro-inflammatory cytokine IL-6 could interfere with adult neurogenesis (Vallières et al., 2002). It was suggested that IL-6 represses neurogenesis in the mature brain by acting directly on hippocampal cells (Vallières et al., 2002), or indirectly by leading to enhanced production of glucocorticosteroids (Turnbull and Rivier, 1999), which were reported to inhibit neurogenesis in the dentate gyrus (Gould et al., 1992; Cameron and Gould, 1994). In accordance, we demonstrated that treatment of SLE-affected (NZBxNZW)F1 mice with hCDR1 led to diminished production of IL-6 in the hippocampi of the treated mice (Lapier et al., 2009). BAFF is a TNF-like cytokine that plays a role in peripheral B cell maturation and survival (Mackay et al., 2005). It has been reported that BAFF-transgenic mice manifest prominent neuropsychiatric signs (Mackay et al., 1999), and exhibit reduced neurogenesis and altered emotional behavior (Crupi et al., 2010). Recently, we showed that treatment of lupus-affected mice with hCDR1 down-regulated BAFF production as well in association with amelioration of the disease manifestations (Parameswaran et al., 2009). It is therefore possible that the down-regulation of BAFF production following treatment with hCDR1 contributed to the increased neurogenesis observed in the hCDR1-treated, SLE-affected mice.

Similarly to the tolerogenic peptide hCDR1 that affects neurogenesis in a mouse model of lupus, an approved drug for treating multiple sclerosis, namely glatiramer acetate, was shown to promote neurogenesis in a mouse model of experimental autoimmune encephalomyelitis (Aharoni et al., 2005a). In their report, Aharoni and colleagues showed that glatiramer acetate up-regulated neuronal proliferation, migration, and differentiation. Thus, our findings demonstrating neurogenesis induction by hCDR1 in mice with SLE are supported by similar findings in regard to a different drug for an autoimmune disease.

Altogether, the current study demonstrated that tolerogenic treatment of SLE-affected mice with hCDR1 resulted in hippocampal neurogenesis at least partially through the induction of growth and neurotrophic factors, and the activation of ERK and AKT pro-survival pathways. These findings suggest that hCDR1 may be a potential drug for the treatment of neuropsychiatric SLE.

References


