PAPER

Somatostatin treatment attenuates proteinuria and prevents weight loss in NZB/W F1 mice

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Somatostatin, a naturally occurring neuropeptide, is an immunomodulator which inhibits humoral and cell mediated immunity as well as secretion of proinflammatory cytokines. The objective of this study was to examine the effects of a somatostatin analogue on the severity of glomerulonephritis in the female NZB/W F1 murine model of systemic lupus erythematosus (SLE). Twenty female NZB/W F1 mice were treated at 23 weeks of age with 10 mg/kg of the somatostatin analogue Sandostatin- LAR, IM every four weeks. Ten control mice received IM injection of vehicle. Mice were assessed at four-week intervals for weight change, proteinuria, anti-DNA antibodies and splenocyte cytokine profile. The mice were sacrificed at age 34.5 weeks. Kidneys were collected and evaluated by light and immunofluorescence (IF) microscopy. Spleens were collected and splenocyte intracellular cytokines were measured by FACS analysis. In the treatment group significantly less proteinuria was observed four weeks after the second somatostatin analogue injection (dipstik scale: $+2.07 \pm 0.95$ versus $+3.5 \pm 1.08$, P = 0.0002). The treated mice did not lose weight while the control group lost weight over time (P = 0.016). No differences were noted between the groups in anti-DNA antibody titres, cytokine profile or the severity of lupus nephritis as assessed by light and IF microscopy. Somatostatin analogue treatment attenuated proteinuria and prevented weight loss in NZB/W F1 mice, suggesting a possible beneficial effect on renal parameters and systemic manifestations of the disease. Further studies will be needed to assess the value of somatostatin analogue treatment in lupus nephritis, utilizing higher doses, at different stages of the disease, for longer periods. *Lupus* (2006) **15**, 526–531.

Key words: NZB/W F1 mice; somatostatin; systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE) is a multi-system, inflammatory autoimmune disease that is caused by tissue damage mediated by autoantibodies and immune complexes.[1] Individuals who develop the disease make pathogenic autoantibodies and immune complexes and cannot regulate their production and clearance properly.[1] There are additional hormonal/cytokine factors that contribute to the pathogenesis of SLE. Some patients have hyperprolactinemia and others inappropriate levels of ADH, suggesting abnormal hypothalamic and/or pituitary function. Neuroendocrine-immune interactions have been shown to contribute to the pathogenesis of auto-immunity. Skewing of cytokine patterns in the immune system also plays a major role in SLE,

supporting emergence of T cells favouring autoantibody production.[1]

Somatostatin (SST) is a widely distributed neuropeptide, first discovered in 1973 as a growth hormone (GH) release-inhibiting factor in the hypothalamus.[2] It inhibits the hypophysial release of GH, thyrotropin-releasing hormone, and prolactin.[3] Prolactin has been shown to play an immunomodulatory role, and suppressive therapy with bromocriptine has been used successfully to treat autoimmune diseases.[4] In addition to its central hypothalamic/hypophysial axis mediated actions, SST elicits direct peripheral inhibitory effects on immune and inflammatory responses as well.[5,6] It inhibits immunoglobulin synthesis by human circulating B lymphocytes, proliferation of human T lymphocytes[7] and neutrophil chemotaxis.[8] SST is also an antagonist of substance P, having a role in the modulation of neurogenic inflammation and pain perception.[8] Moreover, SST has anti-proliferative, anti-angiogenic and apoptotic inducing effects in various tumour cells, explaining why several studies have reported the use of SST analogues in cancer.[9–11] These diverse effects of

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SST are mediated by a family of five cell surface receptors that selectively bind SST with high affinity.[9,12] SST receptors have been identified in normal and tumor tissues and on more than 95% of normal mitogen-activated human peripheral lymphocytes.[13] SST receptor scintigraphy in patients with immune-mediated diseases revealed receptor expression in 75% of patients with Sjogren syndrome and 50% of patients with SLE.[13] Unlike the native SST hormone which binds to all five SST receptors and has a short half life, the SST-analogue octreotide binds selectively to the receptor subtypes 2 and 5 and has a half life of two hours.[5,14] The anti-inflammatory and immunomodulatory effects of SST are exerted through receptor subtype 2.[15]

Taking into account the impact of SST on the immune system and on cell proliferation and apoptosis, we hypothesized that treatment with a SST analogue with affinity for the receptor subtype 2 may have a beneficial effect in a murine model of SLE. In humans glomerulonephritis is a frequent cause of morbidity in SLE. Treatment modalities available at present for this life threatening disease, corticosteroids, cyclophosphamide or other immunosuppressants are successful but their potential side effects are grave. SST analogues are not cytotoxic and do not induce immunosuppression and the known side effects, as seen in the long term treatment of acromegaly are mild making this a potentially favourable agent for the treatment of SLE.[5,9]

This preliminary study presents the effects of SST analogue therapy on the course of disease in a murine model of SLE, utilizing one dose of the SST analogue administered once a month for a period of three months.

Materials and methods

Thirty female New Zealand Black X New Zealand White (NZB/W) F1 mice at the age of 12 weeks were obtained from Harlan Laboratories, Jerusalem, Israel. Mice were 23 weeks old at the start of the experiment.

The research protocol was carried out according to the guidelines for the care and use of laboratory animals and was approved by the Meir Medical Center institutional animal care and use committee. Mice were fed a standard commercial diet and had free access to water.

Study groups

Mice were divided into two groups: 20 NZB/W F1 mice were treated with a long acting Somatostatin analogue; 10 mice served as controls.

Treatment

Sandostatin-LAR® (Novartis, Basel, Switzerland) consists of octreotide acetate, a SST analogue, incorporated into microspheres of a bio-degradable polymer (poly-(DL-lactide-co-glycolide-glucose)), designed to provide long term, controlled-release of octreotide after a single intra-muscular injection. Therapeutic levels of octreotide are obtained for a period of 4 weeks post injection.[16]

Treatment was started in both groups at the age of 23 weeks when all the mice had proteinuria on dipstick testing. In the treatment group sandostatin-LAR® was administered IM once every four weeks (three injections), for a period of 12 weeks, at a dose of 10 mg/kg per injection as used in previous studies.[17,18] Control mice were treated with IM vehicle every four weeks.

Clinical and laboratory assessment

All mice were assessed at four-week intervals for weight change, proteinuria and anti-DNA antibodies. Proteinuria was measured semiquantitively on fresh urine samples by dipstick on a scale of 0 to +4, using a commercial dipstick method (Multistix® 10SG, Bayer) where 0: no proteinuria; +1: >30 mg/dL; +2: >100 mg/dL; +3: >300 mg/dL; +4 >2000 mg/dL. At least two measurements were performed at each assessment to ensure true proteinuria was recorded. Blood samples were collected at four-week intervals by retro-orbital sinus puncture and sera were stored at -70°C for autoantibody analysis. Mice were sacrificed at the end of study at the age of 8.5 months (34.5 weeks), when 30% of mice died in the control group due to the disease. Kidneys were removed and cleaned from all surviving mice at the end of the study for histological assessment by light microscopy and immuno-fluorescence. Spleens were also collected and supernatants were prepared for the measurement of intracellular cytokines by FACS analysis (TNF-α, IL-4, IL-10, IL-6, IFN-γ, IL-1ra).

Histopathological analysis

Light microscopy. Histological kidney sections were analysed by light microscopy in a blinded fashion by a pathologist. Kidney specimens were fixed in 10% buffered formaldehyde and embedded in paraffin. Histological sections of 4–5 μm thickness were stained with hematoxylin-eosin (HES), periodic acid Schiff (PAS) and Masson trichrome (light green). Glomerular lesions were semi-quantitatively graded from 0 to 3 for hypercellularity and mesangial matrix expansion (0 = no changes; 1 = mild; 2 = moderate; 3 = severe), as well as for the percentage of sclerotic glomeruli (0 = less than 10%; 1 = 10–25%; 2 = 25–40%; 3 = more than 40%). Tubular (atrophy, casts, and dilation) and interstitial changes (fibrosis and inflammation)

were graded from 0 to 3 (0 = no changes; 1 = changes affecting less than 25% of the sample; 2 = 25–50% of the sample; 3 = more than 50% of the sample). At least 60 glomeruli were examined for each kidney sample. At least 10 fields per sample were examined at low magnification (×10) for histological scoring of the interstitium.

Immunohistology. Frozen cryostat kidney sections (5 μm) were air dried for at least one hour and fixed in acetone for 10 minutes. Immunoglobulin (IgG) deposits were detected with fluorescein isothiocyanate (FITC) labelled goat anti-mouse IgG (Jackson ImmunoResearch Laboratory, West Grove, PA) as described previously.[19,20] Sections were visualized using a fluorescence microscope (Zeiss Axioskop 2 Germany).

Anti-DNA antibodies

For measuring anti-dsDNA Abs, 96-well Maxisorb microtiter plates (Nunc) were coated with poly L-lysine 5 μg/mL (Sigma), followed by coating with λ-phage double stranded DNA (Boehringer Mannheim, 5 μg/mL). Plates were blocked with 10% fetal calf serum (FCS) in PBS and the sera (diluted 1:10–1:1250) were incubated for two hours. Goat anti-mouse IgG (γ-chain specific) conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratory, West Grove, PA) was added to the plates, followed by the addition of the substrate, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (Sigma). Results were read at 405 nm by using an ELISA reader (Tecan Spectra Classic, Austria).

Determination of cytokines in splenocytes

Methanol fixation. An appropriate number of cells (5–10 × 10^6) were fixed by methanol using the following protocol: cells were washed with cold PBS (4°C) and centrifuged at 4°C and 1500 rpm for seven minutes. The cells were shaken extensively with 300 μL of cold PBS (4°C) and then fixed by dripping 5 mL of cold methanol (−20°C) into the tube containing the cells while using slow vortex.

The cells were then incubated (with the methanol) for one hour in −20°C and centrifuged at 4°C and 2000 rpm, for five minutes and resuspended in 100 μL cold PBS followed by addition of 900 μL of cold PBS and incubation for 30 minutes at 4°C.

Flow cytometry staining. For flow cytometry, 10^6 cells were incubated with PRE-conjugated rat-anti-mouse-TNF-α (MCA 1488, Serotec Company), PE-conjugated rat-anti-mouse-IL-10 (MCA 1302, Serotec Company), PE-conjugated rat-anti-mouse-IL-6 (12-7061-81, Bioscience Company), FITC-conjugated rat-anti-mouse-INF-γ (MCA 1548F, Serotec Company) or PRE-conjugated rat-anti-mouse-IL-1ra (sc-25444, Santa Cruz Biotechnology Company) monoclonal antibodies for 45 minutes on ice. The cells were then washed and analysed with a Flow-Cytometer (Becton Dickinson Immunocytometry Systems, San Jose, California, USA).

Statistical analysis

For statistical analysis of proteinuria and weight, one way analysis of variance with repeated measures with the mixed model was used. A P-value of less or equal to 0.05 was considered statistically significant.

The Mann–Whitney test was used for analyses of the histopathology data.

Results

### Effect of SST treatment on clinical manifestations

All mice had proteinuria of at least +1 on dipstick testing at the beginning of the experiment (at 23 weeks of age). Four weeks after the second dose of SST the treated mice had significantly less proteinuria on dipstick analysis (2.07 ± 0.95 in the treated group versus. 3.5 ± 1.08 in the control group, P = 0.0002) as shown in Figure 1. At six weeks after the second dose of SST the difference nearly reached significance (2.46 ± 0.87 versus 3.1 ± 0.99, P = 0.057) (Figure 1). The treated mice did not lose weight over time while in the control group the mice continued to lose weight, reaching a significant difference six weeks after the second dose of SST (34.9 ± 3.96 g versus 30 ± 7 g, P = 0.016) (Figure 2). Macroscopic evaluation was performed in all mice at the time of sacrifice. Ascites was not seen in any of the mice. Animal survival until the end of the experiment was 70% in both groups. This data has taken into account only the occurrence of natural death of mice (three mice from the SST group died during the study due to technical reasons).

Effect of SST treatment on histolpathological findings of glomerulonephritis

Light microscopy. Histological sections of 11 treated mice and seven control mice were scored. Glomerular lesions were present in both groups and were characterized by enlarged glomeruli with cellular proliferation as well as mesangial expansion. One mouse in each group
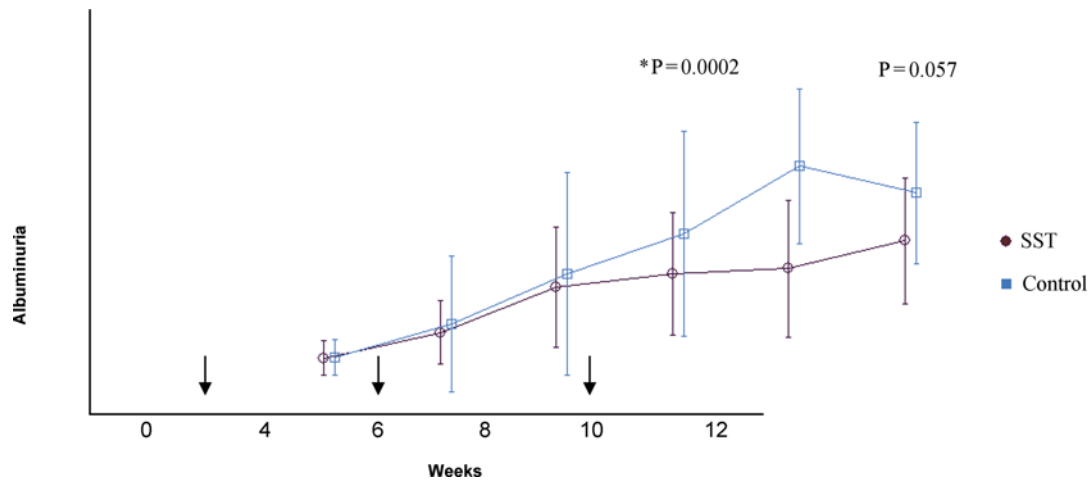
Figure 1 Effect of Somatostatin on proteinuria. Somatostatin treatment attenuated proteinuria in NZB/W F1 mice as measured by dip-stick ($+2.07 \pm 0.95$ in the SST treated group versus $+3.5 \pm 1.08$ in the control group; P = 0.0002). This difference was seen four weeks after the second SST injection (● SST, ■ control; arrows indicate timing of SST injections).
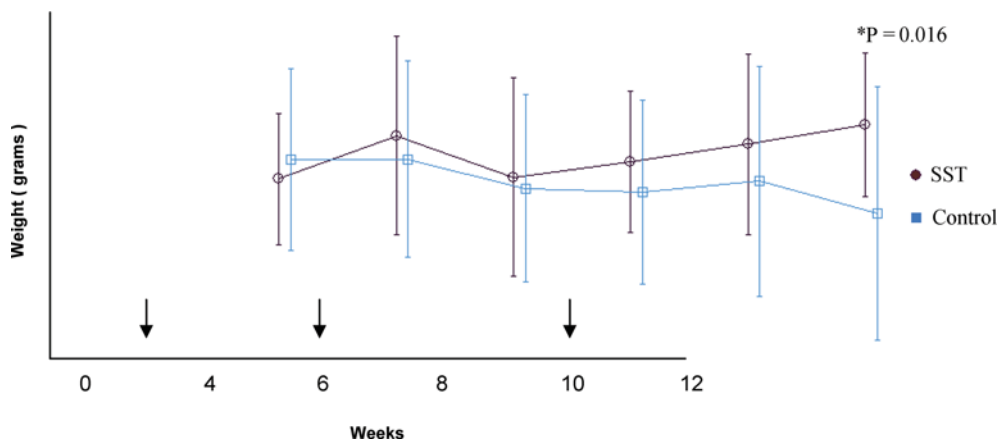

Figure 2 Effect of somatostatin on body weight. Somatostatin treatment prevented weight loss in NZB/W F1 mice (34.9 ± 3.96 g versus 30 ± 7 g; P = 0.016). This difference was seen after the third SST injection (● SST, ■ control; arrows indicate timing of SST injections).

exhibited a mild degree of glomerulosclerosis (grade 1). One mouse in the control group exhibited severe tubulointerstitial damage (grade 3) giving the highest score in this group but on the whole tubulointerstitial changes were mild in both groups with a trend towards less damage in the treatment group (SST 0.36 ± 0.5 versus control 1.14 ± 1.06, P = 0.08) (Figure 3).

Beyond the presence of characteristic signs of lupus nephritis, no significant differences were seen between the two groups regarding glomerular mesangial

expansion (SST 1.32 ± 0.38 versus control 1.48 ± 0.68, P = 0.8) and glomerular cellularity (SST 2.05 ± 0.54 versus control 1.48 ± 0.6, P = 0.07) (Figure 3).

Immunohistology. Histological frozen cryostat kidney sections (5 μm) of seven treated mice and three control mice were available for immunohistology. In four of seven treated mice kidney sections showed intense immune complex deposits and two of the three available

Renal morphologic parameters
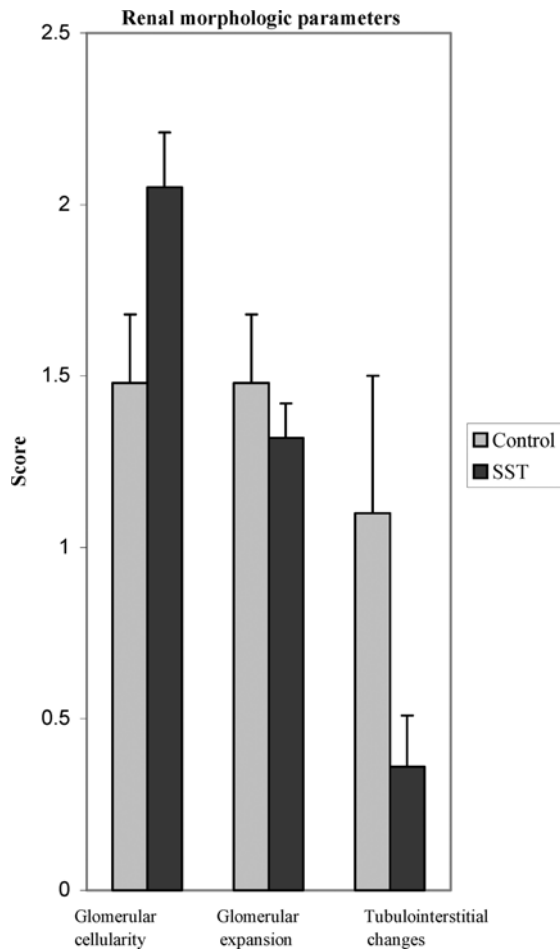
Figure 3 Effect of Somatostatin on renal morphologic parameters. Somatostatin treatment did not lead to significant histopathological improvement of glomerulonephritis when looking at glomerular cellularity and glomerular expansion. A trend towards less tubulointerstitial changes was seen in the SST treated group (P = 0.08).

control mice kidney sections showed immune complex depositions with the same intensity. The number of sections examined was too small to allow for meaningful conclusions but no differences could be noted between the groups.

Effect of SST treatment on anti-DNA antibodies

No effect was seen on anti-DNA antibody titres in the treatment group as compared to the control mice.

Effect of SST treatment on cytokines in splenocytes

No consistent effect on the profile of the following cytokines: TNF-α, IL-4, IL-10, IL-6, IFN-γ, IL-1ra was noted in the splenocytes collected.

Discussion

When designing this study we hypothesized that treatment with a SST analogue may have a beneficial effect on the severity of the lupus nephritis lesions found in NZB/W F1 mice. This was expected knowing the multiple effects of SST on the immune system. Moreover, SST has anti-proliferative actions and may induce apoptosis. In fact we have found that somatostatin analogue treatment attenuated proteinuria and prevented weight loss in these NZB/W F1 mice. The histopathological assessment of kidney sections did not show significant differences between controls and SST treated mice regarding the severity of glomerular mesangial expansion and glomerular cellularity. However, there was a trend towards less tubulointerstitial damage in the SST treated group which may be due to SST's immunomodulatory and anti-proliferative effects.[5,21,22] Albeit, the mildly attenuated tubulointerstitial damage may not entirely explain the decrease in proteinuria in the treated mice. SST treatment may have also reduced the proteinuria via its renal hemodynamic effects. Indeed SST's effects on renal function have been studied extensively in diabetes. Chronic administration of octreotide to streptozotocin induced diabetic rats could prevent renal hyperfiltration by increasing afferent arteriolar resistance.[23] In the NOD mouse with diabetic kidney, SST analogues exert beneficial effects similar to those of an ACE inhibitor. Diabetic hyperfiltration and overt proteinuria are similarly blunted by SST or ACE inhibitors alone or in combination. Renal hypertrophy and depositions of type IV collagen and PAS material are reduced only in SST treated mice.[24] The beneficial effect of SST on glomerular hypertrophy, albuminuria and GFR is associated with a reduction in serum and renal insulin-like growth factor-1(IGF-1).[25]

In humans, Vora *et al.* and Pedersen *et al.* could show that native SST or octreotide induced an immediate reduction in renal plasma flow (RPF) and glomerular filtration rate (GFR) in normal subjects and in diabetic patients.[26,27]

In our study the insufficient number of kidney sections for immunofluorescence evaluation precluded an adequate assessment of a possible effect of SST on immune complex deposition. We did not find any meaningful effect on the cytokines studied when looking at splenocyte cytokine profiles. Cytokine expression was measured only as intracellular expression, and not as secreted protein. In other models of inflammation SST has been shown to inhibit IL-6 and IL-8 mRNA expression as well as the protein synthesis of these pro-inflammatory cytokines.[28,29] The lack of an anti-inflammatory effect in the present study, as reflected by the cytokines analysis may due to inadequate dosing and/or inappropriate timing of SST

administration during the evolution of the disease. The beneficial effect on weight gain seen in the treated animals may reflect a general 'well-being effect' related to a better protein balance, but also a possible systemic neuroendocrine effect via suppression of prolactin and GH secretion.[30] Despite the beneficial effect on proteinuria and body weight no significant action was found on anti-DNA titres. Other agents used to treat SLE have shown clinical efficacy not always accompanied by a change in anti-DNA titres.[31–33]

In conclusion, our study has shown a beneficial effect of SST treatment on proteinuria and weight loss in NZB/W F1 mice, but no clear histological or serological effects were seen. The effect of SST was studied when lupus nephritis was well established, at an advanced stage of the disease. Following the results of this preliminary study, further studies will be needed to assess when and at what dose SST may be more efficient.

Acknowledgement

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