CLINICAL STUDY

***In vivo* and *in vitro* effects of statins on lymphocytes in patients with Hashimoto's thyroiditis**

Sevim Gullu, Rifat Emral, Mehmet Bastemir, Arthur B Parkes¹ and John H Lazarus¹Ankara University, School of Medicine, Department of Endocrinology and Metabolic Diseases, Ibn-i Sina Hospital, 10th floor, D-Block, 06100 Sıhhiye, Ankara, Turkey and ¹School of Medicine, Cardiff University, Cardiff, UK

(Correspondence should be addressed to S Gullu; Email: gullu@medicine.ankara.edu.tr)

Abstract

Background: Statins have apoptotic effects on many cell types. Hashimoto's thyroiditis (HT) is an autoimmune disease in which cell-mediated autoimmune mechanisms are pathogenetically involved.

Objective: The aim of this study was to evaluate the *in vivo* effects of Simvastatin on thyroid function, lymphocyte subtypes and also to investigate the apoptotic effects of Simvastatin, Mevastatin, Pravastatin and Cerivastatin on lymphocytes from patients with HT.

Methods: In the first part of the study, 11 patients with HT and subclinical hypothyroidism (SH) were given Simvastatin (20 mg/day) for 8 weeks. Ten patients with SH and HT served as the control group. No treatment was given to controls. Thyroid function, C-reactive protein (CRP) levels and lymphocyte subtypes of both groups were determined before the study and after 8 weeks. In the second part of the study, the apoptotic effects of statins on lymphocytes were evaluated in patients with HT ($n = 10$) and normal subjects ($n = 10$) *in vitro*. Apoptosis was investigated by using Annexin-V and propidium iodide. Lymphocytes from patients and controls were incubated with different concentrations of Simvastatin, Cerivastatin, Mevastatin and Pravastatin.

Results: An increase in serum free tri-iodothyronine and free thyroxine levels and a decrease in TSH levels were observed ($P < 0.05$) with Simvastatin treatment. CD4⁺ cells and B lymphocytes increased whilst CD8⁺ cells, natural killer cells and activated T lymphocytes decreased significantly in the treatment group ($P < 0.05$). The CRP level of the group also decreased with Simvastatin but it did not reach significance ($P = 0.057$). None of parameters was found to be different from the baseline in the control group. In *in vitro* experiments, apoptosis was observed in CD3⁺ (both in CD8⁺ and CD4⁺ cells) with all statins in both patient and control samples. Mevalonate, which was used in experiments, reversed apoptosis in some but not all samples.

Conclusions: The results of this study suggested that Simvastatin is an immune modulatory agent and improves thyroid function in patients with HT. This effect is probably mediated via lymphocyte apoptosis as demonstrated with *in vitro* experiments and is not confined to Simvastatin since Mevastatin, Pravastatin and Cerivastatin also induced apoptosis in lymphocytes.

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Introduction

Hashimoto's thyroiditis (HT) is an inflammatory disorder of the thyroid gland characterized by an enlargement of the gland with lymphocytic infiltration, various degrees of thyroid hypofunction and circulating antibodies to thyroid antigens (1).

Thyroid cell destruction is observed in HT but there is debate regarding the exact mechanism of this destruction. Recently, a link with thyrocyte apoptosis has been observed (2–8). Although a B-cell response may have role in the process, a T-cell response is much more prominent in HT (1, 9–14). A large number of intrathyroidal lymphocytes in patients with HT are actually thyrocyte-specific cytotoxic T lymphocytes

(CTLs) (10, 12, 13). Both CD8⁺ CTLs and CD4⁺ CTLs have a role in apoptosis through different pathways. Because CTLs kill target cells by inducing apoptosis and because increased apoptosis is observed in thyrocytes proximal to infiltrating lymphocytes, this activity has been proposed as a major mechanism for the thyrocyte destruction observed in HT (2–14). In some studies, an increase in activated T cells, a decrease in CD4/CD8 ratio, an increase in proliferative response to mitogens and an increase in natural killer cell activity have been demonstrated in patients with HT (14–24). In HT, the immunologic attack appears to be typically aggressive and destructive (1).

Statins competitively inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity and

reduce mevalonate synthesis (25–27). As mevalonate and other intermediates of cholesterol synthesis (isoprenoids) are necessary for cell proliferation and other important cell functions, the effects of statins, other than cholesterol reduction, can be expected and this has been confirmed by recent studies (27–47). Statins inhibit the proliferation and migration of vascular smooth muscle cells and macrophages. They also sensitize human smooth muscle cells to apoptosis and affect vascular endothelial cells (26, 27, 30, 31, 33, 35, 36). Their growth arrest and apoptotic properties in some benign and malignant cells, including breast cancer, prostate cancer, prostate hyperplasia, renal mesangial cells, neuroblastoma, leukaemic cells, malignant mesothelioma and malignant glioma cells, have been reported (27–30). Statins may also induce apoptosis in proliferating thyroid cells and in thyroid cancer cells (37–40).

Some recent clinical trials indicate that statins have immunomodulatory effects (41–47). As a result of immunosuppressive actions, statins may also reduce the incidence of rejection following organ transplantation in some inflammatory and autoimmune disorders (41–43). The cytotoxicity of natural killer cells was found to be lower in patients taking statin (30, 44, 47). Inhibition of lymphocyte proliferation responses to non-specific mitogens has also been reported (44–47). Reduction in C-reactive protein (CRP), an acute phase protein, has also been reported in some studies (34).

Since T lymphocyte response is thought to be the major factor responsible for thyroid cell destruction in HT and some degree of altered lymphocyte numbers, lymphocyte and natural killer cell function can be seen in these patients and, since statins appear to have an inhibitory effect on lymphocytes, it is logical to consider whether statins could have some effects on disease progression in HT.

The aim of this study was to investigate the effects of Simvastatin, an HMG-CoA reductase inhibitor, on thyroid function, lymphocyte function and the distribution of lymphocyte subtypes in patients with HT. The *in vitro* effects of Pravastatin, Cerivastatin, Mevastatin and Simvastatin on lymphocytes from patients with HT were also investigated.

Materials and methods

Part 1: clinical trial

Patients and methods Twenty-one patients with HT (19 women and two men) aged between 28 and 48 years were included in the study. All the patients had had primary subclinical hypothyroidism for at least 6 months prior to the study and were not taking any medications including thyroid hormones. Diagnostic criteria for subclinical hypothyroidism were an elevated serum thyrotrophin (TSH) level with normal serum free

thyroxine (FT4) and free tri-iodothyronine (FT3) levels. The diagnosis of HT was made by the existence of a firm goiter, thyroid dysfunction, elevated anti-thyroid peroxidase (anti-TPO) and/or anti-thyroglobulin (anti-Tg) antibodies, a low ultrasonographic echogenicity of the gland and demonstration of lymphocytic infiltration by fine-needle aspiration biopsy. Patients with other medical diseases or using drugs for any other conditions were excluded from the study. The study was approved by the local ethical committee and informed written consent was obtained from the patients.

Serum TSH, FT4, FT3, anti-TPO and anti-Tg antibody levels, CRP levels, percentages of T lymphocytes (T-suppressor and T-helper), natural killer cells, B lymphocytes and activated T lymphocytes of the patients were measured. After these baseline evaluations patients were randomly assigned into two groups. Simvastatin was started at a dosage of 20 mg/day to 11 patients (Simvastatin group). No treatment was given to ten patients (control group). All the parameters were re-evaluated after 8 weeks in both groups. Lymphocyte subtype analyses were done by the same technician who was blinded to the patient groups.

Serum FT4, FT3 and TSH levels were measured using a chemiluminescence immunoassay kit (ACS:180; Chiron Diagnostics, Bayer Corporation, New York, NY, USA). Anti-TPO and anti-Tg levels were measured by RIA (Brahms Diagnostics GMBH, Berlin, Germany). Lymphocyte subpopulations were evaluated by flow cytometry. Proportions of T-helper cells (CD3 + CD4 +), T-suppressor/cytotoxic cells (CD3 + CD8 +), B lymphocytes (CD3(CD19 +)), natural killer cells (CD3(CD16 + CD56 +)) and activated T lymphocytes (CD3 + CD25 +) were measured using a FacSort machine (Becton-Dickinson Co., Franklin Lakes, NJ, USA). The transferred data were then analyzed using the Win MDI 2.8 software (provided by J Trotter, Scripps Research Institute, La Jolla, CA, USA). T lymphocytes were gated on the basis of their staining characteristics.

Statistical analysis Data are given as a means $\pm$ S.D. or as median. The paired *t*-test, Wilcoxon matched-pairs test or Mann–Whitney U test were used for statistical analysis. *P* values less than 0.05 were accepted as indicating statistical significance.

Part 2: in vitro experiments

Cell isolation Blood samples were obtained from ten untreated patients with subclinical hypothyroidism and HT. Ten healthy sex- and age-matched subjects served as the control group. Peripheral blood mononuclear cells were isolated from the blood by histopaque (Sigma Chemical Co., St Louis, MO, USA) density-gradient centrifugation. After three washes in RPMI 1640 (Gibco-BRL, Grand Island, NY, USA), cells were frozen and stored in liquid nitrogen prior to assay. Cells were used for flow cytometry.

Monoclonal antibodies Allophycocyanin (APC)-labelled CD3 (T cells) (Immunotech, Marseille, France), R-phycoerythrin cyanin-labelled CD8 (cytotoxic T cells) (Immunotech), R-phycoerythrin-labelled CD4 (T-helper cells) (Immunotech), fluorescein isothiocyanate (FITC)-labelled Annexin-V (early apoptosis marker) (Immunotech) were used for flow cytometry analysis using a FACS-can flow cytometer (Becton Dickinson Co.).

Drugs Pravastatin (a gift from Bristol-Myers Squibb, Hounslow, UK) and Cerivastatin (a gift from Bayer, Mid-glamorgan, Glamorgan, UK) were dissolved in RPMI 1640. Simvastatin (a gift from Merck Sharp and Dohme, Leopardstown, Dublin, Southern Ireland) was dissolved in ethanol. Mevastatin was purchased (Sigma Chemical Co., Gillingham, Dorset, UK) and dissolved in ethanol. Mevalonate was obtained from Sigma Chemical Co. (UK).

Flow cytometry analyses Cytofluorometric estimation of lymphocyte subsets and apoptosis was performed as follows. Cells were taken from storage in liquid nitrogen and defrosted rapidly. After washing three times with RPMI 1640, cells were resuspended in RPMI 1640 containing 10% heat-inactivated fetal calf serum, 2 mmol/l L-glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml) and were seeded at a density of 0.5×10^6 cells/ml in 24-well plates. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂ without or with different concentrations of drugs for 72 h (final concentrations of 10, 50, 100 µmol/l for Simvastatin, Pravastatin and Mevastatin and 10, 50, 100 nmol/l for Cerivastatin). Mevalonate was added to culture medium at a final concentration of 100 µmol/l in the different experiments.

For apoptosis and cell death analysis, cells were removed from culture and put into the tubes (100 µl/tube). Labelled CD3, CD4 and CD8 were added to the tubes and cells were incubated for 15 min in the dark at room temperature (RT). After washing with phosphate-buffered saline containing 0.1% bovine serum albumin, cells were re-suspended in 100 µl Annexin V-binding buffer and incubated with Annexin V-FITC for 15 min in the dark at RT. Cell membrane permeability was assessed by determining the ability of cells to exclude the DNA-binding fluorescent dye, propidium iodide (PI). To distinguish cells that had lost membrane integrity, PI was added to a final concentration of 10 µg/ml 10 min before analysis. Twenty thousand cells were counted in each analysis.

Results

Clinical study

Demographic characteristics of the patients are given in Table 1. No statistical differences were seen between the data for the treatment and control groups of patients.

Table 1 Demographic characteristics of the patients. Data are given as means $\pm$ S.D. for age, FT3 and FT4 and as median for TSH.

	Control group	Simvastatin group	P
Number (F/M)	10 (9/1)	11 (10/1)	
Age (years)	32 $\pm$ 4	34 $\pm$ 3	NS
FT3 (pmol/l)	4.5 $\pm$ 0.58	4.18 $\pm$ 0.28	NS
FT4 (pmol/l)	12.4 $\pm$ 1.9	13.2 $\pm$ 1.7	NS
TSH (mIU/ml) (range)	7.3 (5.8–13.4)	7.7 (6.3–14.6)	NS

NS, not significant.

Data for the Simvastatin and control groups, both before and after 8 weeks of treatment, are given in Table 2. Mean serum FT3 (4.62 ± 0.39 vs 4.18 ± 0.28 pmol/l, $P < 0.05$) and FT4 levels (14.8 ± 2.5 vs 13.2 ± 1.7 pmol/l, $P < 0.05$) increased and median serum TSH level (4.6 vs 7.7 mIU/l, $P < 0.05$) decreased with Simvastatin treatment. Although there were decreases in anti-TPO and anti-Tg antibody levels, these did not reach statistical significance. An increase in CD4 + and a decrease in CD8 + lymphocytes numbers were observed (47 ± 5 vs $44 \pm 6\%$, $P < 0.05$; 32 ± 9 vs $25 \pm 4\%$, $P < 0.05$ respectively). The CD4 to CD8 ratio also increased with therapy (1.8 ± 0.31 vs 1.4 ± 0.45 , $P < 0.05$). A significant increase in B lymphocyte number accompanied by a decrease in natural killer cell and activated T lymphocyte numbers was also detected ($P < 0.05$ for all; Table 2). The mean CRP level decreased with Simvastatin but it was not significant (3.4 ± 0.46 vs 6.5 ± 4.8 mg/l, $P = 0.057$).

None of the parameters changed in the non-treated control group (Table 2).

In vitro apoptosis experiments

To evaluate whether HMG-CoA reductase inhibitors (statins) induce apoptosis in lymphocytes, lymphocytes from a separate group of normal subjects and patients with HT were cultured *in vitro* and exposed to different concentrations of Pravastatin, Mevastatin, Cerivastatin and Simvastatin for 72 h. To exclude the possibility that statins exerted a direct cytotoxic effect on the lymphocytes, cells were simultaneously treated with exogenous mevalonate, the product of HMG-CoA reductase, in some experiments. Statin treatment was associated with an increase in Annexin-V-positive cells (early apoptotic) and/or Annexin-V- and PI-positive cells (late apoptotic) in both control and patient lymphocytes in a dose-dependent manner (Table 3) in some samples. The percentages of CD3-positive cells undergoing apoptosis in the presence of Cerivastatin, Simvastatin, Pravastatin and Mevastatin were 28 ± 3.5 , 30 ± 2.5 , 26 ± 3.7 and 27 ± 4.1 respectively.

Although the number of apoptotic samples were slightly higher in the Cerivastatin and Simvastatin

Table 2 Laboratory findings of the patients before and after study. Data are given as means $\pm$ s.d. except medians for TSH and thyroid autoantibodies.

	Control group (n = 10)			Simvastatin group (n = 11)		
	Before	After	P	Before	After	P
FT3 (pmol/l)	4.5 $\pm$ 0.58	4.5 $\pm$ 0.84	NS	4.18 $\pm$ 0.28	4.62 $\pm$ 0.39	<0.05
FT4 (pmol/l)	12.4 $\pm$ 1.9	13.2 $\pm$ 1.9	NS	13.2 $\pm$ 1.7	14.8 $\pm$ 2.5	<0.05
TSH (mIU/ml) (range)	7.3 (5.8–13.4)	7.8 (6.1–12.8)	NS	7.7 (6.3–14.6)	4.6 (2.8–8.9)	<0.05
Anti-TPO (range)	1162 (138–4326)	1054 (96–4957)	NS	1216 (166–4594)	365 (58–4136)	NS
Anti-Tg (range)	79 (36–224)	86 (38–265)	NS	102 (49–286)	56 (27–229)	NS
B cells (%)	11.3 $\pm$ 5	11.0 $\pm$ 5.4	NS	9.7 $\pm$ 2.2	11.5 $\pm$ 2.2	<0.05
T cells (%)	72 $\pm$ 8	73 $\pm$ 8	NS	72 $\pm$ 7	72 $\pm$ 8	NS
CD4 + (%)	45 $\pm$ 6	44 $\pm$ 6	NS	44 $\pm$ 6	47 $\pm$ 5	<0.05
CD8 + (%)	28 $\pm$ 6	30 $\pm$ 6	NS	32 $\pm$ 9	25 $\pm$ 4	<0.02
CD4/CD8	1.6 $\pm$ 0.49	1.5 $\pm$ 0.38	NS	1.4 $\pm$ 0.45	1.8 $\pm$ 0.31	= 0.006
Activated T (%)	15.3 $\pm$ 11.7	14.5 $\pm$ 10.1	NS	13.4 $\pm$ 8.3	8.7 $\pm$ 6.1	= 0.05
Natural killer (%)	16.3 $\pm$ 6.3	16.3 $\pm$ 6.3	NS	19.6 $\pm$ 7.6	15.4 $\pm$ 7.3	<0.05
CRP (mg/l)	3.65 $\pm$ 1.69	3.8 $\pm$ 1.75	NS	6.5 $\pm$ 4.8	3.4 $\pm$ 0.46	= 0.057

NS, not significant.

experiments than in the Pravastatin and Mevastatin experiments, no difference could be found between patients and controls with any of the four statins.

Both CD3 + and CD3 – cells showed apoptosis in the experiments. CD4 + and CD8 + cells were also affected similarly and decreases in both cell types were observed in the samples in which statins induced apoptosis. Apoptosis in CD3 + cells are given in Figs 1 and 2. Apoptosis was observed with all statins in both patient and control samples in different proportions. Increases in Annexin-V-positive cells are given in Fig. 3.

Mevalonate at a concentration of 100 μ mol/l prevented a cytotoxic effect of the statins in some experiments, suggesting that the effects of statins were indeed related to the inhibition of the mevalonate pathway. Inhibition of apoptosis by mevalonate is also shown in Figs 1 and 2.

Discussion

In the clinical part of the present study, Simvastatin, an HMG-CoA inhibitor, changed the distribution of the lymphocyte subpopulations in a group of patients with HT. Along with these alterations, an improvement in the thyroid function was also demonstrated. We have

also shown that Simvastatin, Cerivastatin, Mevastatin and Pravastatin induced apoptosis in peripheral blood T lymphocytes from some normal individuals and some patients with HT *in vitro*.

HT may manifest as hypothyroidism that is accompanied by massive infiltration of lymphoid cells (1). A large number of the intrathyroidal lymphocytes in patients with HT are thyrocyte-specific CTLs (10–12). In HT, increased apoptosis of the thyrocytes has been proposed as the mechanism of thyroid destruction characteristic of the disease (2–8). CD8 + CTLs and CD4 + CTLs have been proposed to play role in cytokine-mediated thyrocyte apoptosis in HT (1–8). It seems that cytokines, produced by the intrathyroidal lymphocytes, up-regulate apoptosis via increasing the expression of proteins that have a role in the apoptotic pathways.

Statins are used clinically for lowering hypercholesterolaemia because of their inhibitory effect on hepatic biosynthesis of cholesterol at the mevalonate step (25). Recently, however, it was found that their effects are not limited to lipid lowering (26–48). Apoptotic effects of the drugs on several cell lines have been demonstrated. Anti-inflammatory effects, including decreases in acute phase proteins, have also been found in some patient groups, especially in patients with atherosclerosis (34). Inhibition of leukocyte rolling, adherence and transmigration in acute inflammatory states, inhibition of chemotaxis by monocytes, regulation of natural killer cell cytotoxicity and inhibition of antibody-dependent cellular cytotoxicity have been reported (41–47).

In contrast to a study reporting no change in total lymphocyte numbers and lymphocyte subtypes, performed in hypercholesterolaemic subjects with Lovastatin (49), we found changes in lymphocyte subtypes with Simvastatin in patients with HT. Similar to our results, the effects of statins on lymphocyte functions have been reported previously (50–53). Decreases in

Table 3 Number of lymphocyte samples that showed apoptosis in patients with HT and controls (*in vitro* experiments).

	No. of samples with apoptosis/total no.	
	Patients	Control
Pravastatin	6/10	5/10
Cerivastatin	8/10	7/10
Mevastatin	4/10	4/10
Simvastatin	8/10	8/10

Figure 1 CD4 + cell apoptosis with statins in a typical patient. The control bar represents the cells incubated without any drug. The open bars show the data obtained with cells incubated with mevalonate.

CD8 + lymphocytes, activated T lymphocytes, natural killer cells and CRP levels and improvement in thyroid function in our study have suggested that these drugs have immune modulatory effects. Although it is difficult to make a strong suggestion with the rather small number of patients in our study, improvement in thyroid function was probably mediated through apoptosis of the lymphocytes as indicated by the present *in vitro* study. Although only Simvastatin was used for the clinical trial, the effect does not appear to be confined to Simvastatin since Cerivastatin, Mevastatin and Pravastatin also showed apoptotic effects on lymphocytes *in vitro*.

The first direct scientific and mechanistic evidence that statins might have a role in immunomodulation indicated that statins inhibited the expression of major histocompatibility complex class II genes (47, 54). This finding led to the suggestion that statins might become novel therapeutic agents in the area of immunosuppression, anti-inflammation and immune-related disorders such as auto-immune diseases (47). Several

publications have identified a therapeutic use for statins in the treatment of multiple sclerosis, rheumatoid arthritis, autoimmune encephalomyelitis (42, 43, 55, 56). Statins have also been found to be beneficial in decreasing the rejection rates and graft survival in organ transplantation (41, 44). To our knowledge, this is the first study to show *in vivo* and *in vitro* effects of statins on lymphocytes and thyroid functions in patients with HT. We observed improvements in the thyroid function tests in patients with HT and subclinical hypothyroidism with Simvastatin treatment. If this observation is validated, statins may become the drug of choice in the treatment of hyperlipidaemic patients with subclinical hypothyroidism, in whom thyroxine therapy may not be safe. However, since the number of patients in the clinical part of the study was small and the study was conducted in an open rather than a placebo-controlled manner, it is obvious that longitudinal placebo-controlled double-blind studies with a larger patient population are now required to determine the role of statins in the treatment of HT.

Figure 2 CD8 + cell apoptosis with statins in a typical patient. The control bar represents the cells incubated without any drug. The open bars show the data obtained with cells incubated with mevalonate.

Figure 3 Graphical output from the FACScan analyzer. Before analysis, lymphocytes were stained with CD3-APC mouse antihuman CD3 and FITC-labelled Annexin-V was used as apoptosis marker. (A) Cells without any drug, (B) cells plus 10 µmol/l Simvastatin, (C) cells plus 50 µmol/l Simvastatin and (D) cells plus 100 µmol/l Simvastatin. Increases in Annexin-V-positive cells with the increases in drug concentration can be seen in the lower right quadrants. SSC, side scatter.

CD4⁺ cells and B lymphocytes showed increases in the clinical study but, on the contrary, increases in apoptosis in these subtypes were found in the *in vitro* part of the study. Although it is difficult to explain this discrepancy, it may be related to the methods used in *in vivo* and *in vitro* experiments. Another explanation for the discrepancy may be that HT is accepted as a Th1-mediated disease and, in a very recent study (50), inhibition of HMG-CoA reductase reduced Th1 and promoted Th2 development, which may, in part, explain our results. We did not analyse the Th subtypes but it may be speculated that the increase observed in our *in vivo* study might result from an increase in Th2 cells.

Apoptosis could not be demonstrated in some samples in the present study and mevalonate did not inhibit the apoptosis in some samples. The latter finding suggests that mechanisms other than inhibition of the mevalonate pathway have a role in the lymphocyte apoptosis with statins. Why did apoptosis not occur in every sample with statins? It might be speculated that lymphocytes may have been resistant to statins in some individuals.

In conclusion, statins may alter the number and type of lymphocyte through apoptosis and may improve thyroid functions in patients with HT. These newly observed effects may represent a new strategy for treatment of cell-mediated autoimmune diseases including HT. The preliminary data of this study provide some

evidence that statins may also have immunomodulatory properties in addition to their lipid-lowering effects in patients with HT, but larger scale, randomized, double-blind trials are needed to validate the role of statins for the treatment of this autoimmune disease.

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