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Body surface area is an independent factor contributing to the effects of lamivudine treatment

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Abstract

Background: It has been suggested that lamivudine therapy may be even more effective if administered at higher doses than is dictated by the current standard regimen. We analyzed the correlation between the effects of lamivudine and body surface area (BSA).
Method: We evaluated 134 patients with chronic hepatitis B who had been treated with lamivudine for more than 12 months. The effect of the treatment was evaluated from the levels of serum alanine aminotransferase (ALT) and HBV–DNA. Several variables that could influence the response to treatment, including ALT, albumin, and bilirubin levels, platelet counts, BSA, HBV–DNA, and HBeAg were analyzed.
Results: Univariate logistic analysis selected platelet counts, BSA, HBV–DNA and HBeAg in the biological evaluation, and bilirubin, BSA, HBV–DNA and HBeAg in the virological evaluation ($\chi^2 > 1.0$). Using these factors, multivariate analysis revealed that BSA ($\chi^2 = 12.8$, $p = 0.0004$) was the only factor that could contribute significantly to the improvement of ALT levels, and that BSA ($\chi^2 = 4.4$, $p = 0.0354$) and HBeAg ($\chi^2 = 8.1$, $p = 0.0044$) were independent factors that could influence the suppression of HBV–DNA.

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Conclusion: We revealed that BSA is a significantly predictor of the effect of lamivudine therapy, suggesting that lamivudine dosage should be based on the individual BSA.

Keywords: Lamivudine; Hepatitis B virus (HBV); Body surface area (BSA); Dose

1. Introduction

Chronic hepatitis B is an important cause of morbidity and mortality resulting from cirrhosis-related liver failure and hepatocelluar carcinoma (HCC) [1,2]. Lamivudine is a nucleoside analogue with activity against hepatitis B virus (HBV) replication. A daily dosage of lamivudine of 100 mg has been accepted worldwide for the treatment for chronic hepatitis B, since early studies showed that there was no significant difference in the effect of lamivudine at doses of 100 mg and 300 mg [3,4]. However, to establish ideal dosages in those studies, efficacy was mainly evaluated by measuring HBV–DNA, and the assay used was not as sensitive as the PCR assay [5,6]. Studies in which HBV–DNA was measured by PCR assay reported an additional viral suppressive activity with high doses (300 mg) of lamivudine for 24 weeks [7]. In addition to limits imposed by the assay methods used, the observation periods in the studies on lamivudine doses of 100 mg to 300 mg were limited to a period of 12 [3] or 24 weeks [4], because 1 year treatment with lamivudine often resulted in the relapse of HBV viremia [8]. Furthermore, the major drawback of lamivudine monotherapy is the emergence of resistant HBV with mutations of the tyrosine–methionine–aspartate–aspartate (YMDD) motif. The incidence of these mutants rises from 15 to 20% in the first year of therapy to 40% by the second year, and to 67% by the fourth year [9]. Although the effect of doses greater than 100 mg on the emergence of YMDD mutants has not been evaluated, base line body mass index has been reported to be significantly related to the emergence of mutation of HBV during lamivudine treatment in patients with co-infection of HBV and human immunodeficiency virus-1 HIV-1 [10]. Therefore, we evaluated the relationship between lamivudine effects and body surface are (BSA).

2. Patients and methods

2.1. Patients

A total of 134 patients with chronic hepatitis B were evaluated. Patients with fatty liver, patients with viral hepatitis C, patients with alcoholic abuser and patients with autoimmune disorder such as autoimmune hepatitis and primary biliary cirrhosis were excluded. They had been treated with 100 mg of lamivudine for more than 12 months at Kyushu University Hospital and its related hospitals (Table 1). For all patients, the existence of serum HBV–DNA was confirmed by TMA assay ($10^{3.7}$–$10^{8.7}$ genome equivalents/mL; 3.7–8.7 log genome equivalents [LGE]/mL) (Chugai Diagnostic Science, Tokyo, Japan) or by a Roche Monitor kit ($10^{2.6}$–$10^{7.6}$ copies/ml; 2.6–7.6 log copies/mL) (Roche Diagnostics, Tokyo, Japan) before treatment. None of the patients dropped out and all were treated with 100 mg/day lamivudine until the end of the observation period. After the start of medication, basic hepatic function and serum levels of HBV–DNA were measured at least every 3 months for all patients. The efficacy of lamivudine was evaluated from the serum levels of alanine aminotransferase (ALT) and HBV–DNA, as biological and virological effects, respectively. The evaluation using serum ALT was done as follows: (1) sustained responder- (SR-) ALT: the serum levels of ALT decreased, and remained at less than 30 U/L continuously during the observation; (2) transient responder- (TR-) ALT: the serum ALT decreased to less than 30 U/L, but increased to more than 30 U/L again during the subsequent observation; (3) non-responder- (NR-) ALT: the serum ALT always remained at more than 30 U/L throughout the observation. Similarly, the virological evaluation by HBV–DNA was done as follows, using a Roche Monitor kit: (1) SR-HBV: serum HBV–DNA decreased to levels undetectable by PCR (<2.6 log copies/mL), and remained negative continuously during the observation; (2) TR-HBV: the serum HBV–DNA decreased to undetectable levels once (<2.6 log copies/mL), but become positive again during the subsequent observation; (3) NR-HBV: the serum HBV–DNA was never negative throughout the observation (>2.6 log copies/mL). BSA was calculated using the method of DuBois.

Table 1
Characteristics of the 134 patients at base line[a]

	Total
Age	48.9 ± 11.4 (21–70)
Male/Female	98/36
CH/LC (Child A/B/C)	83/51 (35/9/7)
Observation period (month)	23.1 ± 7.9 (12–40)
ALT (U/L)	184.6 ± 243.4 (17–1722)
Albumin (g/dl)	3.76 ± 0.57 (2.3–4.9)
Bilirubin (mg/dl)	1.41 ± 1.79 (0.4–12.9)
Platelet (10^4/μl)	13.62 ± 6.19 (2.6–29.9)
BSA (m^2)[a]	1.702 ± 0.189 (1.28–2.24)
Height (m)	1.644 ± 0.084 (1.44–1.85)
Weight (kg)	64.2 ± 11.7 (38.0–105.0)
<5.0 (LEG/ml)	24
5.0 < ≤ 6.0	15
6.0 < ≤ 7.0	35
>7.0	48
Positive	77
Negative	57

[a] Plus-minus values are means ± S.D.

2.2. Statistical analysis

The backgrounds of the patients at the beginning of lamivudine treatment are shown as mean ± S.D. for the quantitative variables. Differences in the backgrounds of the SR, TR and NR-patients were examined by one-way ANOVA or χ^2-test. In order to confirm the contribution of the variables toward the effect of the treatment, univariate and multivariate logistic analysis were performed. For multivariate logistic analysis, we analyzed BSA as an independent factor contributing to effects of lamivudine treatment variables that showed χ^2 values of more than 1.0 in the univariate logistic model.

3. Results

Differences in background at the beginning of lamivudine treatment among the variables were evaluated by one-way ANOVA or χ^2-test. In the studies evaluating ALT (the biological response), the mean values of BSA and body weight were significantly lower in the SR-ALT group than in the other groups, and there was no significant difference in sex, observation period, height, ALT, albumin, bilirubin, platelet counts, HBV–DNA, or HBeAg among the groups (Table 2). Fig. 1 shows the distribution of BSA in each group, and demonstrated that there was an inverse relationship between BSA and the effects of lamivudine ($p = 0.0394$ at SR versus TR and $p = 0.0004$ at SR versus NR by Fisher's PLSD test). Similarly, the evaluation of HBV–DNA (the virological response) showed that BSA and body weight were also significantly lower in the SR-HBV group than in NR-HBV group (Table 3 and Fig. 1 [BSA: $p = 0.0087$ at SR versus NR by Fisher's PLSD test]). The prevalence of HBeAg differed significantly among the groups ($p < 0.005$ by χ^2-test), and in the NR-DNA group, most of the cases were positive for HBeAg (Table 3). The observation period also differed significantly among the groups ($p < 0.05$), and the SR-DNA group had longer observation period than the others. There was no difference in sex, height, ALT, alubumin, bilirubin, platelet counts, or HBV–DNA among the groups.

Table 2
Evaluation using the serum levels of ALT

	SR	TR	NR
Number	60	43	31
Male/Female	39/21	31/12	28/3
Observation period (month)	22.3 ± 8.8	23.9 ± 7.1	23.5 ± 7.3
ALT (U/L)	196.7 ± 195.2	181.2 ± 263.9	165.6 ± 299.6
Albumin (g/dl)	3.82 ± 0.51	3.66 ± 0.67	3.80 ± 0.54
Bilirubin (mg/dl)	1.42 ± 2.18	1.58 ± 1.75	1.16 ± 0.70
Platelet ($\times 10^4\ \mu l^{-1}$)	14.49 ± 6.25	13.36 ± 6.31	12.30 ± 5.83
BSA (m^2)*	1.644 ± 0.184	1.719 ± 0.184	1.790 ± 0.172
Height (m)	1.629 ± 0.087	1.650 ± 0.091	1.667 ± 0.060
Weight (kg)*	60.45 ± 10.41	65.24 ± 10.53	70.10 ± 13.27
HBV–DNA			
<5.0 (LEG/ml)	11	8	5
5.0 < ≤ 6.0	6	1	8
6.0 < ≤ 7.0	17	11	7
>7.0	26	23	11
HBeAg			
Positive	32	25	20
Negative	28	18	11

* $p < 0.005$.

In order to confirm the contribution of the variables toward the treatment, univariate and multivariate logistic analysis were performed. In univariate logistic analysis, platelet counts, BSA, body weight, height, HBV–DNA and HBeAg in the biological evaluation, and bilirubin, BSA, body weight, height, HBV–DNA and HBeAg in the virological evaluation, had χ^2 values of more than 1.0 (Table 4). Therefore, we used these factors except body weight and height as variables for multivariate logistic analysis, because there was a strong correlation between BSA and body weight ($R^2 = 0.89072$, $p < 0.0001$), or BSA and height ($R^2 = 0.67708$, $p < 0.0001$). The results of multivariate analysis revealed that BSA was the only significant factor that could contribute to the improvement of ALT levels ($\chi^2 = 12.8$, $p = 0.0004$), and BSA and HBeAg were independent factors that could influence the disappearance of serum HBV–DNA (BSA: $\chi^2 = 4.4$, $p = 0.0354$ and HBeAg: $\chi^2 = 8.1$, $p = 0.0044$) (Table 5).

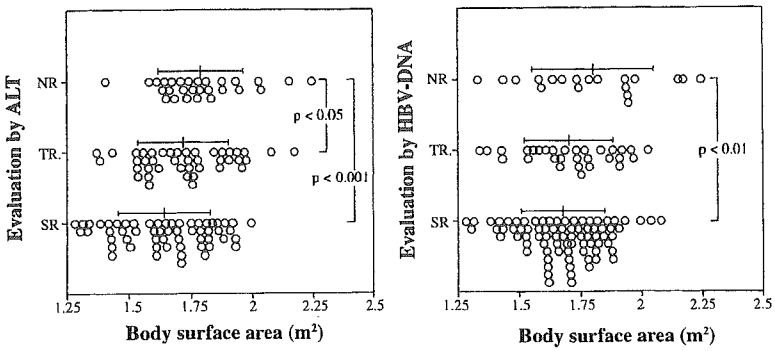

Fig. 1. The distribution of BSA according to the effect evaluated by ALT (left panel) and by HBV–DNA (right panel) was shown (open circle). The bars represent mean ± S.D. Non responders had significantly larger BSA than sustained responders in both evaluations.

Table 3
Evaluation using the serum levels of HBV–DNA

	SR	TR	NR
Number	83	31	20
Sex	60/23	22/9	16/4
Observation period (month)*	22.6 ± 7.5	26.7 ± 7.7	19.6 ± 7.7
ALT (U/L)	193.7 ± 246.6	188.5 ± 296.3	138.2 ± 91.4
Albumin (g/dl)	3.74 ± 0.58	3.78 ± 0.52	3.83 ± 0.61
Bilirubin (mg/dl)	1.56 ± 2.16	1.30 ± 0.98	0.92 ± 0.43
Platelet ($\times 10^4\ \mu l^{-1}$)	13.51 ± 6.05	13.04 ± 6.32	15.04 ± 6.70
BSA (m^2)*	1.676 ± 0.170	1.702 ± 0.181	1.801 ± 0.248
Height (m)	1.629 ± 0.087	1.650 ± 0.091	1.667 ± 0.060
Weight (kg)*	62.40 ± 10.03	64.33 ± 10.14	71.59 ± 17.26
HBV–DNA			
<5.0 (LEG/ml)	18	6	0
5.0 < ≤ 6.0	10	4	1
6.0 < ≤ 7.0	20	8	7
>7.0	35	13	12
HBeAg**			
Positive	40	19	18
Negative	43	12	2

* $p < 0.05$.
** $p < 0.005$.

Table 4
Univariate analysis on the lamivudine effects

Variable	χ^2	p-value
ALT evaluation		
ALT	0.35543766	0.5511
Albumin	0.31157046	0.5767
Bilirubin	0.19658636	0.6575
Platelet	2.68462623	0.1013
BSA	13.060725	0.0003
Height	4.09176033	0.0431
Weight	12.734481	0.0004
HBV–DNA	3.05562744	0.3831
HBeAg	1.02785409	0.3107
HBV–DNA evaluation		
ALT	0.4754853	0.4905
Albumin	0.39014806	0.5322
Bilirubin	2.42720044	0.1192
Platelet	0.25597276	0.6129
BSA	5.54675909	0.0185
Height	1.24367306	0.2648
Weight	5.45864661	0.0380
HBV–DNA	4.18232253	0.2424
HBeAg	10.2577192	0.0014

4. Discussion

In this present study, we found that BSA was a significant factor that could contribute to both the improvement of ALT levels (biological response) and the disappearance of serum HBV–DNA (virological response). Because the pharmacokinetics of lamivudine correlate with body weight, as is the case with many other drugs [11], it is reasonable to conclude that patients with lower BSA would have the higher blood concentration of lamivudine, although we did not actually monitor the lamivudine blood concentration. Recent reports suggest

Table 5
Multivariate analysis of the effects of lamivudine using potential univariate predictors

Variable	χ^2	p-value
ALT evaluation		
Platelet	3.2287548	0.0724
BSA	12.7808491	0.0004
HBV–DNA	2.42555739	0.4889
HBeAg	0.93377434	0.3339
HBV–DNA evaluation		
Bilirubin	3.76147767	0.0524
BSA	4.42516561	0.0354
HBV–DNA	2.23355431	0.5254
HBeAg	8.10893404	0.0044

that baseline body mass index is significantly related to the emergence of HBV mutation during lamivudine treatment (300 mg/day, >6 months) in patients coinfected with HBV and HIV-1 [10]. Therefore, the results of our study question whether a lamivudine dosage of 100 mg/day is adequate, particularly for long-term treatment.

The standard lamivudine dose of 100 mg daily was based on early studies in which doses of 25, 100, 300 mg were compared for 12 [3] or 24 weeks [4]. Dienstag et al. [3] reported that levels of HBV–DNA became undetectable in 70% of the patients who received the 25 mg dose of lamivudine ($n = 10$) and 100% of those treated with doses of 100 mg ($n = 11$) or 300 mg ($n = 11$). Nevens et al. [4] reported that HBV–DNA was undetectable at the end of 24 weeks of lamivudine treatment in 58% (25 mg), 93% (100 mg), and 88% (300 mg) of patients ($n = 16$, 19 and 19, respectively), and that abnormal ALT levels at baseline were normalized in 64% (25 mg), 45% (100 mg), and 36% (300 mg) of the patients at treatment completion. Because there were no significant differences reported in the rates of non-detection of HBV–DNA and nomalization of ALT levels between the 100 and 300 mg dose, the dose of 100 mg has become accepted as a therapeutic standard. However, several issues should be considered when evaluating the results of these studies, including: (1) number of patients; (2) period of treatment; (3) emergence of lamivudine-resistant mutants in long-term treatment; and (4) detection limit of HBV–DNA.

As we showed in the present study of 134 cases, there was a significant difference in the effect of BSA on both the biological and virological response at the end of observation period (mean = 2 years), although the differences in the mean values of BSA in each group were relatively small. Therefore, it is possible that previous studies failed to detect a significant contribution of BSA to the effects of lamivudine because of the smaller numbers of patients examined.

Observation periods of 24 weeks are not adequate for detecting the emergence of lamivudine-resistant mutants, since the incidence of the mutants rises from 15 to 20% in the first year to 40% by the second year and to 67% by the fourth year of treatment [9]. In our present study of HBV–DNA evaluation, the rate of TR-DNA and NR-DNA was 38% in patients

who were positive for HBV–DNA (>2.6 log copies/mL) at the end of observation (average of about 2 years). We did not confirm the YMDD mutation in all cases of TR-DNA and NR-DNA, and further study will be needed to confirm whether BSA affects the incidence of YMDD mutants.

It is important to consider the sensitivity of measurement of HBV–DNA in evaluating the effects of lamivudine, because a decrease in HBV–DNA is reported to be associated with a lower incidence of YMDD mutants [12]. In previous studies, which showed no difference in the effects of 100 and 300 mg lamivudine on HBV–DNA reduction (as described above) [3,4], HBV–DNA was measured quantitatively by liquid hybridization assay (Abbott Laboratories), which has a detection limit of 10^7 geq/mL [13]. Honkoop et al. studied the efficacy of 100 and 300 mg lamivudine in viral suppression for 24 weeks using a semi-quantitative PCR method with a detection limit of 10^2–10^3 geq/mL [7]. They showed that 29 and 37% patients were HBV–DNA negative after 24 week treatment with 100 and 300 mg lamivudine, respectively, indicating the possibility of additional viral suppressive activity with higher doses and longer-term therapy with lamivudine. Furthermore, using a more sensitive real-time quantitative PCR method with a detection limit of 1.7 log copies/mL, Ide et al. [12] reported that neither emergence of YMDD mutants nor a virological breakthrough of serum HBV DNA was observed in patients with <1.7 copies/mL. In our study, we measured HBV–DNA with a Roche Monitor kit, which has detection limit of 2.6 log copies/mL; 23% patients (TR-DNA) were HBV–DNA negative once, but became positive again during the subsequent observation. The TR-DNA group had a longer observation period than the other groups, this difference might affect the result that there was no significant difference in BSA between TR-DNA and SR-DNA groups, or TR-DNA and NR-DNA groups in the evaluation of HBV–DNA (Fig. 1). More precise analysis with longer observation period and/or using the real-time PCR method might show a clearer relationship between BSA and virological response with lamivudine.

In conclusion, we have revealed that BSA is a statistically significant and potentially important factor that can predict the effect of lamivudine therapy for chronic hepatitis B. A noteworthy finding in our study was that small differences in BSA could significantly influence the effect of the lamivudine treatment, suggesting that a small dose increase might increase the efficacy of lamivudine therapy. We believe that a long-term clinical trial with higher dose lamivudine treatment and a large number of cases is warranted, since lamivudine will continue to be a first-line treatment for HBV.

Appendix A

In addition to the authors, the Kyushu University Liver Disease Study Group includes the following individuals: R. Sugimoto (Harasanshin Hospital, Fukuoka), H. Amagase and S. Tominaga (Mihagino Hospital, Kitakyushu), K. Yanagita (Saiseikai Karatus Hospital, Karatsu), K. Ogiwara (Kyushu Rosai Hospital, Kitakyushu), M. Tokumatsu (Saiseikai Fukuoka Hospital, Fukuoka), S. Tabata (Hayashi Hospital, Fukuoka), M. Yokota (National Kyushu Cancer Center, Fukuoka), H. Tanaka (Chihaya Hospital, Fukuoka), S. Nagase (Fukuoka Teishin Hospital, Fukuoka), S. Tsuruta (Nakabaru Hospital, Fukuoka), S. Tada (Moji Rosai Hospital, Kitakyushu), M. Nagano (Kyushu Koseinenkin Hospital, Kitakyushu), M. Honda (Nishi-Fukuoka Hospital, Fukuoka), T. Umeno (Sawara Hospital, Fukuoka), T. Sugimura (National Hospital Organization Fukuoka Higashi Hospital, Fukuoka), S. Ueno (Kitakyushu Municipal Wakamatsu Hospital, Kitakyushu), K. Miki (Kitakyushu Municipal Moji Hospital, Kitakyushu), H. Okubo (Shineikai Hospital, Kitakyushu), H. Fujimoto (Mitusbishikagaku Hospital, Kitakyushu), N. Higuchi (Shin-Nakama Hospital, Kitakyushu), S. Shigematsu (Kouseikan Hospital, Saga), N. Higashi (National Hospital Organization Beppu Hospital, Ohita).

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NOTE

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Serum antibody response to tuberculosis-associated glycolipid antigen after BCG vaccination in adults

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Abstract Tuberculous glycolipid antigens (TBGLs) are derived from the cell walls of *Mycobacterium tuberculosis*. Detection of anti-TBGL antibody in serum has recently become possible for the serodiagnosis of active tuberculosis. TBGL is not indigenous to *M. tuberculosis*, but it is widely found in *Mycobacterium* species. To elucidate the influence of *M. bovis* bacille Calinette-Guérin (BCG) vaccination, we assayed serum anti-TBGL antibody after BCG vaccination in adults. BCG vaccination was done for 20 Japanese healthcare workers with a negative tuberculin skin test reaction, and serum was collected 0, 2, 4, and 8 weeks and 1 year after vaccination. The tuberculin skin test became positive in 85% of the subjects. The mean anti-TBGL antibody titer remained negative throughout the observation period, but was elevated significantly compared with the pre-vaccination level, peaking at week 4 and showing a reduced level 1 year post-vaccination. These results showed that serological diagnosis using anti-TBGL antibody was not influenced by prior BCG vaccination.

Key words Tuberculosis · TBGL · Serodiagnosis · BCG

Tuberculosis remains a major global public health problem. Rapid diagnosis and treatment of tuberculosis are important for prevention of the spread of the disease. A diagnosis of mycobacterial infection is based on finding bacilli in the sputum of patients, by smear staining or isolation by culture. However, the detection rate of bacilli using the conventional method is low, and culturing requires 4–6 weeks. Modern gene-based technologies such as the polymerase chain reaction (PCR) are useful, but their usefulness is reduced in patients without sputum expectoration. There is strong demand for the establishment of a rapid and easy method to diagnose tuberculosis.

Recently, the serodiagnosis of tuberculosis using mycobacterial antigens has been studied for rapid diagnosis.[1–5] The cell walls of Mycobacteria contain peptidoglycolipids and cord factor (trehalose-6, 6′-dimycolate) and other glycolipids abundant in mycolic acids. Serum antibody against cord factor has been detected in patients with active tuberculosis.[6] Tuberculous glycolipid antigens (TBGLs), including cord factor, are reported to be suitable for serodiagnosis by enzyme-linked immunosorbent assay (ELISA).[1,7–9] Clinical validation of the assay was confirmed by the measurement of IgG antibody to TBGL, in which patients with active tuberculosis showed significantly higher antibody titers than patients with inactive tuberculosis and healthy adults.[1]

Although TBGL is purified from *Mycobacterium tuberculosis*, TBGL is not indigenous to *M. tuberculosis*, but is widely found in *Mycobacterium* species.[2,10–13] Cross-reaction of antibody to TBGL is considered to occur in subjects vaccinated with bacille Calinette Guérin (BCG) or patients with nontuberculous mycobacteriosis. In Japan, for several decades BCG vaccinations have been given to infants and revaccinations have been performed in tuberculin skin test-negative children at the ages of 6 and 12 (revaccination was discontinued at the start of 2003). If the BCG vaccination influences the serum antibody titer to TBGL, the clinical benefits of using the ELISA for serodiagnosis are reduced although the cross-reaction between BCG and *M. tuberculosis* is reported to be less than expected.[6] In this context, our study was designed to document, over time, serum antibody titers to TBGL in adults after BCG vaccination.

In 2000, the infection control team of Haradoi Hospital (Fukuoka, Japan) carried out tuberculin skin test screening of 449 healthcare workers to identify skin test-negative indi-

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viduals and to immunize them with BCG to prevent nosocomial infection by tuberculosis, as described in a previous report.[14] After two-step tuberculin skin testing, 36 (8.0%) subjects were found to be negative, of whom 26 were then given intradermal vaccinations with BCG. Of the vaccinees, 20 (mean age, 27.8 years; male, 6; female, 14) were enrolled in this study after the obtaining of informed consent. Because most Japanese were immunized with BCG as infants or in childhood, the present vaccination was considered a revaccination. Patients with pulmonary tuberculosis ($n = 7$) were also enrolled in the study. The diagnosis of active tuberculosis was based on culture, radiographic findings, and clinical symptoms. Serum specimens from patents with pulmonary tuberculosis were collected before the start of chemotherapy. All BCG-vaccinated subjects were negative for HIV infection and were not medicated with immunosuppressive drugs. Patients with diabetes mellitus and cancer were not enrolled in this study.

ELISA was performed according to the manufacturer's instructions (Determiner TBGL; Kyowa Medex, Tokyo, Japan) as previously described.[1,8] Briefly, 96-well polystyrene plates were coated with TBGL from *M. tuberculosis* strain H37Rv by placing 25 µl of antigen solution in each well and allowing the plate to dry at room temperature. All samples were diluted 1:41 with dilution buffer, and were added to each well in a volume of 100 µl. After incubation for 60 min at room temperature, the plates were washed with washing buffer, and horseradish peroxidase-conjugated rabbit Fab' raised against human IgG was added to each well. The plates were again incubated for 60 min at room temperature and washed with washing buffer. Then, 100 µl of 3,3',5,5'-tetramethylbenzine solution was added to each well and the plates were incubated for 15 min at room temperature. Stop solution (1 M H2SO4) was added to each well and absorbance at 450 nm was read with a Spectrafluor plate reader (Maennedorf, Switzerland). Samples with at least 2.0 U/ml of antibody per milliliter were considered positive. Parametric analysis (paired Student's *t*-test) was used to determine the significance of differences. A P value of less than 0.05 was considered significant.

To investigate the anti-TBGL antibody response to BCG vaccination, the serum anti-TBGL antibody titer was analyzed before vaccination and for 1 year after vaccination. As we reported previously, the tuberculin skin test 1 year after the BCG vaccination showed that 17 of the 20 subjects (85%) had become positive.[14] Figure 1 shows the serum anti-TBGL antibody titers before (week 0) and at weeks 2, 4, and 8, and 1 year post-vaccination. The mean antibody titers (U/ml) at weeks 0, 2, 4, and 8, and 1 year post-vaccination were 0.51, 0.74, 0.86, 0.73, and 0.61, respectively. The mean antibody titers at 2, 4, and 8 weeks post-vaccination were significantly elevated compared with that at week 0. On the other hand, the mean antibody titer of serum from patients with tuberculosis was 3.46 U/ml. Before the BCG vaccination, all but one of the subjects were negative for anti-TBGL antibody, with one being positive for anti-TBGL antibody at the cutoff level (2.0 U/ml). After the BCG vaccination, one subject was positive for anti-TBGL antibody at week 4 (from 0.5 U/ml to 2.9 U/ml), and then was negative again at week 8 and 1 year post-vaccination. This subject showed no signs or symptoms of active tuberculosis or mycobacteriosis during the study period.

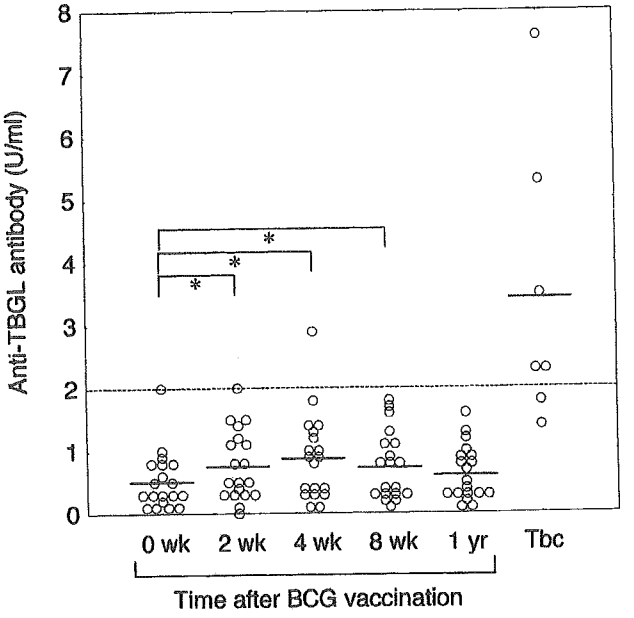

Fig. 1. Serum anti-tuberculous glycolipid antigen (*TBGL*) antibody response to bacille Calinette Guérin (*BCG*) vaccination. BCG was given to 20 Japanese healthcare workers with a negative tuberculin skin-test reaction, and serum specimens were collected at 0, 2, 4, and 8 weeks (*wk*) and 1 year (*yr*) post-vaccination. *Horizontal bars* indicate mean values, and the *dotted line* shows the cutoff level of the anti-TBGL assay kit. *Tbc*, patients with active tuberculosis ($n = 7$). $^*P < 0.05$

Antibodies to *M. tuberculosis*-associated antigens, such as PPD, antigen 60, lipoarabinomannan, and TBGL, have been available for the diagnosis of tuberculosis since the establishment of the appropriate ELISAs.[1–5] The efficacy of these ELISAs ranges from 50% to 90% sensitivity and 75% to 98% specificity. Detection of serum antibody to *M. tuberculosis*-associated antigens is useful for the diagnosis of culture-negative tuberculosis, which has hitherto been diagnosed by chest X-ray and clinical manifestations. TBGL is a cell-wall antigen from *M. tuberculosis*, and serum antibody to TBGL has been well characterized and is commonly used for the diagnosis of tuberculosis. Maekura et al.[1] reported that the sensitivity of this diagnosis for patients with active tuberculosis was 81.1% and the specificity was 95.7%. Even in patients with culture-negative active tuberculosis, the sensitivity was 73.5%.

Although cross-reaction between anti-TBGL antibody and other *Mycobacterium* species has been reported,[2,10–13] it is not known whether BCG vaccination influences the antibody titer to TBGL. *M. bovis* BCG has been used for vaccination in infants and tuberculin skin test-negative children in Japan. In the present study, the anti-TBGL antibody titer was elevated 2–8 weeks after BCG vaccination, but it was still below the cutoff point (2.0 U/ml). Although one subject showed transient elevation of the anti-TBGL titer

(2.9 U/ml) 4 weeks after vaccination, the titer had decreased to 1.2 U/ml at 1 year post-vaccination. The low response of the anti-TBGL antibody to BCG vaccination is not due to anergy to *M. tuberculosis*, because 85% of the vaccinated subjects showed a positive tuberculin skin test at 1 year post-vaccination. The present study indicates that serological diagnosis using anti-TBGL antibody was not influenced by prior BCG vaccination. The use of anti-TBGL antibody was also shown to be of value in the diagnosis of active tuberculosis, even in people previously vaccinated with BCG.

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Association between chronic *Helicobacter pylori* infection and acute ischemic stroke: Fukuoka Harasanshin Atherosclerosis Trial (FHAT)

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Abstract

Helicobacter pylori (*H. pylori*) have been associated both epidemiologically and pathogenetically with coronary atherosclerosis, but data on the relationship between chronic *H. pylori* infection and stroke are lacking. Therefore, we investigated the relationship between *H. pylori* infection and acute ischemic stroke in 62 patients with their first stroke and 143 controls. The stroke patients were all admitted to Harasanshin General Hospital (Fukuoka, Japan) and the controls were asymptomatic age-matched outpatients with hyperlipidemia who did not have cardiac disease or infections. All patients underwent cranial CT scanning and/or brain magnetic resonance imaging, duplex ultrasonography of the extracranial carotid arteries, and transthoracic echocardiography. *H. pylori* infection was diagnosed by detection of anti-*H. pylori* IgG antibodies, the ^{13}C-urea breath test, and histology. Conditional logistic regression analysis was performed to analyze the data. The 62 stroke patients and 143 controls were aged from 41 to 92 years. Chronic *H. pylori* infection was associated with a higher risk of stroke due to small artery occlusion (odds ratio: 9.68; 95% CI: 3.56–33.08, $P < 0.001$) and a lower risk of cardioembolic stroke (odds ratio: 0.27; 95% CI: 0.03–1.53). Chronic *H. pylori* infection still showed an overall association with ischemic stroke (odds ratio for all subtypes combined: 2.57; 95% CI: 1.09–6.08) after adjusting for major cardiovascular risk factors. These results suggest that chronic *H. pylori* infection may be a triggering factor that increases the risk of acute ischemic stroke.

Keywords: *Helicobacter pylori*; Ischemic stroke; Stroke subtypes; Carotid atherosclerosis

1. Introduction

Atherosclerosis is a highly prevalent disease, and is currently the greatest cause of morbidity and mortality in developed societies. Many risk factors are involved in the development of atherosclerosis, which manifests as coronary artery disease (CAD) and myocardial infarction (MI), including hyperlipidemia, hypertension, smoking, and diabetes mellitus [1], but much of the risk remains unexplained. The pathogenesis of atherosclerosis involves the processes of vascular injury, inflammation, degeneration, and thrombosis, but the stimulus that triggers the inflammatory response is largely unclear.

The pulse wave velocity can be used as an indicator of arterial stiffness [2,3], and it is regarded as a marker of vascular damage [4,5]. An instrument was recently developed that can measure the branchial-ankle pulse wave velocity (baPWV) by the volume rendering method. Yamashina et al. have reported a high validity and reproducibility of baPWV measurements, suggesting that this parameter may be an acceptable indicator of vascular damage and may be suitable

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for screening large populations to detect vascular disease [6].

Chronic infection with *Helicobacter pylori* (*H. pylori*) has been seroepidemiologically linked to CAD and atherosclerosis [1,7]. Ischemic stroke comprises a heterogeneous mixture of different stroke subtypes caused by atherosclerotic as well as non-atherosclerotic mechanisms [9]. Although stroke is pathogenetically related to coronary atherosclerosis, data about the association between chronic *H. pylori* infection and cerebrovascular disease are limited [9–14]. It appears that the first paper regarding ischemic cerebrovascular disease and *H. pylori* was published in 1998 [14], and it was only a small study. To make a valid and reliable assessment of the role of chronic *H. pylori* infection in cerebrovascular disease, the underlying mechanism of ischemic stroke must be taken into consideration by stratifying the subjects into different etiologic subtypes. Because direct detection of *H. pylori* in the cerebral vasculature would require samples of cerebral vessels, which are not clinically available, surrogate markers such as the anti-*H. pylori* antibody titer must be used to assess the association between stroke and infection with this organism.

The present case-control study was performed to investigate whether *H. pylori* infection was an independent risk factor for various etiologic subtypes of ischemic stroke.

2. Methods

2.1. Subjects and methods

All of the patients with acute cerebrovascular disease admitted to the Division of General Medicine at Harasanshin General Hospital (Fukuoka, Japan) during the year 2002 were considered eligible for the present study. Between July 1 and December 31, 2002, a total of 62 patients who suffered their first ischemic stroke were registered for this study.

2.2. Selection of stroke patients

Patients with their first stroke were enrolled in the study if they met the following criteria: (a) first ischemic stroke, (b) admission to hospital for treatment, and (c) admission within 72 h of the onset. Stroke was defined according to World Health Organization criteria [15]. Cerebral infarction was diagnosed on the basis of the initial CT and MRI data. All patients underwent ultrasonography of the neck and intracranial arteries. The carotid arteries were assessed by color flow B-mode Doppler ultrasound (SONOS 5500, PHILIP) according to the standard method [16,17]. The vertebrobasilar system was evaluated as described by Bartels [18]. In some cases, the ultrasound images were unsatisfactory, so MRI angiography was performed to determine the presence/absence of atherosclerotic lesions.

Patients without clinical or imaging evidence of atherosclerosis who had atrial fibrillation and/or echocardiographic findings suggestive of possible cardiogenic embolism were classified as having thromboembolic stroke. The other patients were diagnosed as having large artery stroke if there was >50% stenosis of the extracranial carotid or an intracranial artery, small artery occlusion if they had a clinical lacunar syndrome associated with appropriate CT changes or a typical clinical syndrome despite normal CT scans, or undefined stroke if their condition was not due to either of these mechanisms [8]. The present study only included patients with large vessel stroke and cardioembolic stroke, while the other subtypes were excluded because both atherosclerotic and non-atherosclerotic mechanisms might be involved.

During hospitalization, neurological evaluation was always done by one neurologist who applied the specified study criteria for classification of the patients. All evaluations were performed at the Department of Neuroradiology.

2.3. Selection of controls

The control subjects were chosen from among asymptomatic age-matched outpatients with hyperlipidemia who did not have cardiac disease or infections. The absence of atherosclerosis in the control subjects was assessed as follows: normal 12-lead ECG, normal echocardiography findings, <25% stenosis of the carotid arteries on Doppler ultrasonography, and normal lower limb arteries on physical examination. A history of cardiac disease meant exclusion from the control group.

2.4. Baseline evaluation

Data were collected by interview, physical examination, and neurological examination performed by trained health professionals, detailed review of all available medical records, and laboratory tests of fasting blood samples. Stroke patients were evaluated on day 7 after the onset of symptoms, while blood samples were taken within 24 h of admission (86% of the blood samples were obtained within 48 h after stroke onset). Control subjects were evaluated in the same manner as the stroke patients at the Stroke Prevention Clinic of the Department of Neurology between July 2002 and January 2003. Patients and control subjects were defined as hypertensive if they had a diastolic blood pressure >90 mmHg and a systolic blood pressure >140 mmHg or if they had been treated with antihypertensive therapy for at least 1 year. Patients were classified as diabetic if they had a fasting glucose level >126 mg/dL on two occasions or if they had been treated with antidiabetic drugs for at least 1 year. Patients were defined as smokers if they reported daily smoking of >10 cigarettes for at least 1 year during the last 10 years, and they were considered to be hyperlipidemic if the total cholesterol level was >220 mg/dL or if they had been treated with lipid-lowering drugs for at least 1 year. The BMI (kg/m^2) was calculated as a measure of obesity. All subjects reporting previous *H. pylori* eradication therapy were excluded from the study. The stroke patients

and controls lived in the same geographic area (Fukuoka City).

The type of ischemic stroke was classified according to Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria into large artery atherosclerosis, cardiogenic embolism, small artery occlusion, other etiologies, and undefined. Classification was performed by one neurologist on the basis of clinical findings and the results of standardized diagnostic tests, including CT or MRI, vascular imaging, and ECG or echocardiography.

2.5. Laboratory tests

All samples were stored at −80 °C and were analyzed simultaneously by technicians who were unaware of whether each sample belonged to a patient or a control.

2.6. IgG antibody to H. pylori

Serum IgG antibody to *H. pylori* was detected by ELISA [19] according to the manufacturer's instructions. Samples were tested in duplicate and results were expressed in arbitrary units. The reference limits were previously determined in our laboratory using serum samples from persons with or without *H. pylori* infections. A value >7.5 U was defined as positive, whereas values <3.0 U were negative (sensitivity and specificity >95%). Values between 3.0 and 7.5 U were defined as borderline and were excluded from the study.

2.7. ^{13}C-urea breath test

Each patient underwent a ^{13}C-urea breath test (UBT) by drinking 100 mg of ^{13}C-urea in water after an overnight fast. Breath samples were collected before and 20 min after the administration of ^{13}C-urea. The $^{13}CO_2/^{12}CO_2$ ratio ($\Delta^{13}CO_2$) in the breath bag was analyzed by a small infrared spectrometer (UBiT-200; Otsuka Electronics Co., Hirakata, Japan) and the results were expressed as the percent excess (parts per thousand) of $^{13}CO_2$ ($\Delta^{13}CO_2$). This method has been shown to have an excellent correlation (r = 0.996) with mass spectrometric measurement of $^{13}CO_2$ [20,21]. ^{13}C-UBT values below 3.5% were considered negative for *H. pylori*.

2.8. Brachial-ankle pulse wave velocity (baPWV)

The baPWV was measured using a volume plethymograph (PWV/ABI; Colin, Co., Ltd., Komaki, Japan), which simultaneously recorded the PWV, blood pressure, electrocardiogram, and heart sounds [6]. Each subject was examined in the supine position, with the electrocardiographic leads on both wrists, a microphone for detecting heart sounds taped at the left sternal edge, and cuffs on both arms and ankles. The cuffs were connected to a plethymographic sensor that determined the pulse volume waveform and to an oscillometric pressure sensor that measured the blood pressure. Pulse volume waveforms were recorded using a semiconductor pressure sensor with the acquisition frequency set at 1200 Hz. Waveforms for the arm and ankle were stored in 10 s batches with automatic gain analysis and quality adjustment. In this study, baPWV data were obtained after at least 5 min of rest. The coefficient of variation for reproducibility of baPWV values in healthy subjects was reported to be 2.4% for the interobserver coefficient of variation (n = 15) and 5.8% for the intraobserver coefficient of variation (n = 17) [22].

2.9. Detection of H. pylori infection

H. pylori infection was identified by histologic examination, the ^{13}C-UBT, and serologic evaluation. Patients in whom at least one of these three tests was positive were classified as *H. pylori*-positive and those in whom all three tests were negative were considered to be *H. pylori*-negative.

2.10. Statistical analysis

Odds ratios (ORs) and 95% confidence intervals (CIs) for the risk of ischemic stroke associated with *H. pylori* infection were estimated by univariate analysis, as well as by multiple logistic regression analysis with adjustment for age, sex, smoking history, diabetes, and hypertension.

Because C-reactive protein (CRP) values do not show a normal distribution, a non-parametric test (the Mann–Whitney U-test) was used to compare this variable between groups. All analyses were performed with SPSS software (ver. 8). When not otherwise stated, data are presented as the mean ± S.D. and P = 0.05 (two-tailed) was considered to indicate statistical significance.

2.11. Ethics

The design of this study was approved by the Ethics Committee and the Data Protection Committee of Harasanshin General Hospital (Fukuoka, Japan). Informed consent for the collection of blood was obtained from all patients (or their closest relatives).

3. Results

One hundred and three patients with stroke and 281 control subjects were considered for the study, but 51 patients and 138 control subjects were excluded for the following reasons: unclassified stroke subtype (13 patients), refusal to participate in the study (19 patients and 38 control subjects), previous *H. pylori* eradication therapy (9 patients and 21 control subjects), abnormal ECG (27 control subjects), abnormal echocardiography findings (31 control subjects), and asymptomatic carotid stenosis (21 control subjects). Therefore, 62 patients and 143 control subjects were investigated further.

Table 1
Characteristics of the subjects

	Stroke patients (n = 62)	Controls (n = 143)	P value
Age [years, mean ± S.D.]	71.5 ± 11.3	69 ± 9.3	N.S.
Male sex [%]	40 (65%)	48 (34%)	$P < 0.001$
Blood pressure [mean ± S.D., mmHg]			
Systolic	161.4 ± 31.4	132 ± 20.4	$P < 0.001$
Diastolic	84.2 ± 13.5	78.1 ± 11.1	$P < 0.01$
Smoking [%]			
Ever	31 (50%)	65 (46%)	N.S.
Never	31 (50%)	78 (55%)	N.S.
History [%]			
Hypertension	55 (89%)	47 (33%)	$P < 0.001$
Diabetes mellitus	26 (42%)	16 (11%)	$P < 0.001$
IMT [mean ± S.D., mm]	1.22 ± 0.35	1.13 ± 0.41	N.S.
baPWV [mean ± S.D., cm/s]	2,292.8 ± 665.1	1,527.4 ± 314.8	$P < 0.001$

3.1. Characteristics of the subjects

The clinical characteristics of the stroke patients and control subjects are displayed in Table 1. The mean age of the 62 stroke patients was 71.5 years (S.D. 11.3), and 40 of them (65%) were men. Their average systolic blood pressure and diastolic blood pressure was 161.4 and 84.2 mmHg, respectively. Among the 62 patients, 50% were recent or former smokers, 89% had a history of hypertension, and 42% had diabetes. The mean carotid intima/media thickness (IMT) was 1.22 mm and baPWV was 2292.8 cm/s.

3.2. Prevalence of chronic H. pylori infection

Table 2 shows the prevalence of seropositivity to *H. pylori* and *H. pylori* infection. Infection was detected in 48/62 stroke patients (77%) compared with 64/143 controls (45%) and the difference was significant ($P < 0.0001$). When analyzed for sex, 34/40 male stroke patients (85%) were infected by *H. pylori* compared with 18/48 controls (38%) ($P < 0.0001$). Among the women, 14 out of 22 stroke patients (64%) were infected compared with 46 out of 95 controls (48%) ($P < 0.05$). Chronic *H. pylori* infection was found in 40 patients (89%) with small artery occlusion, 6 patients (67%) with large artery atherosclerosis, and 2 patients (25%) with cardiogenic embolism.

Table 2
Crude prevalence of chronic *H. pylori* infection in each group

	Stroke patients (n = 62)	Controls (n = 143)	P value
All	48 (77%)	64(45%)	$P < 0.0001$
Age (years)			
<70	19 (74%)	31(40%)	$P < 0.01$
≥70	29 (81%)	33(50%)	$P < 0.01$
Sex			
Male	34 (85%)	18(38%)	$P < 0.0001$
Female	14 (64%)	46(48%)	$P < 0.05$
Stroke subtype			
Small artery occlusion	40 (89%)		
Large artery atherosclerosis	6 (67%)		
Cardioembolic	2 (25%)		

3.3. ORs for H. pylori infection in stroke patients and controls after adjustment for possible confounding factors (Table 3)

Chronic *H. pylori* infection showed an overall association with ischemic stroke when all of the stroke subtypes were combined. There was no significant difference in the prevalence of chronic *H. pylori* infection between patients with cardiogenic embolism and the control subjects, whereas the prevalence of infection was significantly higher in the patients with small artery occlusion than in the controls (univariate analysis showed OR: 9.68 and CI: 3.56–33.08 ($P < 0.0001$)). However, there was no significance difference in the prevalence of chronic *H. pylori* infection between the patients with large artery atherosclerosis and the control subjects.

3.4. ORs for association between chronic H. pylori infection and stroke subtypes

Conditional logistic regression analysis (Table 4) showed that chronic *H. pylori* infection was significantly associated with a higher risk of stroke due to small artery occlusion and large artery atherosclerosis. In contrast, there was a significant inverse correlation between chronic *H. pylori* infection and cardiogenic embolism (adjusted OR: 0.137; 95% CI: 0.0236–0.796). Despite these differential associations with the stroke subtypes, chronic *H. pylori* infection also showed an overall association with ischemic stroke (all subtypes combined).

3.5. C-reactive protein (CRP)

Measurement of CRP was performed in all stroke patients and control subjects. The CRP level was 1.13 ± 2.03 mg/dL in the stroke patients (all subtype combined) and 0.32 ± 0.87 mg/dL in the control subjects, showing a significant difference ($P < 0.001$). There was no significant difference of CRP between the patients with cardiogenic embolism and the control subjects, whereas the CRP level was significantly higher in patients with small artery occlusion and large

Table 3
Odds ratios for *H. pylori* infection after adjustment for possible confounding factors

	Ischemic stroke (all subtypes)				Stroke subtype									
					Small artery occulusion				Large artery atherosclerosis				Cardioembolic			
	OR	RR	95%	CI	OR	RR	95%	CI	OR	RR	95%	CI	OR	RR	95%	CI
Age (years)																
<70	0.62	0.72	0.32	1.18	0.74	0.79	0.36	1.52	0.27	0.28	0.03	1.47	0.99	0.99	0.18	5.47
≥70	0.61	1.40	0.85	3.09	1.35	1.26	0.66	2.78	4.10	3.90	0.76	41.41	1.01	0.18	5.59	6.18
Male sex	3.57	2.42	1.84	7.09***	3.49	2.66	1.66	7.61**	2.77	2.66	0.57	17.59	1.34	1.33	0.24	7.43
Smoking	1.20	1.14	0.63	2.28	0.89	0.91	0.43	1.81	1.44	1.42	0.30	7.48	3.55	3.41	0.62	36.72
Hypertension	15.8	7.93	6.53	44.4***	9.90	6.56	3.86	30.31***	8.61	8.08	1.12	388.17*				
Diabetes mellitus	5.67	2.80	2.62	12.67***	2.45	1.94	1.07	5.48*	15.78	13.58	2.85	161.89**	4.15	3.88	0.74	23.33
large IMT	1.68	1.43	0.88	3.20	1.31	1.24	0.64	2.69	4.36	4.13	0.80	44.01	1.19	1.18	0.22	6.57
high baPWV	5.84	2.77	2.56	13.82***	6.07	3.43	2.62	14.34***	1.36	1.34	0.13	7.57*	1.59	1.57	1.15	9.42
high CRP	21.39	4.88	8.60	59.67***	11.31	5.16	4.94	26.90***	5.14	4.71	1.05	27.21*	4.02	3.77	0.72	22.57
H. pylori infection	4.20	2.85	2.06	9.03***	9.68	6.64	3.56	33.08***	1.69	1.66	0.35	10.77	0.27	0.28	0.03	1.53

* $P < 0.05$.
** $P < 0.001$.
*** $P < 0.0001$.

artery atherosclerosis than in the controls (univariate analysis showed OR: 11.31 and CI: 4.94–26.90 ($P < 0.0001$); OR: 5.14 and CI: 1.05–27.21 ($P < 0.05$), respectively) (Table 3).

4. Discussion

4.1. Main findings

In the present case-control study, *H. pylori* infection was associated with an increased risk of ischemic stroke due to small artery occlusion or large artery atherosclerosis versus a decreased risk of stroke caused by cardiogenic embolism. There was also a strong association between *H. pylori* infection and the overall risk of stroke. Moreover, we found no difference in the prevalence of *H. pylori* infection among patients with two different stroke subtypes (large artery and small artery stroke) and controls. Our study showed that the presence of *H. pylori* infection might be increased in patients with stroke that is due to large artery and small artery disease but not in patients with cardiogenic embolism.

Although CRP (a sensitive marker of systemic inflammation) was increased in both groups of stroke patients compared with control subjects, the *H. pylori*-positive patients showed significantly higher CRP levels than the *H. pylori*-negative patients.

4.2. Chronic H. pylori infection and stroke subtype

Our findings were consistent with the results of some previous studies that have addressed the relationship between chronic *H. pylori* infection and ischemic stroke.

Markus and Mendall [14] reported elevated levels of IgG antibody for *H. pylori* in patients with lacunar stroke, which is comparable to small artery occlusion [8]. The adjusted OR was 2.51 (95% CI: 1.19–5.28), which was compatible with our findings (OR: 9.68; 95% CI: 3.56–33.08). The classic lacunar hypothesis is that lipohyalinosis of small arteries caused by diabetes mellitus and hypertension represents the underlying pathogenesis of this stroke subtype [21]. In recent years, however, evidence has been obtained that lacunar infarcts also share the mechanisms involved in atherosclerotic disease [23]. Similar trends were demonstrated for stroke caused by large artery atherosclerosis in the study performed by Markus and Mendall [14] and the present study (adjusted OR: 2.17; 95% CI: 1.11–4.21 and adjusted OR: 1.69; 95% CI: 0.35–10.77, respectively), although the association did not reach statistical significance in our study, possibly because of the small number of patients with this stroke subtype.

Unfortunately, comparison of the role of *H. pylori* in cardioembolic stroke could not be performed between the two studies because Markus and Mendall [14] combined their data for stroke due to cardiogenic embolism and stroke due to undefined causes. Since cardioembolic stroke is mainly caused by disorders such as atrial fibrillation that lead to thromboembolic occlusion of the cerebral arteries [8], our

Table 4
Odds ratios for association of chronic *H. pylori* infection with ischemic stroke subtypes

	Unadjusted OR[a]			Adjusted OR[b]		
	OR	95% CI	*P* value	OR	95% CI	*P* value
Ischemic Stroke (all subtypes)	2.22	1.04–4.72	$P < 0.05$	2.57	1.09–6.08	$P < 0.05$
Stroke Subtypes						
Small artery occlusion	1.00		$P < 0.05$	1.00		$P < 0.05$
Large artery atherosclerosis	0.215	0.0430–1.07		0.215	0.0430–1.07	
Cardioembolic	0.137	0.0236–0.796		0.137	0.0236–0.796	

ORs and 95% CIs were calculated by conditional logistic regression analysis.
[a] Matched for age and sex.
[b] Matched for age, sex, smoking, hypertension, and diabetes.

findings are consistent with a positive association between chronic *H. pylori* infection and ischemic stroke caused by cerebral atherosclerosis. When stroke is defined as a non-homogeneous condition and stratified analysis of various stroke subtypes is performed, this often results in a small number of patients in each subgroup. Therefore, the lack of a significant association with large artery atherosclerosis needs further investigation in a larger population. Furthermore, it is possible that the apparent association of stroke with *H. pylori* infection was attributable to residual confounding factors related to socioeconomic status, although we tried to control them. This might be of particular relevance with regard to reports of *H. pylori* infection as a marker of lower socioeconomic status [24], which seems to be an independent risk factor for vascular disease [25].

4.3. Chronic H. pylori infection and mechanism of atherogenesis

In recent years, it has been hypothesized that only certain *H. pylori* strains, which express the cytotoxin-associated gene A (Cag A) encoding the Cag A protein, may have a link with atherosclerosis. Several mechanisms have been hypothesized, including inflammation, hyperhomocysteinemia, immune-mediated vascular damage, and direct bacterial invasion of atherosclerosis plaques [26]. Although we did not investigate antibody for Cag A protein, Maeda et al. have reported that most *H. pylori* strains in Japan are capable of producing Cag A protein [27]. Compared with *H. pylori*-negative patients, the *H. pylori*-positive patients showed more evidence of systemic inflammation (higher CRP levels), which gives some support to the hypothesis that *H. pylori* infection may induce generalized inflammation, a recognized risk factor for atherosclerosis [7]. The strong non-specific inflammatory response to acute tissue ischemia associated with stroke may well have minimized a pre-existing difference of chronic inflammation between the patients with and without *H. pylori* infection. Other mechanisms may also be suggested that could link infection with *H. pylori* to atherosclerotic stroke.

Amerisco et al. [26] recently reported that *H. pylori* might be present in carotid plaques because chronic *H. pylori* infection elicits a strong local inflammatory response; it is possible that the presence of bacteria may contribute to plaque instability and the onset of acute ischemic stroke through a local vascular effect.

In any case, our data suggest that infection with *H. pylori* represents a risk factor for the development of atherosclerotic stroke.

4.4. Limitations

The main limitation of the present study was its case-control design. Although care was taken to avoid potential biases, it is well known that prospective studies are often unable to confirm the associations detected in case-control studies, so further prospective studies are needed to confirm our findings.

In addition, the cross-sectional nature of the present study did not allow us to establish whether or not *H. pylori* played a causative role in atherosclerotic stroke. Furthermore, given the wide prevalence of *H. pylori* infection, it is possible that unknown factors having an independent association with both this organism and ischemic stroke may have produced a spurious association in our study. Finally, our control subjects were not representative of the general population and the enrollment criteria may have led to selection of a population with a low risk of *H. pylori* infection. However, it should be noted that the subjects with and without ischemic stroke showed almost identical rates of *H. pylori* infection.

5. Conclusion

The present study suggested that *H. pylori* infection might be associated with ischemic cerebrovascular disease due to an increased prevalence of this organism in patients with ischemic stroke. The pathophysiological mechanism underlying this association seems likely to be a chronic inflammatory response to bacterial infection.

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Original Article

Pravastatin Improves Insulin Resistance in Dyslipidemic Patients

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To evaluate the effect of pravastatin on both lipid and glucose metabolism, twenty-two consecutive dyslipidemic patients treated with pravastatin at 10 mg/day for one year were enrolled in this study. The meal test, which consisted of 115 g of cookies (energy 560 kcal; glucose 75 g; protein 7 g; fat 24 g), was conducted before and after one year of treatment. Insulin resistance was assessed by the homeostasis model assessment of insulin resistance (HOMA-IR), by the area under the IRI curve (AUC-IRI), and by the formula AUC-IRI × AUC-PG. After one year of treatment with pravastatin, the plasma glucose (PG), immunoreactive insulin (IRI) and C-peptide levels were unchanged after fasting and at 120 minutes after the meal test; however, PG, IRI and C-peptide levels at 60 minutes after the meal were all significantly decreased from baseline ($p < 0.05$). AUC-IRI and AUC-IRI × AUC-PG were also significantly decreased ($p < 0.05$). HOMA-IR was reduced by 26.8%, but the reduction was not significant. The triglyceride (TG) level was decreased after fasting and increased at 60 and 120 minutes after the meal test, but not significantly. This study demonstrated that pravastatin not only reduced serum lipids, but also improved the glucose metabolism, including insulin resistance, of dyslipidemic patients. *J Atheroscler Thromb, 2005; 12: 322–329.*

Key words: Statin, meal test, Postprandial change, Area under the IRI curve (AUC-IRI)

Introduction

Metabolic syndrome is a disorder in which insulin resistance is the main component of the etiology. In 1988, Reaven (1) defined syndrome X, which consists of insulin resistance, hyperinsulinemia, glucose intolerance, hypertriglycemia, decreased HDL-C and hypertension. He suggested that the major feature of the syndrome is insulin resistance, and that all other changes are likely to follow this abnormality. He also suggested that such a state might increase the risk of coronary artery disease (CAD). Several subsequent studies also revealed that insulin resistance increases the risk of CAD (2–4). Therefore, it is important to know whether or not the drugs used to treat these diseases have a secondary effect, either favorable or unfavorable, on insulin resistance.

Statins have multiple actions, independent of their classical effects on serum lipids. These actions include the modulation of endothelial function, the stabilization of plaques, an attenuation of atherogenesis, and anti-inflammatory and antithrombotic effects. However, research into the effect of statins on insulin resistance is inconclusive. Some reports indicate that statins worsen the insulin action (5), or have no effect on the plasma insulin level (6). Other reports indicate that statins improve insulin sensitivity (7–10). In the West of Scotland Coronary Prevention Study (WOSCOPS) (10), the incidence of type 2 diabetes mellitus was found to be thirty percent lower in pravastatin-treated patients than in controls, suggesting that the anti-inflammatory and endothelial effects of pravastatin, in addition to the lipid lowering effect, may have been a factor. This also implies that pravastatin

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might improve insulin sensitivity. To our knowledge, no previous reports have compared the effect of statins using a meal test to reveal postprandial change in both glucose and lipids.

The purpose of this study was to evaluate the effect of pravastatin on insulin resistance in a selected group of dyslipidemic patients.

Methods

Subjects and study design

The study included 32 (12 male, 20 female) consecutive dyslipidemic patients at the outpatient clinic of the Department of General Medicine, Kyushu University, between September 2001 and April 2002. The examination consisted of a general physical examination, a questionnaire, carotid ultrasound, and a meal test. Eligibility criteria included (a) 40 to 75 years; (b) BMI (body mass index) < 30 kg/m^2; (c) fasting total cholesterol (TC) ≥ 220 mg/dl and/ or fasting triglyceride levels between 150 and 350 mg/dl; (d) fasting plasma glucose (PG) < 126 mg/dl (e) plasma glucose < 200 mg/dl at 2 hours after the meal test; (f) no evidence of hypertension (systolic blood pressure < 140 mmHg, diastolic blood pressure < 90 mmHg, and no treatment with antihypertensive medications), and no renal, hepatic, endocrine, or cancer diseases or severe allergies as determined by medical history, physical examination, and routine laboratory tests; and (g) no history of taking any medicine known to affect glucose and lipid metabolism. The purpose, nature, and potential risk of the study were explained to all patients and written informed consent was obtained before enrollment. Of the potential subjects, ten were omitted because of withdrawal of consent, or ineligibility, leaving 22 patients (7 male, 15 female) who were followed up for one year.

After the baseline measurements were taken, the administration of pravastatin at 10 mg per day was begun. Monitoring visits were scheduled 4 weeks after the baseline data was gathered and every 2 months thereafter. At each visit, a brief physical examination was conducted and drug compliance was confirmed.

Meal test

After a 10- to-14- hour overnight fast, a meal test, similar to that conducted in numerous recent studies (11, 12), was done by all the patients between 8:30 and 9:30 AM. Ingested within 15 minutes, the test meal was a cookie (Abilit co., ltd, Osaka, Japan) consisting of the following: energy 560 kcal; carbohydrate 75 g; protein 7 g; fat 24 g (13). The amount of carbohydrate in this "cookie test" is equivalent to that (75 g) in the standard oral glucose tolerance test (OGTT) (Trelan- G, Shimizu Pharmaceutical, Shimizu, Japan). Patients were asked not to do any unusual exercise or drink alcohol the day before the test. Water was allowed, but no other beverages or foods were permitted during the test. Walking, but no strenuous exercise or smoking, was allowed. Blood samples were taken at 0, 60, and 120 minutes for the measurement of PG, immunoreactive insulin (IRI), C-peptide, and TG, at 0 and 120 minutes for TC and HDL-C, and in a fasting state for hs CRP and hemoglobin A_{1c} (HbA_{1c}).

Biochemical measurements

PG was measured by the glucose oxidase method and immunoreactive insulin (IRI) and C-peptide were measured by radioimmunoassay at a commercial laboratory (MBC Laboratories, Inc., Tokyo, Japan). TC, HDL-C, TG, and HbA1c were measured by standard laboratory techniques at the above laboratory. High-sensitivity CRP (hs CRP) was measured by high-sensitivity latex-enhanced immunonephelometics. LDL-C was calculated according to the Friedewald formula.

Assessment of insulin resistance

Homeostasis model assessment of insulin resistance (HOMA-IR), a widely advocated index that reflects insulin resistance, was calculated with the formula: HOMA-IR = [fasting PG (mg/dl) × fasting IRI (μU/ml]/405] (14). The area under the PG and IRI curves (AUC) during the meal test were estimated by the linear trapezoidal method. AUC-IRI, and AUC-IRI × AUC-PG are recommended for determining possible insulin resistance in non-diabetic subjects (15, 16), so, in addition to HOMA-IR, these two factors were used to evaluate insulin resistance.

Carotid ultrasound measurements

Both carotid arteries scanned with high-resolution B-mode ultrasound using a 7.5 MHz mechanical sector transducer on the Aloka SSD-2000 (Aloka co., Ltd., Tokyo, Japan) by four trained physicians as described previously (17–20). The maximum IMT was measured 2 to 3 cm proximal to the flow divider on the far wall of the right and left common carotid arteries at the end of the diastolic phase. All assessments of the carotid arteries were done blinded to a knowledge of the clinical history or risk factor profile.

An analysis of within- and between-reader (reading of a duplicate set of 25 scans) and -observer (duplicate mean IMT measurements of five subjects) variability was done (20). The Spearman correlation coefficients for intra-observer and intra-reader measurements were > 0.95, and the mean differences (± 2 SD) were < 1% (10%). The Spearman correlation coefficients for between-observer and between-reader variability were > 0.95 and > 0.95, respectively, and the mean differences (± 2 SD) were < 5% (15%).

Statistical analysis

All data were reported in standardized forms, which were

then entered into a database. Categorical variables among the groups were assessed using the chi-square test or Fisher's exact test. The mean levels of variables between two groups were compared with the unpaired *t*-test or Mann-Whitney *U*-test. Comparisons between more than two groups were made using the Kruskal-Wallis test. Differences in BMI, HbA_{1c}, blood pressure, serum lipids, hs CRP, max IMT, PG, IRI, C-peptide, AUC-IRI, AUC-IRI × AUC-PG, and HOMA-IR between baseline and after 12 months were compared using the paired *t*-test or Wilcoxon's signed-ranks test. Between-group changes in each variable from 0 to 60 min and from 0 to 120 min from baseline to 12 months were compared using the Bonferroni correction method. Percent change was calculated with the following formula: % change = (value at 12 months – baseline value) / baseline value × 100. *P* values < 0.05 were considered statistically significant in all analyses.

Table 1. Baseline characteristics of the 22 study subjects

Characteristic	
Age (years)	58.0 ± 9.2
Sex (males/females)	10 /12
Body mass index (kg/m2)	25.4 ± 2.4
Blood pressure (mmHg)	
Systolic	119.8 ± 8.1
Diastolic	70.2 ± 8.2
Medical history (%)	
Hypertension	0 (0)
Diabetes mellitus	0 (0)
Cardiovascular disease	1 (4.5)
Cerebrovascular disease	0 (0)

Data represents the mean value ± S.D. or number (%) of subjects.

Results

Baseline characteristics

The baseline characteristics of the study subjects are shown in Table 1. The mean age ± S.D. of the patients was 58.0 ± 9.2 years. Of the 22 patients, 10 were men (45.5%). The average BMI was 25.4 ± 2.4 kg/m^2. The average systolic and diastolic blood pressures were 119.8 ± 8.1 and 70.2 ± 8.2 mmHg, respectively. Of the 22 patients (n = 1, 4.5%) had a history of cardiovascular disease, and none had a history of hypertension, diabetes mellitus, or cerebrovascular disease.

Change in variables from baseline to the 12 month follow-up

The changes in BMI, HbA_{1c}, blood pressure, serum lipids, high-sensitivity CRP, and maximum IMT are shown in Table 2.

No significant change in BMI, HbA_{1c}, blood pressure, TG, or maximum IMT was found from baseline to the 12 month follow-up. TC levels decreased after 12 months, falling by 13.6% in a fasting state ($p < 0.001$) and 12.7% ($p < 0.001$) at 120 minutes after the meal test. LDL-C levels also decreased after 12 months, falling by 23.3% in a fasting state ($p < 0.001$) and 26.4% ($p < 0.001$) at 120 minutes after the meal test. HDL-C levels significantly increased, by 8.5% in a fasting state ($p = 0.030$) and 8.9% at 120 minutes ($p = 0.033$). A significant decrease (46.1%) in the fasting state hs CRP was found after 12 months ($p = 0.048$).

Change in glucose metabolism and insulin sensitivity from baseline to the 12 month follow-up

PG, IRI, and C-peptide levels in response to the "cookie test" at baseline and after 12 months of pravastatin treatment are shown in Table 3. These results showed that the fasting PG, IRI, and C-peptide levels did not change, even after the patients were treated with pravastatin. However, the PG, IRI, and C-peptide levels at 60 minutes in response to the cookie test were all significantly decreased after treatment: PG, from 171.1 mg/dl to 152.6 mg/dl (– 10.8%), $p = 0.017$; IRI, from 66.8 mg/dl to 50.3 mg/dl (– 24.7%), $p < 0.035$; and C-peptide, from 8.52 mg/dl to 7.10 mg/dl (– 16.7%), $p = 0.019$. PG, IRI, and C-peptide levels at 120 minutes in response to the test were decreased after treatment, but not significantly. The change in PG levels from 0 to 60 min was significantly geater at baseline than at 12 months (72.2 mg/dl vs. 49.6 mg/dl, $p = 0.004$), but the change from 0 to 120 min did not differ between baseline and the 12 month follow up. The change in IRI levels from 0 to 60 min was significantly greater at baseline than at 12 months (55.5 mg/dl vs. 41.6 mg/dl, $p = 0.098$), but again that from 0 to 120 min did not differ between baseline and 12 months. The change in C-peptide levels from 0 to 60 min was significantly greater at baseline than at 12 months (6.0 mg/dl vs. 4.7 mg/dl, $p = 0.019$), but once again the change from 0 to 120 min did not differ significantly between baseline and 12 months.

Insulin resistance, as indicated by HOMA-IR, was reduced after 12 months of pravastatin treatment, from 2.88 to 2.11 (– 26.7%), although the reduction was not significant (Fig. 1). AUC-IRI was significantly decreased after the treatment, from 99.5 to 77.9 (– 21.7%) ($p = 0.020$) (Fig. 2). AUC-IRI × AUC-PG was also significantly decreased after 12 months of pravastatin treatment, from 29215.5 to 21478.6 (– 26.5%) ($p = 0.018$) (Fig. 2).

Discussion

This study evaluated the effect of pravastatin on both

Table 2. Changes in the tested variables from baseline to 12 months of follow-up.

	Baseline mean ± S.D.	12 months mean ± S.D.	Percentage change (%)	*p*-value
BMI (kg/m^2)	25.4 ± 2.4	25.5 ± 2.7	0.3	0.189
HbA_{1c} (%)	5.15 ± 0.37	5.27 ± 0.46	2.3	0.383
Blood pressure				
Systolic	119.8 ± 8.1	128.4 ± 15.4	7.2	0.188
Diastolic	70.2 ± 8.2	75.2 ± 8.5	7.1	0.250
Serum lipids				
TC (mg/dl)				
0 min	226.3 ± 30.1	195.5 ± 26.5	− 13.6	< 0.001
120 min	221.6 ± 33.0	193.5 ± 25.3	− 12.7	<0.001
change from 0 to 120 min	− 4.7 ± 7.1	− 2.4 ± 5.0		0.101
LDL-C (mg/dl)				
0 min	144.5 ± 28.0	110.8 ± 20.3	− 23.3	< 0.001
120 min	135.2 ± 26.0	99.5 ± 18.9	− 26.4	< 0.001
change from 0 to 120 min	− 9.3 ± 7.3	− 11.6 ± 8.5		0.318
HDL-C (mg/dl)				
0 min	56.2 ± 10.5	61.0 ± 13.1	8.5	0.030
120 min	54.0 ± 10.4	58.8 ± 12.3	8.9	0.033
change from 0 to 120 min	− 2.3 ± 2.2	− 2.4 ± 2.7		0.881
TG (mg/dl)				
0 min	127.8 ± 65.7	118.5 ± 56.0	− 7.3	0.494
60 min	151.0 ± 63.4	145.0 ± 69.7	− 4	0.540
change from 0 to 60 min	23.3 ± 45.1	27.0 ± 34.5		0.680
120 min	162.4 ± 58.5	175.8 ± 72.6	8.3	0.293
change from 0 to 120 min	34.6 ± 48.5	57.8 ± 39.0		0.063
high-sensitivity CRP (mg/dl)	0.13 ± 0.15	0.07 ± 0.06	− 46.1	0.048
maximum IMT (mm)	1.47 ± 0.92	1.42 ± 0.83	− 3.4	0.220

BMI: body mass index, HbA_{1c}: hemoglobin A_{1c}, TC: total cholesterol, LDL-C: LDL- cholesterol, HDL-C: HDL-cholesterol, TG: triglyceride, CRP: C-reactive protein, IMT: intima-media thickness.

lipid and glucose metabolism in dyslipidemic patients. We used a new meal test that provides a natural source of carbohydrates and lipids that is more similar to our daily food intake than the regular OGTT (13). This test, using the same criteria as OGTT, can evaluate glucose tolerance in subjects without endocrine pancreatic diseases, and also reveals hyperinsulinemia, insulin resistance and postprandial dyslipidemia more effectively than an OGTT or fat loading test. In addition, while 30% of the healthy subjects tested showed reactive hypoglycemia (2h PG below 80 mg/dl) in response to OGTT, none showed hypoglycemia or adverse effects to this meal test (13). Our study demonstrated that pravastatin can improve both lipid and glucose metabolism.

Large-polulation studies of the effect of statin administration on glucose metabolism have produced conflicting results (10, 21). In the WOSCOPS report (10), the incidence of type 2 diabetes mellitus was found to be thirty percent lower in pravastatin-treated patients than in controls; by contrast, the Anglo-Scandinavian Cardiac Out-

Table 3. Change in glucose metabolism from baseline to12 months of follow-up.

	Baseline mean ± S.D.	12 months mean ± S.D.	Percentage change (%)	*p*-value
PG (mg/dl)				
0 min	98.9 ± 11.3	101.6 ± 13.3	2.7	0.137
60 min	171.1 ± 47.3	152.6 ± 41.6	− 10.8	0.017
change from 0 to 60 min	72.2 ± 39.5	49.6 ± 30.9		0.004
120 min	137.9 ± 42.0	139.0 ± 36.9	0.8	0.654
change from 0 to 120 min	39.0 ± 34.6	36.2 ± 27.7		0.775
IRI (mg/dl)				
0 min	11.3 ± 12.3	8.3 ± 4.7	− 26.5	0.291
60 min	66.8 ± 37.2	50.3 ± 26.3	− 24.7	0.035
change from 0 to 60 min	55.5 ± 37.9	41.6 ± 22.3		0.098
120 min	54.2 ± 27.7	46.8 ± 30.8	− 13.7	0.235
change from 0 to 120 min	42.8 ± 26.6	37.9 ± 27.7		0.487
C-peptide (mg/dl)				
0 min	2.6 ± 0.8	2.4 ± 0.9	− 7.8	0.230
60 min	8.5 ± 2.2	7.1 ± 3.0	− 16.7	0.019
change from 0 to 60 min	6.0 ± 2.1	4.7 ± 2.5		0.026
120 min	8.7 ± 2.1	7.9 ± 2.4	− 9.5	0.124
change from 0 to 120 min	6.2 ± 2.3	5.5 ± 2.0		0.253

PG: plasma glucose, IRI: immunoreactive insulin

comes Trial-Lipid Lowering Arm (ASCOT-LLA) (21) revealed the incidence of diabetes to be significantly higher in an atorvastatin group than in a placebo group.

An important effect of statins on glucose metabolism is the anti-inflammatory effect. Pravastatin has been shown to reduce circulating levels of interleukin-6 (IL-6) and TNF-α (22). It has been assumed that these cyotokines, derived in part from adipose tissue, may be responsible for the metabolic syndrome associated with insulin resistance (23). Hs CRP, an inflammatory marker, has been reported to be a strong independent predictor of risk of future myocardial infarction (24). A nested, case-control study in the secondary-prevention Cholesterol and Recurrent Events (CARE) trial (25) showed that the relative risk of recurrent events was reduced to a greater degree in pravastatin versus placebo-administered patients who had elevated levels of hs CRP that in pravastatin versus placebo-administered patients with normal hs CRP levels. These results support the idea that statins have an inflammatory effect which results in a reduction in the incidence of cardiovascular events. In our study, a significant decrease in hs CRP, by 46.1%, was found after 12 months of pravastatin treatment. This decrease may have been the cause of the reduction in insulin resis-

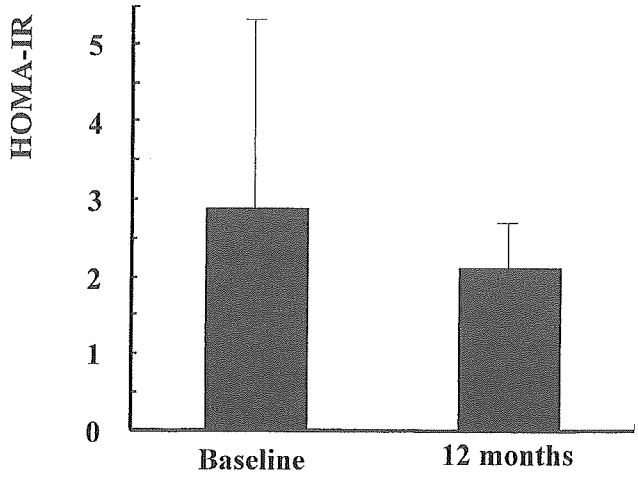

Fig. 1. Change in HOMA- IR over the 12 months of pravastatin treatment.