INFLUENCE OF HMG-COA REDUCTASE INHIBITORS ON PLAQUE-FORMING CELL (PFC) RESPONSE IN MICE

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ABSTRACT — Effects of pravastatin and simvastatin on the humoral immune response were studied using Cunningham’s method of the IgM plaque-forming cell (PFC) assay system in mice. The vehicle (0.5% CMC), pravastatin (1 mg/body : about 50 mg/kg) or simvastatin (0.5 mg/body : about 25 mg/kg) were given orally for 14 days in experiment(Exp)-I and Exp-II. The vehicle, pravastatin (0.5 mg/body : about 25 mg/kg) or simvastatin (0.25 mg/body : about 12.5 mg/kg) were given orally 29 times for 32 days in Exp-III. There were no significant differences in the mean numbers of PFCs per 10^6 splenocytes between the pravastatin groups and the vehicle groups. In the simvastatin groups, however, there were significant reductions of the numbers of PFCs in Exp-I, Exp-II and Exp-III, as compared with the vehicle group. Based on these results, it was considered that suppressive effect on IgM antibody production was induced by simvastatin, whereas such a suppression was not observed with pravastatin.

KEY WORDS : HMG-CoA reductase inhibitor, Pravastatin, Simvastatin, Immunotoxicity, Plaque-forming cell (PFC), BALB/c mouse.

INTRODUCTION

Pravastatin and simvastatin, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, have been used for the therapy of hypercholesterolemia. It is desirable for these inhibitors to act selectively on the target organ, the liver, to allow normal cellular functions in non-hepatic tissue such as the spleen, and to minimize potential side effects. It has been reported that the relative hydrophilicity of pravastatin, due to its 6'-hydroxy group, is related to its hepatocyte selectivity (Watanabe, 1990). Komai and Tsujita (1994) reported that pravastatin was distinguished from the hydrophobic simvastatin with respect to its selective inhibition of cholesterol synthesis in the liver, and this tissue selectivity of pravastatin could be ascribed to its less efficient uptake by tissues other than the liver such as spleen and testis. Recently, there have been a number of attempts to establish methods for the evaluation of immunotoxicity of drugs such as anticancer agents with respect to two
major components, humoral immunity and cell-mediated immunity. PFC assay is a suitable method which is able to evaluate the humoral immunity from the recognition of antigen to the production of antibody by counting the antibody-forming cells in the spleen following immunization with sheep red blood cells (SRBC) (Maki et al., 1989; Doi et al., 1992). The present experiments were undertaken to clarify the difference of the effects of pravastatin and simvastatin on the humoral immune response using IgM PFC assay system of murine splenocytes from an immunotoxicological point of view.

MATERIALS AND METHODS

Test substances: Pravastatin (lot No.: Q039) and simvastatin (lot No.: SN4-6-2) synthesized by Sankyo Co., Ltd. were used in the present experiments as test substances. Distilled water solution of sodium carboxymethylcellulose (CMC, lot No.: STN1487, Wako Pure Chemical Industries, Ltd.) was used as a vehicle control at the concentration of 0.5%.

Animals: BALB/c mice (female, 7 weeks old) were purchased from Japan SLC, Inc. In each group, six animals were housed together in S-cages (Clea Japan, Inc.) having Clean Chip (Clea Japan, Inc.) in an animal room, where temperature, humidity, lighting and ventilation were respectively controlled using an environment controlling equipment Ebac (Clea Japan, Inc.) at 23±3°C, 55±15%, 12 hr (7:00-19:00)/day with a luminous intensity of about 200 luxes, and about 40 times/hr. They had free access to solid food NMF (Oriental Yeast Co., Ltd.) and tap water.

Materials: After 4.7 g of Eagle’s minimum essential medium (EMEM) powder (Nissui Pharmaceutical Co., Ltd.) was dissolved in 500 ml of distilled water, this solution was autoclaved at 121°C for 15 min. The pH was then adjusted to about 7.2 with a 10% solution of sodium bicarbonate. L-glutamine was added to the EMEM to give a final concentration of 0.03% just before use.

Fetal calf serum (FCS, Life Technologies, Inc.) was added to the RPMI medium 1640 (Life Technologies, Inc.) to give a final concentration of 10% [10% FCS-RPMI medium].

SRBC were obtained from sheep reared at the Laboratory Animal Science and Toxicology Laboratories, Sankyo Co., Ltd. SRBC for sensitization and PFC assay were washed with a 0.9% saline solution and EMEM, respectively, and then centrifuged twice at 3,000 rpm for 15 min.

Grouping: According to the test substances, dosages and times of administration, 3 groups, each of which consists of 6 animals, were set up in Exp-I, Exp-II and Exp-III. Details of each group are described below:

Exp-I: Group 1, Vehicle (0.5% CMC)×14;
Group 2, Pravastatin 1 mg/body×14;
Group 3, Simvastatin 0.5 mg/body×14

Exp-II: Group 4, Vehicle (0.5% CMC)×14;
Group 5, Pravastatin 1 mg/body×14;
Group 6, Simvastatin 0.5 mg/body×14

Exp-III: Group 7, Vehicle (0.5% CMC)×29;
Group 8, Pravastatin 0.5 mg/body×29;
Group 9, Simvastatin 0.25 mg/body×29

Dosage: The toxicities of pravastatin were evaluated in the male and female F344 rats administered orally for 13 weeks followed by 4 weeks withdrawal at doses of 0.8, 4, 20, 100 and 500 mg/kg/day in the previous toxicological studies (Kimura et al., 1987). There were no significant toxicological changes in hematological or serum biochemical data, gross necropsy observations, weight of organs or histopathological observations in any of the animals of either sex at the end of the 13 weeks’ treatment and 4 weeks’ withdrawal periods. Based on the above toxicological data and clinical dose (0.2–0.4 mg/kg) of pravastatin, a dose 100–200 times that of the clinical dose, 0.5 mg/body (about 25 mg/kg) or 1 mg/body (about 50 mg/kg) was used. Simvastatin was administered at a dose of 0.25 mg/body (about 12.5 mg/kg) or 0.5 mg/body (about 25 mg/kg) based on the clinical dose (0.1–0.2 mg/kg).

Preparation of test samples: Solutions of pravastatin at 0.5% and 0.25% in distilled water were prepared and used in Groups 2, 5 and 8, respectively. Suspensions of simvastatin at 0.25% and 0.125% in 0.5% CMC solution were prepared and used in Groups 3, 6 and 9, respectively. These samples were prepared once a
Effects of pravastatin and simvastatin on PFC response.

week and stored in a refrigerator. An administration volume was fixed to be 0.2 ml/body.

**Schedules of Exp-I and Exp-II**: Animals in Groups 1, 2 and 3 of Exp-I were daily treated per oral with 0.5% CMC (0.2 ml/body), pravastatin at 1 mg/body and simvastatin at 0.5 mg/body from day 0 to day 13, respectively. Animals were immunized intraperitoneally with $1 \times 10^8$ of SRBC on day 10 and PFC assay was performed on day 14. The same schedule as in Exp-I was repeated in Exp-II (Groups 4, 5 and 6).

**Schedule of Exp-III**: Animals in Groups 7, 8 and 9 were daily treated per oral with 0.5% CMC (0.2 ml/body), pravastatin at 0.5 mg/body and simvastatin at 0.25 mg/body, respectively, 29 times for 32 days. Animals were immunized intraperitoneally with $2 \times 10^8$ of SRBC on day 32 and PFC assay was performed on day 36.

**Body weight**: The animals were weighed using an electrobalance (EB-3200D, Shimazu Corporation) on day 0 and 14 in Exp-I or Exp-II. According to the same manner, the animals in Exp-III were weighed on day 0 and 36.

**Measurement of weights of thymus and spleen and determination of the number of PFCs**: The PFC assay was conducted according to the method of Cunningham and Szenberg (1968). Mice were sacrificed by exsanguination on day 14 in Exp-I and Exp-II or on day 36 in Exp-III, and spleen and thymus were removed. After their wet weights were measured, the spleen was put into a Petri dish filled with chilled 10% FCS-RPMI medium and its capsule was broken by carefully pressing it with forceps in order to release splenocytes from it. Released splenocytes were transferred to a centrifuge tube and washed twice by centrifugation (950 rpm, 8 min) at 4°C. The pellets were resuspended with 2 ml of 10% FCS-RPMI medium. The number of splenocytes was counted with a hemocytometer after trypan blue staining.

SRBC were washed twice with EMEM, and a 40% suspension was prepared in EMEM.

A 400 μl portion of each splenocyte suspension, 50 μl of the 40% SRBC and 50 μl of guinea pig serum were mixed in a test tube, and 100 μl of the mixture was put in a Cunningham’s chamber (Takahashi Giken Glass Co., Ltd.). The chamber was sealed with parafilm and incubated at 37°C for 1 hr. After incubation, PFCs were counted under a microscope.

**Statistical analysis**: Data on the number of PFCs were analyzed by use of the Student’s t-test.

**RESULTS AND DISCUSSION**

The results of body weights, thymus and spleen weights in mice treated orally with pravastatin or simvastatin in Exp-I, Exp-II and Exp-III are shown in Tables 1, 2 and 3, respectively. There were no abnormalities in body weights, thymus and spleen weights in any group of Exp-I, Exp-II and Exp-III. There was a mild decrease in thymus weight of the mice given 0.5 mg/body of simvastatin (Group 6), but no significant difference from that of the vehicle control (Group 4).

The numbers of PFCs of mice given either

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**Table 1. Body weights, thymus and spleen weights in BALB/c mice treated orally with pravastatin or simvastatin in Exp-I.**

<table>
<thead>
<tr>
<th>Group &amp; Compound</th>
<th>Dose (mg/body)</th>
<th>No. of mice</th>
<th>Body Weight (g)</th>
<th>Thymus (mg)</th>
<th>Spleen (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 0</td>
<td>Day 14</td>
<td></td>
</tr>
<tr>
<td>Group 1:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle (0.5% CMC)</td>
<td>0</td>
<td>6</td>
<td>22.4 ± 0.83</td>
<td>22.0 ± 0.92</td>
<td>48.5 ± 6.2</td>
</tr>
<tr>
<td>Group 2:</td>
<td>1</td>
<td>6</td>
<td>22.5 ± 1.17</td>
<td>22.4 ± 0.81</td>
<td>44.3 ± 5.6</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>0.5</td>
<td>6</td>
<td>21.7 ± 0.90</td>
<td>21.1 ± 0.49</td>
<td>45.2 ± 7.5</td>
</tr>
<tr>
<td>Simvastatin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) Each value is the mean ± S.D.
pravastatin or simvastatin are shown in Tables 4, 5 and 6. There were no changes in the mean numbers of PFCs per $10^6$ splenocytes in the pravastatin groups compared to those in the vehicle group in Exp-I, Exp-II or Exp-III. On the other hand, there was a significant reduction of the numbers of PFCs in the simvastatin groups in Exp-I, Exp-II and Exp-III, as compared with those in the vehicle and pravastatin groups.

According to the study of Tsujita et al. (1986), pravastatin inhibited strongly the sterol synthesis in cell-free enzyme systems prepared from the rat liver and in freshly isolated rat hepatocytes. However, such an inhibition by pravastatin was much less in the cells from non-hepatic tissues such as freshly isolated rat splenocytes. Koga et al. (1990) reported that about 90% inhibition of cholesterol synthesis was observed in the liver, but the inhibition was less than 14% in the spleen when pravastatin at a dose of 20 mg/kg was orally administered to mice. Simvastatin did not show such a tissue-selective inhibition of sterol synthesis under the same conditions. In these respects, it was reported that pravastatin inhibited de novo sterol synthesis in the liver but minimally in the spleen and testis, whereas simvastatin inhibited the synthesis in all three tissues in rats after a single oral administration (25 mg/kg) of these drugs (Koga et al., 1992).

Thus, the results obtained in the present study might be related to the difference of the tissue-selective uptake for two drugs, pravastatin and simvastatin. Since it was not delineated which of two lymphocytes, B and T cells, was

Table 2. Body weights, thymus and spleen weights in BALB/c mice treated orally with pravastatin or simvastatin in Exp-II.

<table>
<thead>
<tr>
<th>Group &amp; Compound</th>
<th>Dose (mg/body)</th>
<th>No. of mice</th>
<th>Body Weight (g) a)</th>
<th>Thymus a)</th>
<th>Spleen a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 0</td>
<td>Day 14</td>
<td></td>
</tr>
<tr>
<td>Group 4: Vehicle (0.5% CMC)</td>
<td>-</td>
<td>6</td>
<td>22.4 ± 0.94</td>
<td>22.6 ± 0.67</td>
<td>47.5 ± 7.1</td>
</tr>
<tr>
<td>Group 5: Pravastatin</td>
<td>1</td>
<td>5 b)</td>
<td>21.6 ± 1.35</td>
<td>21.8 ± 0.79</td>
<td>44.4 ± 6.2</td>
</tr>
<tr>
<td>Group 6: Simvastatin</td>
<td>0.5</td>
<td>4 b)</td>
<td>21.4 ± 0.94</td>
<td>21.6 ± 0.85</td>
<td>33.5 ± 11.9</td>
</tr>
</tbody>
</table>

a): Each value is the mean ± S.D.
b): Data of one mouse in Group 5 and two mice in Group 6 were not employed, since SRBC-sensitization was a failure in these animals.

Table 3. Body weights, thymus and spleen weights in BALB/c mice treated orally with pravastatin or simvastatin in Exp-III.

<table>
<thead>
<tr>
<th>Group &amp; Compound</th>
<th>Dose (mg/body)</th>
<th>No. of mice</th>
<th>Body Weight (g) a)</th>
<th>Thymus a)</th>
<th>Spleen a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 0</td>
<td>Day 36</td>
<td></td>
</tr>
<tr>
<td>Group 7: Vehicle (0.5% CMC)</td>
<td>-</td>
<td>6</td>
<td>20.9 ± 0.69</td>
<td>21.4 ± 0.50</td>
<td>45.3 ± 10.9</td>
</tr>
<tr>
<td>Group 8: Pravastatin</td>
<td>0.5</td>
<td>6</td>
<td>20.6 ± 0.82</td>
<td>22.4 ± 0.72</td>
<td>49.8 ± 6.9</td>
</tr>
<tr>
<td>Group 9: Simvastatin</td>
<td>0.25</td>
<td>6</td>
<td>19.9 ± 0.28</td>
<td>21.0 ± 0.97</td>
<td>45.1 ± 6.0</td>
</tr>
</tbody>
</table>

a): Each value is the mean ± S.D.
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Table 4. Effects of pravastatin and simvastatin on plaque-forming cells (PFCs) of splenocytes in BALB/c mice in Exp-I.

<table>
<thead>
<tr>
<th>Group &amp; Compound</th>
<th>No. of animals</th>
<th>Number of PFCs per 10^6 splenocytes (Mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1: Vehicle (0.5% CMC) (po)</td>
<td>6</td>
<td>570 ± 165</td>
</tr>
<tr>
<td>Group 2: Pravastatin 1 mg/body (po)</td>
<td>6</td>
<td>672 ± 330</td>
</tr>
<tr>
<td>Group 3: Simvastatin 0.5 mg/body (po)</td>
<td>6</td>
<td>369 ± 197</td>
</tr>
</tbody>
</table>

* : Significant at 5% level compared with the pravastatin group.
** : Significant at 5% level compared with the vehicle (control) group.

Table 6. Effects of pravastatin and simvastatin on plaque-forming cells (PFCs) of splenocytes in BALB/c mice in Exp-III.

<table>
<thead>
<tr>
<th>Group &amp; Compound</th>
<th>No. of animals</th>
<th>Number of PFCs per 10^6 splenocytes (Mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 7: Vehicle (0.5% CMC) (po)</td>
<td>6</td>
<td>1357 ± 222</td>
</tr>
<tr>
<td>Group 8: Pravastatin 0.5 mg/body (po)</td>
<td>6</td>
<td>1148 ± 296</td>
</tr>
<tr>
<td>Group 9: Simvastatin 0.25 mg/body (po)</td>
<td>6</td>
<td>695 ± 167</td>
</tr>
</tbody>
</table>

* : Significant at 1% level compared with the pravastatin group.
** : Significant at 1% level compared with the vehicle (control) group.

responsible for the above effects of the drugs on PFC assay system, influence of pravastatin and simvastatin on responses of B and T cell mitogens will be examined to clarify this point using human or animal lymphocytes in vitro.

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