Effects of Simvastatin on Alveolar Regeneration and Its Relationship to Exposure in Mice with Dexamethasone-Induced Emphysema

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In the present study, the relationship between systemic exposure of simvastatin (SV) hydroxy acid (SV-acid), an active form of SV, and its alveolar regeneration rates was investigated using emphysema model mice created by postnatal treatment of dexamethasone. In a model with young animals, the mice were treated with SV for 10 d from postnatal day 42. Similar alveolar regeneration with a % mean linear intercept ($L_m$) recovery of 60 to 70% by histochemical observation was observed in mice in intraperitoneal administration at dose in the range of 4–100 µg/mouse. The % $L_m$ recovery after oral administration of 20 µg/mouse was comparable with that after intraperitoneal administration at a dose of 4 µg/mouse, when their exposure of SV-acid was almost similar in both treated groups. Regardless of the route of administration, the recovery can depend on the exposure level of SV-acid, and to the maximum was about 60–70%. On the other hand, in a model with adult animals, the mice were intraperitoneally administrated SV at a dose of 4 µg/mouse for 10 d from postnatal day 152. Compared to young animals, less % $L_m$ recovery was observed in adult mice even their systemic exposures of SV-acid were similar.

Key words simvastatin; pharmacokinetics; alveolar regeneration; mouse

Emphysema is one of the two major symptoms of chronic obstructive pulmonary disease (COPD), and is characterized by irreversible destruction of the alveolar walls caused by chronic exposure to toxic substances such as cigarette smoke and polluted air. In consequence, since alveoli are enlarged abnormally and their surface area is decreased, the gas exchange capacity is markedly reduced. Therefore, pulmonary function impairment develops with some symptoms such as cough, abnormal sputum, shortness of breath and/or difficulty breathing. In general, once alveoli are damaged, it is extremely difficult for them to recover. As one of the main reasons, there is a very slow turn-over of elastin, which is an essential component of alveoli for their elasticity. At the present time, only symptomatic therapies such as bronchodilator and expectorant agents and/or oxygen inhalation as supportive care are available. Thus, development of a basic remedy for emphysema is strongly desired.

It has been reported that regeneration of alveoli can be induced in rodents by treatment with some compounds, such as adrenomedullin,1) granulocyte-colony stimulating factor,2) emphysema is strongly desired. However, there are few reports demonstrating the relationship between the alveolar regeneration by SV and exposure levels in plasma and lungs. Thus we cannot estimate whether SV would be useful clinically for the treatment of emphysema at the approved dose and dosing route.

In this study, using the dexamethasone (Dex)-induced model of emphysema in mice, we revealed the relationship between the regeneration rate and pharmacokinetics of SV. In particular, the association between the doses and routes of administration and the regeneration were investigated, and also effects in young and adult mice were compared.

MATERIALS AND METHODS

Chemicals and Reagents

SV was purchased from Tokyo Chemical Industries, Ltd. (Tokyo, Japan). SV-acid ammonium salt, Dex and lovastatin hydroxy acid (LV-acid) sodium salt were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents and solvents in this study were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents and solvents in this study were obtained commercially and were either extra pure, molecular biology or biochemical grade.

Animals

Pregnant ICR mice (day 14 of gestation) were obtained from CLEA Japan, Inc. (Tokyo, Japan). Their male offspring were used for alveolar regeneration experiments, and postnatal day 1 (P1) was defined as the first day after birth, consistent with our previous study about the effects of all-trans retinoic acid on alveolar regeneration.13,14) Male ICR mice (6 weeks old) were obtained also from the same breeder and used for pharmacokinetic (PK) experiments after 5 or

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more days of acclimatization. All mice were housed in a temperature- (23±1°C) and humidity- (55±5%) controlled room with 12-h light/dark cycle. Water and food were available ad libitum for all experiments except for the PK experiment as described below. The protocols were approved by the institutional review committee in the Tokyo University of Science as animal experiment protocols No. Y14005 and Y14036. All experimental animals were handled in accordance with the institutional and national guidelines for the care and use of laboratory animals.

**Preparation of the Drug Solutions** A solution of 20 µg per milliliter of Dex for the intraperitoneal (i.p.) administration was prepared as described previously by Kamei et al. Briefly Dex was dissolved in dimethylsulfoxide and diluted with phosphate buffered saline (pH 7.4) up to 200-fold. All of the bottles and tubes containing Dex were covered by aluminum foil to prevent exposure to light and were stored at 4°C until use. SV solution for intraperitoneal and oral administration was prepared as follows; SV was dissolved in ethanol to a final concentration of 50 mg/mL, and diluted with peanut oil to 1.0, 0.2, and 0.04 mg/mL. All of the formulations containing SV were prepared just before use.

**Creation of Dex-Induced Model of Emphysema in Mice** According to our previous reports, newborn male mice in the emphysema group were subcutaneously administered Dex solution at a dose of 0.4 µg/mouse (20 µL injection) from P3 to P14 daily with a 2-d break on P8 and P9. Only vehicle was administered to mice in the control group.

**Alveolar Regeneration Experiments**

**Dose Proportionality**
Mice with induced emphysema were allocated to 4 groups (n=4 in each group), designated 4-SV, 20-SV, 100-SV, and Dex groups. The mice in the 4-SV, 20-SV, and 100-SV groups were i.p. administered SV at doses of 4, 20 and 100 µg/mouse/d, respectively, from P42 to P53 daily with a 2-d break on P47 and P48. Only vehicle was administered to mice in the Dex and control (not induced emphysema, n=4) groups. At P90 the lungs were isolated from the mice after euthanasia by exsanguination under isoflurane anesthesia, and were used for the evaluation of alveolar regeneration.

**Effect of Administration Route**
Mice with induced emphysema were allocated to 4 groups (n=5 in each group), designated 4-SV-i.p., 4-SV-per os (p.o.), 20-SV-p.o. and Dex groups. The mice in the 4-SV-i.p., 4-SVp.o. and 20-SV-p.o. groups were administered SV at the doses of 4 µg/mouse/d i.p., 4 and 20 µg/mouse/d orally, respectively, from P42 to P53 daily with a 2-d break on P47 and P48. Only vehicle was administered to mice in the Dex and control (n=5) groups. At P90 (37 d after completion of SV treatment) the lungs were isolated as described above.

**In Adult Mice**
Mice with induced emphysema were allocated to 2 groups (n=4 in each group), designated 4-SV and Dex groups. The mice in 4-SV group were administered SV at a dose of 4 µg/mouse/d i.p. from P152 to P163 daily with a 2-d break on P157 and P158. Only vehicle was administered to mice in Dex and control (n=4) groups. At P200 (37 d after completion of SV treatment) the lung was isolated as described above.

**Chronological Changes of Alveoli** New born mice (male) were allocated into 3 groups: control (n=10), Dex (n=10), and SV (n=20). The creation of Dex-induced model of emphysema and the treatment with SV (4 µg/mouse, i.p.) were conducted in the same manner as described above. At P38, the lungs were isolated from 5 mice each in the control (n=4) and Dex groups as described above. At P48, 55, and 63, the lungs were also isolated from 5 mice in SV group at each time point.

**PK Experiments** Male mice (6 weeks old) were allocated into 3 groups (n=6 in each group). The mice in each group were administered SV intraperitoneally at a dose of 4 µg/mouse or orally at doses of 4 or 20 µg/mouse. In addition, 5 mice (21 weeks old) were intraperitoneally administered SV solution at a dose of 4 µg/mouse. At 0 (predose), 15, 30, 45, 60, 90, 120, 240, and 360 min after the administration, blood was collected from the tail vein with a hematocrit tube. Blood samples were centrifuged at 3000×g at 4°C for 10 min to obtain plasma. The plasma was frozen at −80°C until the measurement of SV-acid concentration, which was conducted within a week after collecting the samples.

**Histopathological Analyses** Lung sections were prepared and alveolar mean linear intercept (Lm) was calculated according our previous report. The Lm was calculated from the images of the sections using image analyses software, Image J (National Institutes of Health, U.S.A.), as an index of the distance between alveolar walls, according to previous study by Dunnill. The % Lm recovery was calculated as an index of alveolar regeneration by SV using following equation as described previously; % of Lm recovery=[(Lm,Dex−Lm,SV)/(Lm,Dex−Lm,control)]×100. The subscript notation of Lm shows the group name.

**Determination of SV-Acid Concentration in Plasma and Lungs** According to the method of Germershausen et al., plasma samples were pretreated as described below. A four-fold volume of acetonitrile containing 10 ng/mL of LV-acid (internal standard) was added to thawed plasma, and the mixture was centrifuged at 10000×g at 4°C for 10 min to precipitate the proteins. Then, the supernatant was applied to API3200™LC-MS/MS system (ABSciex, MA, U.S.A.).

The LC was performed on a model 20-A Prominence system (Shimadzu Corporation, Kyoto, Japan) equipped with a Sunfire™ C18 column (2.1×50 mm, i.d. 3.5 µm, Waters, MA, U.S.A.). The column temperature was set at 40°C. Solvent A was 0.1% (v/v) formic acid aqueous solution. Solvent B was acetonitrile (t=0–1.0 min, 10% acetonitrile; t=1.1–1.6 min, 95% acetonitrile; t=1.7–8.0 min, 10% acetonitrile). The flow rate was 0.3 mL/min. Injection volume was 15 µL. Tandem mass spectrometry was performed using an API3200™ tandem quadrupole MS system (ABSciex) with negative ion electrospray ionization(−). The precursor→product ions monitored were m/z 421.2→410.1 (LV-acid) and m/z 435.3→319.2 (SV-acid).

**Pharmacokinetics Analyses** Pharmacokinetics of SV in plasma were characterized by determining the peak concentration (Cmax), time to Cmax (Tmax) and area under the concentration–time curve (AUC) from time zero to last measurable time point (AUC0−last) after single administration of SV. The Cmax and Tmax values were taken directly from the data. AUC0−last was calculated by the linear trapezoidal rule.

**Statistical Analysis** As statistical analyses, Tukey’s and Dunnett’s multiple comparison tests and Student’s t-tests were used to determine the statistical significance using SPSS 17.0 (IBM, Armonk, NY, U.S.A.). Differences were considered statistically significant when p<0.05.
RESULTS

Alveolar Regeneration Experiments

Dose Proportionality after Intraperitoneal Administration

To confirm the alveolar regeneration by SV and to further investigate its dose-proportionality, histological analysis of the lung sections was performed. The mice were intraperitoneally administered at doses of 4, 20, and 100 µg/mouse/d on the above mentioned schedule. The alveoli in lung sections from mice in the SV treated group were smaller than those in the Dex group (Fig. 1), but still larger than those in the control group. The average $L_m$ of each of the 3 different dosage groups was 48 to 58 µm, and significantly shorter than that of the Dex group (about 115 µm) (Fig. 2), and the % $L_m$ recovery was 63–72%. However, there were no significant differences of $L_m$ among the 3 dosage groups, indicating no dose-proportionality within that dose range.

After Oral Administration

The mice were administered intraperitoneally at a dose of 4 (4-SV-i.p.) and orally at the doses of 4 and 20 µg/mouse/d (4-SV-p.o. and 20-SV-p.o., respectively) of SV on the above mentioned schedule. Smaller alveoli were observed in the mice in the 4-SV-i.p. and 20-SV-p.o. groups than in the Dex group (Fig. 3), and the $L_m$ of these SV-treated groups were significantly shorter than that of the Dex group ($p<0.05$). In the 4-SV-p.o. group, although the alveoli looked smaller their $L_m$ was not significantly shorter than that of the Dex group (Table 1).

Chronological Changes of Alveoli

For the evaluation on the process of alveolar regeneration, the lung sections in the SV group were observed for chronological changes on several points from the day just before the start of treatment (P38) to the day for final evaluation (P93). At 10 d after the start, $L_m$ in the treatment group began to decrease (Fig. 4). Finally, at P93 the % $L_m$ recovery was 79.3 in SV group.

In Adult Mice

To clarify whether the effect on alveolar regeneration by SV was different between young and adult mice, similar experiments were also conducted using adult mice. In adult mice, $L_m$ after intraperitoneal administration of SV at a dose of 4 µg/mouse tended to be smaller than that in the Dex group, but the difference was not significant (Fig. 5). The % $L_m$ recovery of alveoli in the adult mice was weaker than that observed in young mice as described above (Table 1). Meanwhile the mean alveolar length in adult mice in the Dex group were significantly larger than those in the control group ($p<0.05$). The $L_m$ in adult mice in the Dex and control groups were relatively shorter and longer, respectively, than that in young mice (Fig. 5).

Exposure of SV-Acid in Normal Mice


![Fig. 1. Representative Micrographs of Lungs from Various Treatments](image-url)

A): Control group, B): Dex group, C): 4-SV group, given 4 µg/mouse of SV (i.p.) for 10 d. At P90 the lungs sections were prepared, and the sections were stained with Elastica-van Gieson. Original magnification, 100×. Bars, 200 µm.

![Fig. 2. Effects of SV Dosage on Alveolar Regeneration in Young Mice](image-url)

Zero (only peanut oil as a vehicle), 4, 20 or 100 µg/mouse/d SV was administered intraperitoneally for 10 d to the mice with induced emphysema in the Dex, 4-SV, 20-SV and 100-SV groups, respectively. $L_m$ was calculated from lung sections from each mouse as an index of the alveolar size. Data are shown as the mean±S.D., n=4. Letters over the bars indicate results of the pairwise comparison as shown in the following sentence. The means are significantly different ($p<0.05$, Tukey’s multiple comparison test) for pairs of bars with different characters.

![Fig. 3. Effects of Administration Route of SV on Alveolar Regeneration in Young Mice](image-url)

Four and 20 µg/mouse/d of SV was administered intraperitoneally for 10 d to mice with induced emphysema in 4-SV-i.p. group. Also 4 and 20 µg/mouse/d of SV was administered orally to the mice in 4-SV-p.o. and 20-SV-p.o. groups, respectively. Then the $L_m$ was calculated from lung sections from each mouse as an index of the alveolar size. Data are shown as the mean±S.D., n=5. Letters over the bars indicate results of the pairwise comparison as shown in the following sentence. The means are significantly different ($p<0.05$, Tukey’s multiple comparison test) for pairs of bars with different characters.
In Young Mice

To investigate systemic exposure of SV under various administration conditions, young normal mice were administered intraperitoneally at 4 µg/mouse and orally at 4 and 20 µg/mouse and their pharmacokinetic parameters of SV-acid were calculated (Fig. 6, Table 2). The concentrations in plasma reached the maximum at about 30 min after dosing for both routes of administration and either dose. The AUC0–last after intraperitoneal administration at 4 µg/mouse and oral administration at 20 µg/mouse were comparable. However, that after oral administration at 4 µg/mouse tended to be lower than those of the other two groups.

In Adult Mice

To investigate whether there was a difference in systemic exposure between young and adult mice, 4 µg/mouse/d of SV was administered intraperitoneally to normal adult mice for calculation of their pharmacokinetic parameters of SV-acid (Fig. 6, Table 2). There was no significant difference in AUC0–last after intraperitoneal administration at 4 µg/mouse and oral administration at 20 µg/mouse were comparable. However, that after oral administration at 4 µg/mouse tended to be lower than those of the other two groups.

DISCUSSION

There have been a few reports that SV inhibited alveolar destruction5) and affected alveolar recovery6) in emphysema model animals. In this study, SV was revealed to have effects also in a Dex-induced model of emphysema, which is an alveolar hypoplastic model, indicating the effect of SV was not dependent on mechanism for production of alveolar damage in the model. After intraperitoneal administration of SV to model mice in the dose range of 4 to 100 µg/mouse/d, alveolar regeneration with % Lm recovery of 63–72% was clearly observed in each group (Figs. 1, 2). These results indicated that there was no clear dose-proportionality in this dose range and a limit of alveolar recovery was up to 60–70% at least for a Dex-induced model. Furthermore, it is suggested that the intraperitoneal administration of 4 µg/mouse/d was sufficient for this treatment.

SV is used clinically as an oral formulation. Thus, it is important to determine whether the same effect can be obtained with clinically feasible oral doses of SV. As the beginning,
whether it was possible to obtain an exposure of SV-acid after oral administration that would be similar to that resulting in alveolar recovery was investigated and also the required dose was investigated. The result, the exposure in plasma after oral treatment at 4 µg/mouse was comparable to that after intraperitoneal administration at 4 µg/mouse (Fig. 6, Table 2). As a matter of fact, the alveolar recovery after these treatments with SV was also comparable (Fig. 3). However, after oral treatment at 4 µg/mouse the alveoli only recovered slightly, when the exposure to SV-acid in plasma was less than half of that after the effective doses. From the results, regardless of the route of administration, almost maximum recovery can be obtained after treatment by SV when the plasma exposure is equal to or higher than that after intraperitoneal administration at a dose of 4 µg/mouse. On the other hand, the AUC of SV-acid in the mice (20 µg/mouse p.o., 4 µg/mouse i.p.) in the present study was a little higher or comparable to that after administered 20 mg of SV to human volunteers, which is within the range of a clinical daily dose of SV in humans (2.5–20 mg/d). If the sensitivity to simvastatin in humans is similar to that in mice, clinical application of SV for the treatment of emphysema might wane with age.

Next, the time–course of alveolar recovery was investigated after intraperitoneal administration of SV at a dose of 4 µg/mouse to young model mice. Based on analysis of the lung tissue sections, reduction of Tm value began 10 d after the completion of the administration of SV, and continued until the end of observation period, P90 (Fig. 4). These results revealed that the effect of SV could be maintained not only in the treatment period for 10 d but also after that period.

It is well known that emphysema often occurs in middle-aged and elderly persons rather than in young ones because it is a respiratory disease mainly caused by long-term smoking. So, the evaluation for therapy of emphysema needs to use not only a model in young animals but also especially a model in adult animals. Using adult-model mice, the alveolar recovery after intraperitoneal administration of SV at a dose of 4 µg/mouse for 10 d from P150. After the treatment of SV the alveoli tended to recover, but the effect was weaker than in young model mice (Fig. 5, Table 1). Meanwhile, no significant difference of plasma concentration profile or exposure was observed between the young (6 week old) and adult (21 week old) mice after the intraperitoneal administration (Fig. 6, Table 2), even though the dose on a µg/kg basis in adult mice was lower because of the difference in body weights. From these results, the sensitivity to alveolar recovery by SV might wane with age.

In these experiments, the alveolar enlargement after Dex treatment, compared to control mice, was observed even in adult mice (P200). However, comparing the young and adults, in the Dex groups, adult mice had shorter Tm values than young mice, which fact suggested that the enlarged alveoli healed naturally with age in mice at least after about P90. Also, in the control groups, adult mice had longer Tm than

![Fig. 6. Time Course of SV-Acid Concentration in Plasma](Image)

Four microgram/mouse of SV was administered i.p. to young (filled square) or adult mice (open square), and orally to young mice (closed circle). Twenty microgram/mouse of SV was administered orally to young mice (closed triangle). Data are shown as the mean ± S.D., n=5 (adult) or 6 (young).

Table 2. PK Parameters of SV-Acid in Mice under Various SV Administration Conditions

<table>
<thead>
<tr>
<th>Age (Young/Adult)</th>
<th>Dose (µg/mouse)</th>
<th>Administration route</th>
<th>Cmax (ng/mL)</th>
<th>Tmax (min)</th>
<th>AUC0–last (ng·min/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>4</td>
<td>i.p.</td>
<td>4.7±1.8a</td>
<td>24.0±8.2a</td>
<td>355.2±164.5a</td>
</tr>
<tr>
<td>Young</td>
<td>4</td>
<td>i.p.</td>
<td>4.8±1.6a</td>
<td>22.5±8.2a</td>
<td>332.0±136.9a</td>
</tr>
<tr>
<td>Young</td>
<td>4</td>
<td>p.o.</td>
<td>1.4±0.5b</td>
<td>37.5±8.2a</td>
<td>194.9±44.9b</td>
</tr>
<tr>
<td>Young</td>
<td>20</td>
<td>p.o.</td>
<td>2.8±0.9b</td>
<td>25.0±12.2a</td>
<td>323.5±29.4b</td>
</tr>
</tbody>
</table>

Data are shown as the mean ± S.D., n=5 (adult) or 6 (young). i.p., intraperitoneally; p.o., oral per os; Cmax, peak concentration; Tmax, time to Cmax; AUC0–last, area under the concentration–time curve from time zero to last measurable time point. Superscript characters indicate results of the pairwise comparison as shown in the following sentence. The means with different superscript characters are significantly different (p<0.05, Tukey’s multiple comparison test) for pairs of values with different characters.
young mice (Figs. 3, 5). With these factors taken into consideration, additional research is needed under other experimental conditions such as higher doses or longer treatment period to clarify the clinically practical effect of SV on emphysema.

CONCLUSION

The alveoli damaged by Dex treatment are recovered after administration of SV at a dose of 4 µg/mouse/d i.p. or 20 µg/mouse/d orally for 10 d to young model mice. The alveolar recovery after SV depends on exposure in plasma regardless of the administration route. On the other hand, in adult mice the recovery is not sufficient after i.p. administration at a dose of 4 µg/mouse/d.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES


