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ORIGINAL ARTICLE

Beneficial Effects of Fluvastatin on Liver Microcirculation and Regeneration After Massive Hepatectomy in Rats

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Abstract Fluvastatin, the first entirely synthetic statin, has a significant cholesterol-lowing effect comparable with other statins. In addition, it has been shown to inhibit oxidative stress and improve vascular endothelial function. The aim of this study was to clarify the pretreatment effects of fluvastatin on liver function after massive hepatectomy in rats. Six-week-old male Wister rats were divided into two groups: a fluvastatin group (group F), pretreated with oral administration of fluvastatin (20 mg/kg per day) for 2 days before 90% hepatectomy; and a control group (group C), pretreated with vehicle for 2 days before hepatectomy. Animals were sacrificed at 0, 12, 24, 48, and 72 h after hepatectomy. The liver regeneration rate, liver function tests, and hepatic stellate cell activation were examined. The liver regeneration rate in group F was significantly higher at 72 h after hepatectomy ($P < 0.05$). The serum level of total bilirubin in group F was significantly lower at 48 h after hepatectomy ($P < 0.05$). Sinusoidal area in group F was maintained histologically. Furthermore, the expression of alpha smooth-muscle actin (α-SMA) protein in the liver was inhibited in group F at 48 h after hepatectomy. This study demonstrated the beneficial effects of fluvastatin in a lethal massive hepatectomy model using rats, with improved hepatic regeneration and microcirculations, by inhibiting the activation of hepatic stellate cells.

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Abbreviations

α-SMA	Alpha smooth-muscle actin
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ECM	Extracellular matrix
FPP	Farnesyl pyrophosphate
GGPP	Geranylgeranyl pyrophosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
PCNA	Proliferating-cell nuclear antigen
ROS	Reactive oxygen species
HMG-CoA	Hydroxy-methyl-glutaryl-coenzyme A

Introduction

Although massive hepatectomy is sometimes required to achieve a curative resection for advanced hepatic malignancies, most instances of mortality after such surgery are still attributed to hepatic failure with small remnant liver volume [1]. Therefore, a novel strategy is still needed for the treatment of hepatic insufficiency after massive hepatectomy.

Various hypotheses have been reported for the mechanisms of hepatic failure after massive hepatectomy; excessive metabolic loads for each remnant hepatocyte and excessive stress on the hepatic sinusoids with portal overflow [2]. From the microenvironmental viewpoint, reactive oxygen species (ROS) have been reported to have a crucial role after such injury [3]. ROS are evolved during the early

posthepatectomy phase, causing initial insults on both parenchymal and nonparenchymal cells, and lead to secondary negative cascades including hepatic hypercytokinemia [3–6]. We previously focused on ROS as the crucial factors in such hepatic injuries, acting via Rho-kinase-mediated pathways [7]. In the study reported here, we focused on the potential role of statins, which are known to deoxidize various enzymes involved in modification of small G proteins such as Rho [8–13]. Although possible anti-inflammatory properties of statins have been reported, no previous reports have evaluated their roles in massive hepatectomy.

Fluvastatin is the first entirely synthetic statin, with the most potent cholesterol reduction property of all the available statins [14, 15]. The aim of the study was to investigate the possible beneficial effects of fluvastatin on hepatic function, including microcirculation and regeneration, using a massive hepatectomy model in rats.

Materials and Methods

Animals

Six-week-old male Wister rats weighing 180–220 g were obtained from Charles River Co., Ltd (Kanagawa, Japan). Throughout the experiment, the animals were maintained behind barriers under controlled conditions and had free access to tap water and diet before and after operation. The University of Tokushima Institutional Animal Care and Use Committee approved all animal protocols, according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy for Sciences and published by the National Institutes of Health (NIH).

Experimental Design

Animals were randomly divided into the following two groups: fluvastatin-administrated group (group F; $n = 9$) and control group (group C; $n = 7$). In group F, the animals were pretreated with oral doses of fluvastatin 20 mg/kg per day in normal saline (Tanabe Seiyaku Co., Ltd. Osaka, Japan) starting from 2 days before hepatectomy and continuing until the day of hepatectomy, whereas in group C, the animals received vehicle in the same manner.

Surgical procedures were performed under general anesthesia with diethyl ether. Removal of 90% of the hepatic tissue was performed using the method of Higgins and Anderson [16], with minor modifications as described by Kubota et al. [17]. The left, middle, and right lobes were sequentially removed without constricting the inferior vena cava or causing bleeding. The caudate lobe was left intact. All surgical procedures were completed within 20 min. Signs of distress and survival were monitored for up to 1 week. Animals were sacrificed at 12, 24, 48, and 72 h after hepatectomy. Blood samples were obtained from the superior vena cava for biochemical analysis. The intact liver was removed and stored for histological examinations after measurement of its wet weight.

Biochemical Analysis

To evaluate liver injury, the level of serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), hyaluronic acid, and total bilirubin were measured using commercially available assay kits (Shikoku Chuken, Inc. Kagawa, Japan).

Histological Examination

Liver tissues were fixed in 10% buffered formalin, embedded in paraffin, cut into sections 4-μm thick, and processed for hematoxylin and eosin (H&E) and immunohistochemical staining. The H&E sections were used to evaluate sinusoidal area. Investigators who were kept blind to the treatment group measured ten areas of sinusoidal area at magnification of ×200 using the NIH image software program (National Institute of Health, MD, USA). The maintenance of sinusoid area was investigated by sinusoidal space rate, which is the area of sinusoid per one view at magnification of ×200. For immunohistochemical evaluations, the sections were incubated overnight with antibodies against proliferating cell nuclear antigen (PCNA, mouse monoclonal) (Santa Cruz, CA, USA) and alpha smooth-muscle actin (α-SMA) diluted 1:100, mouse monoclonal (Abcam, Cambridge, UK) at 4°C. After being washed with phosphate-buffered saline (PBS), bound antibodies were visualized with the Dako Envision system (Dako Chemate EnVision Detection Kit, Peroxidase/DAB, Rabbit/Mouse, Dako Cytomation, Denmark). A normal goat immunoglobulin (Ig)G was used to assess nonspecific reactions. At the magnification of ×200, ten areas of PCNA and α-SMA-positive cells were measured in a blinded fashion for each group.

Statistical Analysis

Data are expressed as mean ± standard deviation (SD). Comparisons in the same group were performed using the paired Student's t test. Comparisons between groups were conducted using the Mann-Whitney U test. Statistical significance was defined as a P value <0.05.

Results

Liver Regeneration

During the first 48 h of the postoperative period, the intact liver/body-weight ratio was not significantly different between groups. However, it was significantly increased in group F at 72 h after hepatectomy compared with group C ($P < 0.05$, Fig. 1). The number of PCNA-positive cells in group F was significantly increased over those in group C at 72 h after hepatectomy (Fig. 2).

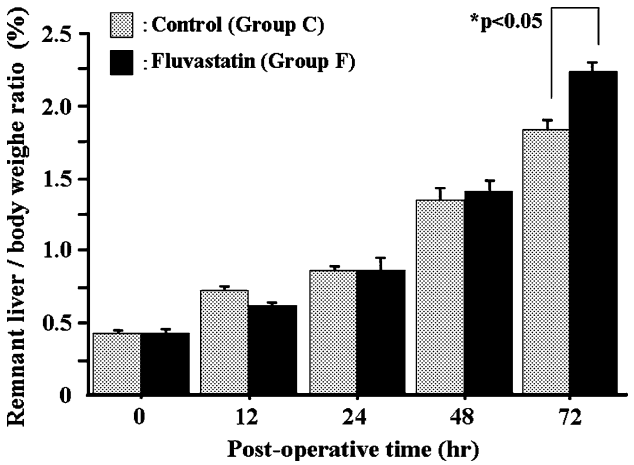

Fig. 1 Liver regeneration. Remnant liver/body-weight ratio in the fluvastatin group was significantly higher at 72 h after hepatectomy compared with the control group (*$P < 0.05$)

Hepatic Chemistries

The serum level of AST, ALT, hyaluronic acid, and total bilirubin in the rats are shown in Table 1. The total bilirubin at 48 h after hepatectomy was significantly lower in group F compared with group C (1.9 ± 0.1 vs. 4.9 ± 1.9 mg/dl, $P < 0.05$). There were no statistically significant differences in the other parameters between the two groups.

Hepatic Sinusoidal Area

Sinusoidal area was maintained in group F compared with group C at 24 h (2.94 ± 1.01 vs. 1.50 ± 0.67%, $P < 0.01$) and 48 h (0.95 ± 0.41 vs. 0.60 ± 0.24%, $P < 0.05$) after hepatectomy (Fig. 3). Sinusoidal areas in the both groups were smallest at 48 h after hepatectomy, but at 72 h after hepatectomy, sinusoidal areas in both groups were restored.

Table 1 Hepatic chemistries (48 h after hepatectomy)

	Group C	Group F	*P* value
AST (IU/l)	1.872 ± 1.103	1,371 ± 261	NS
ALT (IU/l)	683 ± 365	472 ± 114	NS
Hyaluronic acid (ng/ml)	12.383 ± 6.983	1,1084 ± 4,602	NS
Total bilirubin (mg/dl)	4.9 ± 1.9	1.9 ± 0.1	<0.05

AST aspartate aminotransferase, *ALT* alanine aminotransferase

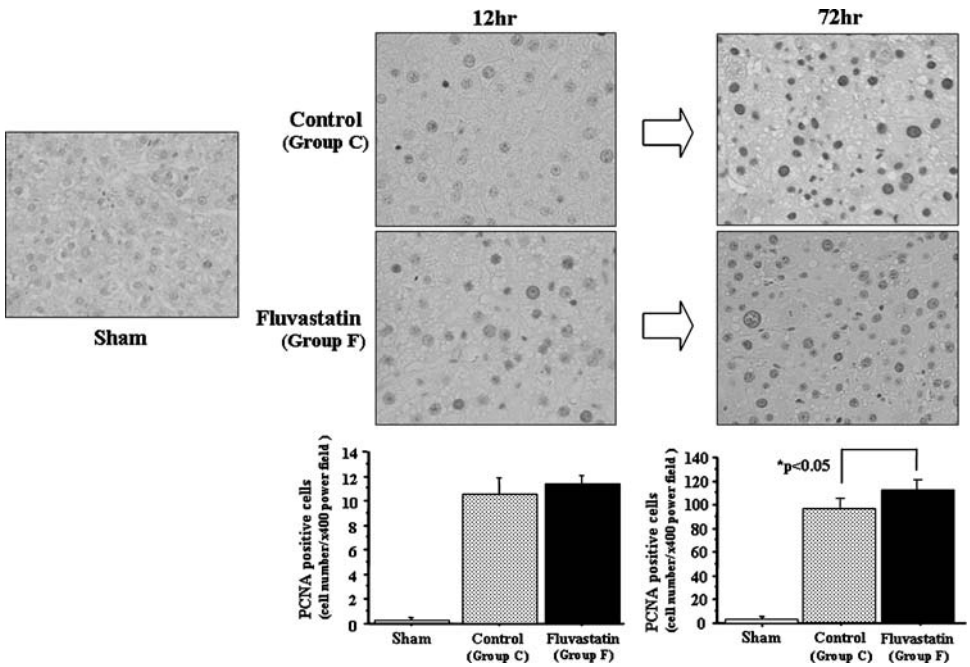

Fig. 2 Proliferating-cell nuclear antigen (PCNA) labeling index. The number of PCNA-positive hepatocytes in the fluvastatin group was significantly higher at 72 h after hepatectomy compared with the control group (*$P < 0.05$)

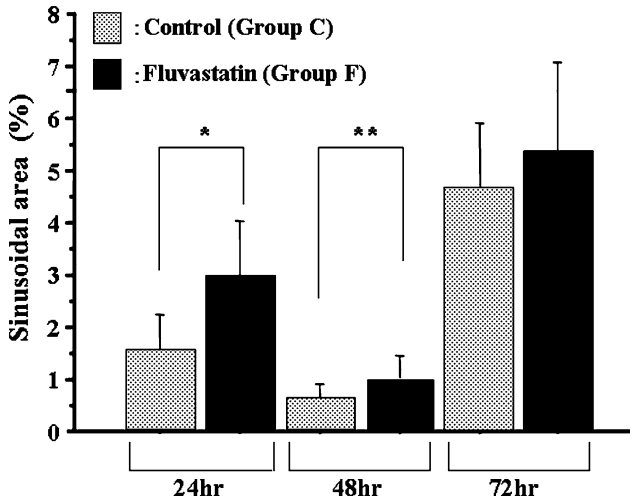

Fig. 3 Sinusoidal area. Sinusoidal space was significantly increased in the fluvastatin group compared with the control group at 24 h and 48 h after hepatectomy (**$P < 0.01$, * $P < 0.05$)

The Expression of α-SMA

Alpha SMA-positive cells were significantly decreased in group F compared with group C (2.7 ± 2.6 vs. 17.6 ± 13.5, $P < 0.01$) at 48 h after hepatectomy (Fig. 4).

Discussion

In this study, pretreatment with fluvastatin accelerated liver regeneration and improved cholestasis by inhibiting the activation of the liver stellate cells. Statins are inhibitors of the enzyme hydroxy-methyl-glutaryl-coenzyme-A (HMG-CoA) reductase and are widely used as lipid-lowering agents [12]. In the previous report, HMG-CoA reductase activity increase prior to onset of DNA synthesis or S phase, and has a stimulatory effect on the initiation of liver regeneration [18–20]. Inhibition of HMG-CoA reductase results in decreased farnesyl pyrophosphate, which not only reduces cholesterol levels but also modifies and inactivates small G proteins, including Rho (Fig. 5). Therefore, fluvastatin has been shown to modify post-translational G-protein-associated phospholipids, resulting in their inactivation, and has been shown to ameliorate ROS production in the liver [12–14]. Although possible anti-inflammatory properties of statins have been reported, no previous reports have evaluated their effects after massive hepatectomy. Therefore, in the study reported here, we investigated the role of fluvastatin, one of the most potent deoxidizer amongst the currently available statins, in an experimental massive hepatectomy model.

The prominent effect of fluvastatin in this study was observed in terms of preserved sinusoidal circulation via the inhibition of hepatic stellate cells, as shown by reduced α-SMA-positive hepatic stellate cells. It has been reported that several factors that improved the microcirculation promote liver regeneration. Wang et al. [21] reported that phosphodiesterase inhibitor, which improves hepatosplanchnic circulation, promotes liver regeneration. Tsujii et al. [22] reported that prostaglandin E2 enhances the proliferation of hepatocytes by increasing intracellular cyclic AMP. Akcan et al. [23] reported that nitric oxide, a flow-dependent factor, is a trigger to initiate the regeneration process. Hepatic stellate cells are located between parenchymal cells and the endothelial lining and have several important functions, such as remodeling of

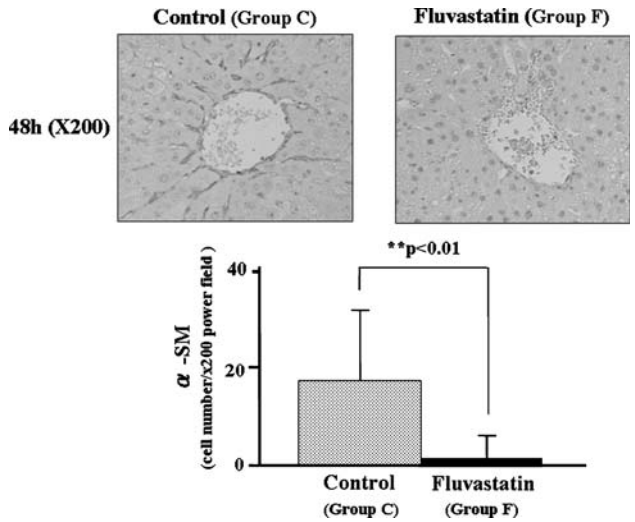

Fig. 4 Expression of alpha smooth-muscle actin (α-SMA). α-SMA positive cells were significantly lower in the fluvastatin group compared with the control group at 48 h after hepatectomy (**$P < 0.01$)

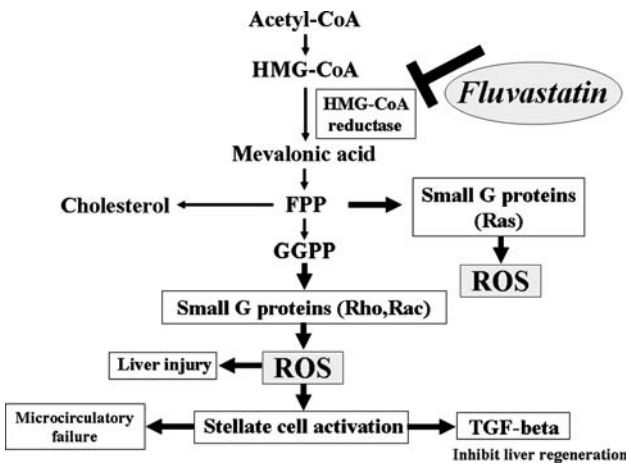

Fig. 5 Effect of fluvastatin in our hypothesis. Inhibition of hydroxy-methyl-glutaryl-coenzyme-A (HMG-CoA) reductase by statins decreases not only the cholesterol production but also reactive oxygen species (ROS) production. *FPP* farnesyl pyrophosphate, *GGPP* geranylgeranyl pyrophosphate

extracellular matrix (ECM) by production of both ECM components and matrix metalloproteinases, production of growth factors and cytokines, and contraction and dilation of the sinusoidal lumen in response to endothelin, angiotensin, thromboxane, and prostaglandins [24–28]. Activation of hepatic stellate cells is mediated by prominent ROS, released by both parenchymal and nonparenchymal cells, in particular by damaged hepatocytes and activated Kupffer cells [24]. It has been reported that the intracellular source of ROS is the intramitochondrial cytochrome P450 system with nicotinamide adenine dinucleotide phosphate and xanthine oxidase, resulting in activation of the extracellular signal-regulated-kinase-signaling pathway [24–28]. Continuing activation of the hepatic stellate cells leads to progressive fibrosis of the liver; fluvastatin, which inhibits activation of hepatic stellate cells, may prevent fibrosis progression.

Among the various factors known to cause hepatic failure after massive hepatectomy, ROS has been reported to play the pivotal role [3, 4]. It is well known that ROS are released by the Kupffer cells via activation by excessive sheer stress and microcirculatory disturbance in the hepatic sinusoids in the intact liver [3–6]. Such ROS cause not only hepatocyte injury but also sinusoidal endothelial cell damage, which further aggravates microcirculatory disturbances. In our previous studies, it was revealed that Rho-kinase, an effector of the small GTPase Rho, plays an important role in ROS production through the nicotinamide adenine dinucleotide phosphate (NADPH) system in the hyperacute phase of hepatic reperfusion [7]. Other studies also showed that Rho-regulated ROS production is involved in the pathogenesis of hepatocellular necrosis and apoptosis [8, 9].

Liver regeneration also might be affected by ROS via indirect or direct effects [29–35]. As an indirect effect, it has been reported that stellate cells activated via the ROS system produce excessive transforming growth factor beta (TGF-β), a potent inhibitory cytokine for liver regeneration [29, 30]. TGF-β has been found to be increased in experimental and human hepatic fibrosis, and TGF-β induces ECM production by hepatic stellate cells. A previous study showed that H_2O_2, as a major source of ROS, induced transient elevation of p53 and p21 proteins, which inhibit the cell cycle, resulting in arrest of the cell cycle at the G1 phase [34]. Another report showed that ROS induce downregulation of cyclin D expression, which is important for G1-phase progression [35].

As a direct effect, ROS caused acute liver injury and increased transaminase [4, 36]. It has been reported that fluvastatin has anti-inflammatory properties and reduces the serum level of AST and ALT on endotoxin-induced liver injury [37]. Another report showed that statin significantly reduced liver injury associated with enhanced endothelial nitric oxide synthase (eNOS) expression [38]. However, in this study the serum level of AST and ALT was not improved significantly. We suppose that the effect of improving liver injury weaken under excessive stress, such as massive hepatectomy.

In conclusion, this study demonstrated the beneficial effects of fluvastatin via improved hepatic regeneration and microcirculation in a lethal massive hepatectomy model using rats. Fluvastatin may be an effective pharmacological strategy to improve outcomes after massive hepatectomy.

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