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Osthole prevents anti-Fas antibody-induced hepatitis in mice by affecting the caspase-3-mediated apoptotic pathway

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Abstract

Fas (Apo-1/CD95) ligand, which is a type II membrane protein, is a major inducer of apoptosis. Osthole is a coumarin derivative present in medicinal plants. The effect of osthole on hepatitis induced by anti-Fas antibody in mice was studied. Pretreatment of mice with osthole (10, 50, and 100 mg/kg, i.p.) prevented the elevation of plasma alanine aminotransferase (ALT) caused by anti-Fas antibody (175 μg/kg, i.v.). Administration of osthole to mice even at a dose of 10 mg/kg significantly inhibited of anti-Fas antibody-induced elevation of plasma ALT. Capase-3 is a cysteine protease, and treatment of mice with anti-Fas antibody caused an elevation of caspase-3 activity at 3.5 and 6 hr. Pretreatment of mice with osthole (100 mg/kg, i.p.) inhibited the elevation of caspase-3 activity caused by anti-Fas antibody. However, the addition of osthole (up to 10^{-4} M) to a liver cytosol fraction isolated from mice treated with anti-Fas antibody did not inhibit caspase-3 activity *in vitro*. Thus, treatment of mice with osthole inhibited caspase-3 activity by an effect upstream of caspase-3 activation. The livers of mice treated with anti-Fas antibody contained apoptotic and dead cells; osthole attenuated the development of this apoptosis and cell death. The present results show that osthole prevented anti-Fas antibody-induced hepatitis by inhibiting the Fas-mediated apoptotic pathway.

Keywords: Hepatitis; Fas; Apoptosis; Coumarin; Osthole; Caspase-3

1. Introduction

Fas (Apo-1/CD95) ligand is a major inducer of apoptosis, and Fas-mediated apoptosis is involved in the development of variety of diseases. In the livers of HCV-induced chronic hepatitis, Fas and Fas ligand are expressed [1,2], and the Fas system is regarded as playing a major role in the development of apoptosis in virus-induced chronic hepatitis [3,4]. Recently, it was indicated that normalization of ALT prevents the development of HCC in HCV-induced chronic hepatitis, even though the virus is not eliminated [5]. Thus, inhibition of Fas-mediated liver injury leads to preventing the development of HCC in virus-induced hepatitis. Furthermore, Fas-mediated apoptosis in hepatocytes plays a role in the development of liver diseases that are not only associated with viral hepatitis, but also autoimmune hepatitis [6], alcohol-induced hepatitis [7], cholestatic hepatitis, and hepatitis in metabolic disorders [8]. Thus, drugs that inhibit Fas-mediated liver injury might be useful not only in preventing the development of HCC, but also in the treatment of a wide variety of liver diseases.

Osthole, a component of medicinal plants, such as *Cnidium monnieri* and *Angelica pubescens*, is a coumarin derivative, which has been administered to humans. Osthole possesses a variety of pharmacological and biochemical properties [9,10], and is considered to have potential therapeutic applications [11]. Con A-induced mouse hepatitis is dependent upon inflammatory cytokines, including tumor necrosis factor-α and interferon-γ [12,13], and it has been reported that osthole prevents Con A-induced cytokine-dependent liver injury in mice [14]. However, the mechanism by which osthole prevents Fas-mediated hepatitis is not known.

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Abbreviations: HCV, hepatitis C virus; ALT, alanine aminotransferase; HCC, hepatocellular carcinoma; Con A, concanavalin A; Ac-DEVD-MCA, Ac-Asp-Glu-Val-Asp-4-methyl-coumaryl-7-amide; and CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate.

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In the present study, we examined the effect of osthole on hepatitis induced by anti-Fas antibody in mice.

2. Materials and methods

2.1. Materials

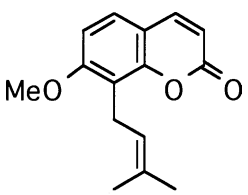
Jo2, an anti-Fas antibody known to induce hepatitis through activation of the Fas receptor [15], was purchased from Pharmingen. The fluorescent substrate Ac-DEVD-MCA was obtained from Peptide Institute Inc. Osthole, a coumarin derivative (Fig. 1), was isolated from *Cnidium monnieri*. The dried fruit of *C. monnieri* was soaked overnight in methanol. The methanol was evaporated, and the crude extract was separated by liquid chromatography. Osthole was identified via nuclear magnetic resonance, and integration of this measurement indicated that the purity of the isolated osthole was more than 99%.

2.2. Animal experiments

Female BALB/c mice obtained from Charles River Japan (Atsugi) were used at 7–10 weeks of age. The animals were kept in an air-conditioned room, and given chow and water *ad lib*. The anti-Fas antibody (175 μg/kg) was administered to the mice via the tail vein (in a volume of 100 μL). Animal experiments were performed according to the experimental protocols approved by the Institutional Ethics Committee. The mice were anesthetized with ether before being killed. Plasma ALT was measured by a standard photometric method [16] using an automatic analyzer (Hitachi 7060).

2.3. Fluorometric analysis

Liver protein extracts were prepared by Dounce homogenization of 100 mg of tissue in a hypotonic buffer [25 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL of both leupeptin and aprotinin]. The homogenates were centrifuged at 15,000 *g* for 10 min. For the measurement of caspase-3 activity, 50-μg aliquots of the extracted proteins were incubated with the fluorescent substrate Ac-DEVD-MCA, at a concentration of 25 μM in 50 mM HEPES, 1% sucrose, 0.1% CHAPS, and 5 mM dithiothreitol, in a final volume of 1.5 mL. The fluorescence of the cleaved substrates was determined with a spectrofluorometer (RF-5000, Shimadzu), at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. One unit corresponds to the activity that cleaves 1 pmol of the respective fluorescent substrate at 25° in 30 min.



Osthole

Fig. 1. Chemical structure of osthole.

2.4. Histological study

For the histological study, the livers were fixed with 10% phosphate-buffered neutral formalin, and paraffin sections (4 μm) were stained with hematoxylin and eosin for microscopic examination.

2.5. Statistical analysis

The results were analyzed by Dunnett's multiple comparison test.

3. Results

3.1. Effect of osthole on anti-Fas antibody-induced elevation of plasma ALT

Mice were pretreated with osthole (10, 50, and 100 mg/kg, i.p.), and then at 30 min the anti-Fas antibody (175 μg/kg, i.v.) was injected. At 3.5 hr after treatment, plasma was sampled. Treatment of mice with the anti-Fas antibody (175 μg/kg, i.v.) caused an elevation of plasma ALT at 3.5 hr (Fig. 2). Osthole treatment inhibited the elevation of plasma ALT caused by anti-Fas antibody dose-dependently (Fig. 2). Osthole, even at a dose of 10 mg/kg, significantly

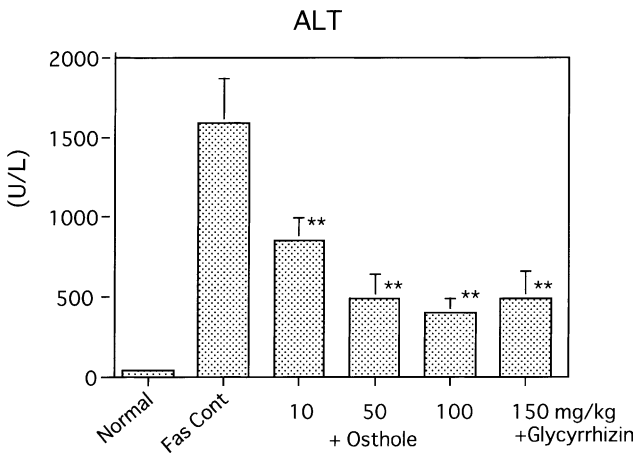


Fig. 2. Effect of osthole on anti-Fas antibody-induced elevation of plasma ALT. The anti-Fas antibody (175 μg/kg, i.v.) was injected, and plasma was obtained from each mouse 3.5 hr later. Osthole was administered 30 min before the anti-Fas antibody treatment. Data represent the means ± SEM of the ALT (U/L) levels in plasma obtained after the various treatments. Normal (N = 3); Fas Cont: anti-Fas antibody-treated (N = 7); +Osthole: anti-Fas antibody + osthole (10 mg/kg, N = 4; 50 mg/kg, N = 5; 100 mg/kg, N = 5) (i.p.); +Glycyrrhizin: anti-Fas antibody + glycyrrhizin (150 mg/kg, i.p., N = 5). Key: (**) $P < 0.01$ vs Fas Cont.

inhibited the elevation of plasma ALT caused by anti-Fas antibody. The inhibition by glycyrrhizin (150 mg/kg) of the anti-Fas antibody-induced elevation of ALT was of a magnitude similar to that induced by osthole (50 mg/kg) (Fig. 2).

3.2. Effect of anti-Fas antibody treatment on caspase-3 activity

Mice were treated with the anti-Fas antibody (175 μg/kg, i.v.); at 1, 2, 3.5, 6, and 24 hr after the treatment their livers were removed and protein was isolated for the measurement of caspase-3 activity. Anti-Fas antibody treatment elevated caspase-3 activity significantly at 3.5 and 6 hr (Fig. 3).

3.3. Effect of osthole on anti-Fas antibody-induced elevation of caspase-3 activity in vivo

Mice were pretreated with osthole (100 mg/kg, i.p.), and at 30 min the anti-Fas antibody (175 μg/kg, i.v.) was administered. At 3.5 hr after treatment, plasma and liver tissue were sampled for measurement of ALT and caspase-3 activity. The osthole treatment inhibited both the anti-Fas antibody-induced elevation of plasma ALT [ALT (U/L): Fas Cont 1593 ± 279, +Osthole 402 ± 87, $N = 4$, $P < 0.01$] and caspase-3 activity (Fig. 4). These results indicated that osthole inhibited anti-Fas antibody-induced hepatitis by affecting caspase-3 activation. Furthermore, pretreatment of mice with osthole prolonged their survival time against a lethal dose of anti-Fas antibody (data not shown).

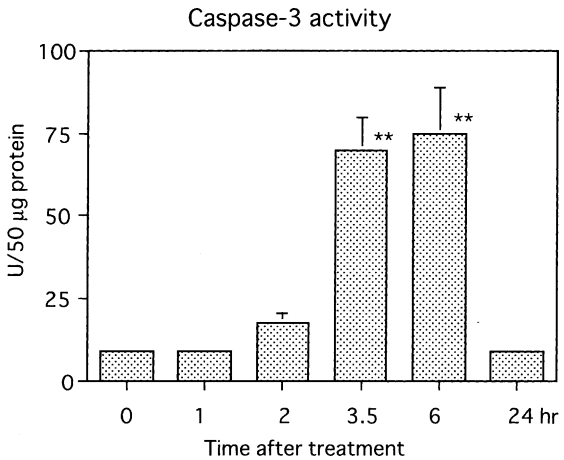


Fig. 3. Effect of anti-Fas antibody treatment on caspase-3 activity. Mice were treated with the anti-Fas antibody (175 μg/kg, i.v.), and then at 1, 2, 3.5, 6, and 24 hr liver tissue was sampled for the preparation of protein extracts ($N = 5$). Caspase-3 activity was measured with the fluorescent substrate Ac-DEVD-MCA (25 μM) in liver cytosol extracts. Caspase-3 activity is expressed as U/50 μg protein. One unit of activity corresponds to the cleavage of 1 pmol of the fluorescent substrate in 30 min at 25°. Data represent the means ± SEM of the caspase-3 activity in the liver cytosol extracts after anti-Fas antibody treatment. Key: (**) $P < 0.01$ vs caspase-3 activity at 0 hr.

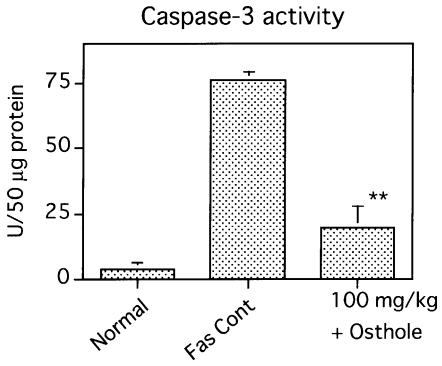


Fig. 4. Effect of osthole on anti-Fas antibody-induced elevation of caspase-3 activity *in vivo*. Osthole treatment was given 30 min before the administration of anti-Fas antibody (175 μg/kg, i.v.), and then 3.5 hr after injection the livers were removed for the preparation of protein extracts. Data represent the means ± SEM of the caspase-3 activity in liver cytosol extracts after the various treatments. Normal: non-treated ($N = 3$); Fas Cont: anti-Fas antibody-treated ($N = 4$); +Osthole: anti-Fas antibody + osthole (100 mg/kg, i.p., $N = 4$). Key: (**) $P < 0.01$ vs liver cytosol extract from mice treated with the anti-Fas antibody alone.

3.4. Effect of osthole on caspase-3 activity in vitro

In the next step, the direct effect of osthole on caspase-3 was studied. Mice were treated with the anti-Fas antibody (175 μg/kg, i.v.), and 3.5 hr later their livers were removed and protein was isolated for the measurement of caspase-3 activity. The anti-Fas antibody treatment elevated caspase-3 activity (Fig. 5). The addition of osthole, at final concentrations of 10^{-6}, 10^{-5} and 10^{-4} M, to the cytosol extract did not inhibit the caspase-3 activity, whereas the activity was abolished by the caspase-3 inhibitor

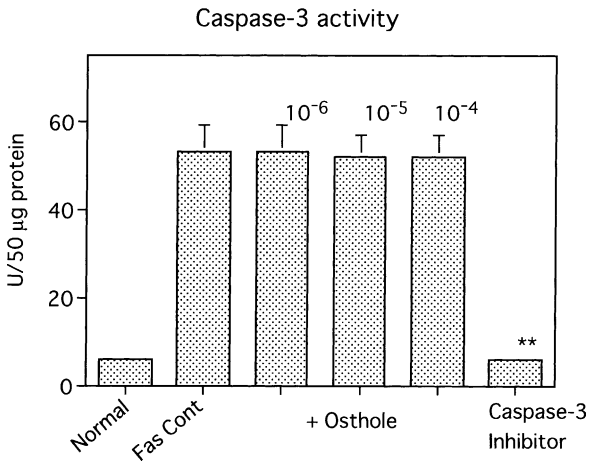


Fig. 5. Effects of osthole and a caspase-3 inhibitor on caspase-3 activity in anti-Fas antibody-treated mouse liver cytosol extracts. Mice were treated with the anti-Fas antibody (175 μg/kg, i.v.), and then 3.5 hr after injection their livers were removed for the preparation of protein extracts. Caspase-3 activity was measured using the fluorescent substrate Ac-DEVD-MCA (25 μM), with the addition of osthole (10^{-6}, 10^{-5}, 10^{-4} M, $N = 5$) or a caspase-3 inhibitor (Ac-DEVD-CHO, 10^{-6} M) ($N = 5$). Normal ($N = 5$). Data represent the means ± SEM of the caspase-3 activity. Key: (**) $P < 0.01$ vs Fas Cont.

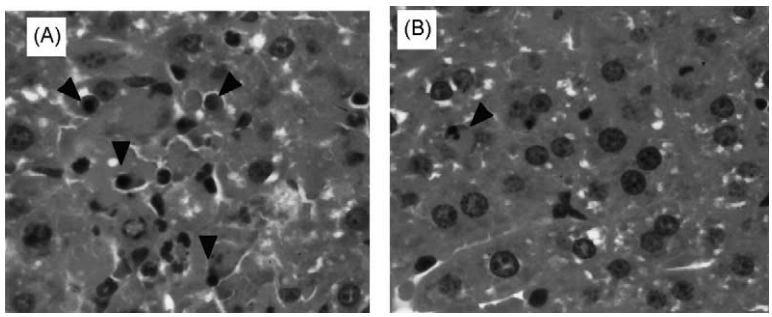


Fig. 6. Effect of osthole on anti-Fas antibody-induced development of apoptosis in the liver. Osthole treatment was given 30 min before the administration of anti-Fas antibody (175 μg/kg, i.v.), and then 3.5 hr after injection the liver was sampled for histological study. (A) Anti-Fas antibody. (B) Anti-Fas antibody + osthole (100 mg/kg, i.p.). An arrowhead indicates apoptosis/cell death.

Ac-DEVD-CHO (Fig. 5). These results indicate that osthole inhibited anti-Fas antibody-induced hepatitis by an effect upstream of caspase-3 activation.

3.5. Histological study

Mice were injected with the anti-Fas antibody (175 μg/kg, i.v.), and 3.5 hr later their livers were sampled and fixed with formaldehyde for histological study. The intralobular regions of the livers from mice treated with the anti-Fas antibody contained apoptotic and dead cells (Fig. 6A). Osthole (100 mg/kg, i.p.) treatment attenuated the development of the anti-Fas antibody-induced apoptosis and cell death (Fig. 6B).

4. Discussion

The Fas system is thought to play a major role in the development of liver injury in virus-induced hepatitis [3,4], and anti-Fas antibody-induced hepatitis represents a hepatitis caused by the Fas system [17]. Osthole is a coumarin compound present in plant medicines as an active component and is considered to have potential therapeutic applications [11]. Osthole dose-dependently inhibited the elevation of plasma ALT caused by anti-Fas antibody. Glycyrrhizin is a hepatoprotective drug used to treat virus-induced chronic hepatitis in humans [18]. Previously, osthole was shown to be more effective than glycyrrhizin in preventing Con A-induced mouse hepatitis [14]. In the present study, osthole, even at a dose of 10 mg/kg, significantly inhibited the elevation of plasma ALT induced by anti-Fas antibody. These results indicated the effectiveness of osthole in preventing anti-Fas antibody-induced hepatitis.

Caspase-3, a cysteine protease involved in the development of apoptosis [19], plays a role as a key enzyme in anti-Fas antibody-induced hepatitis [19,20]. In the present study, pretreatment of mice with osthole inhibited the anti-Fas antibody-induced activation of caspase-3 protease *in vivo*. Thus, osthole seems to prevent anti-Fas antibody-induced hepatitis by inhibiting the activation of caspase-3 protease. In the next step, the direct effect of osthole on caspase-3 was studied *in vitro*. The addition of osthole, at a final concentration of 10^{-4} M, to the liver cytosol fraction isolated from anti-Fas antibody-treated mice did not inhibit caspase-3 activity, whereas addition of the caspase-3 inhibitor Ac-DEVD-CHO, as expected, abolished the activity. These results indicated that osthole inhibited anti-Fas antibody-induced hepatitis by an effect upstream of caspase-3 activation.

Mice treated with anti-Fas antibody were shown to develop apoptosis and cell death in the liver. Osthole (100 mg/kg, i.p.) treatment attenuated the anti-Fas antibody-induced development of apoptosis and cell death. These results support the notion that inhibition of the anti-Fas antibody-induced elevation of plasma ALT by osthole is due to the prevention of apoptosis in the liver.

The present results demonstrated that osthole was effective in preventing anti-Fas antibody-induced hepatitis, suggesting its possible clinical application for the treatment of virus-induced hepatitis. Glycyrrhizin is used to normalize ALT in virus-induced hepatitis in humans [21]. In our screening system, more than 100 mg/kg of glycyrrhizin was required to significantly inhibit anti-Fas antibody-induced mouse hepatitis [17]. In contrast, the present results indicate that osthole was effective in preventing anti-Fas antibody-induced elevation of plasma ALT even at a dose of 10 mg/kg. As a mechanism to prevent anti-Fas antibody-induced hepatitis, osthole seemed to inhibit the Fas-mediated apoptotic pathway by an effect upstream of caspase-3 activation. We are continuing our study to elucidate more details of the mechanism by which osthole inhibits the Fas-mediated apoptotic pathway.

The present results indicated that osthole prevented anti-Fas antibody-induced hepatitis in mice. Fas-mediated apoptosis is involved in the development of various types of liver diseases that include viral hepatitis, autoimmune hepatitis, alcohol-induced hepatitis, cholestatic hepatitis, and hepatitis in metabolic disorders. Thus, osthole might be applicable for the treatment of a wide variety of liver diseases.

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