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European Journal of Pharmacology 541 (2006) 205–210
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Protective effects of α1-acid glycoprotein and serum amyloid A on concanavalin A-induced liver failure via interleukin-6 induction by ME3738

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Received 20 December 2005; received in revised form 2 May 2006; accepted 8 May 2006
Available online 12 May 2006

Abstract

We examined whether the 22β-methoxyolean-12-ene-3β,24(4β)-diol (ME3738)-mediated selective induction of interleukin-6 increased α1-acid glycoprotein and serum amyloid A expression, and whether these proteins protected against liver injury in vitro and in vivo. ME3738 treatment in male mice increased gene expression of α1-acid glycoprotein subtypes and serum amyloid A 2 genes, and plasma concentration of serum amyloid A. Treatment with α1-acid glycoprotein at 5 mg/animal or serum amyloid A at 0.03 and 0.1 mg/animal prior to concanavalin A administration reduced multifocal necrosis in the liver. Treatment with α1-acid glycoprotein and serum amyloid A, but not α1-antitrypsin, protected Hep G2 cells against cell injury. These results suggest that α1-acid glycoprotein and serum amyloid A, increased by ME3738-induced interleukin-6, might protect against concanavalin A-induced liver injury.

Keywords: Protective effect; Acute phase protein; Liver injury; Interleukin-6; ME3738

1. Introduction

Infection and injury provoke a range of physiological changes collectively known as the acute phase response. Part of this response consists of an increased production and release of specific proteins, referred to as the positive acute phase proteins, including α1-acid glycoprotein, serum amyloid A, and C-reactive proteins (Kuebler et al., 2004).

These acute phase proteins have immunomodulating and anticoagulant properties (Fournier et al., 2000). Serum amyloid A is the precursor of amyloid A protein, an insoluble degradation product deposited in major organs in secondary amyloidosis, a progressive, fatal disease that can result from chronic or episodic inflammatory conditions (Thorn et al., 2003). Hepatocytes are the primary source of circulating acute phase proteins (Kuebler et al., 2004). Less is known about the functions of these proteins and their role within the acute phase response of the liver.

Cytokines such as interleukin-6, interleukin-1, and interleukin-10 regulate the production of acute phase proteins (Mejdoubi et al., 1999).

We have identified 22β-methoxyolean-12-ene-3β,24(4β)-diol (ME3738) as a novel compound that ameliorates liver failure in several models of acute and chronic liver injury in animals, as well as in patients with chronic hepatitis C infection (Meiji Seika Kaisha, 2005). We found that the immunomodulatory properties of soyasapogenol extracted from *Glycine max* Merr. could potentially treat hepatic disease resulting from abnormal immune responses (Kuzuhara et al., 2000). ME3738 was selected from a series of soyasapogenol-derived compounds on the basis of its efficacy, toxicity, and pharmacokinetic profile in experimental animals (Kuzuhara et al., 2006; Sasaki et al., 2005). ME3738 was well tolerated at up to

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doi:10.1016/j.ejphar.2006.05.003

800 mg/day in a single- and multiple-dose Phase I trial, and a Phase II trial confirmed the safety and efficacy of ME3738 (Meiji Seika Kaisha, 2005).

ME3738 increases serum interleukin-6 levels and activates signal transducers and activators of transcription 3 (Stat3) DNA binding and target gene transcription in mice (Klein et al., 2003). However, it is not clear whether Stat3-induced proteins ameliorate liver injury.

We first confirmed that ME3738 selectively induces interleukin-6 expression, and then used cDNA microarray analysis to identify serum amyloid A 2 and α1-acid glycoprotein as downstream targets of the interleukin-6 signal. We then showed that serum amyloid A and α1-acid glycoprotein, representative acute phase proteins, could protect against concanavalin A-induced liver injury. Finally we showed that α1-acid glycoprotein and serum amyloid A, but not α1-antitrypsin, directly prevented lesions of Hep G2 cells by aflatoxin B_1.

2. Materials and methods

2.1. Animals

Male BALB/c mice weighing 21 to 25 g were purchased from Charles River Laboratory (Yokohama, Japan), and maintained on a commercial diet (NMF, Oriental Yeast Co., Tokyo, Japan). Mice received water ad libitum. The animals were housed in a temperature- and light-controlled room as previously reported (Kuzuhara et al., 2000). All experiments were conducted in accordance with the local institutional guidelines for the care and use of laboratory animals.

2.2. Chemicals and cells

ME3738 (Fig. 1) having chemical formula defined as 22β-methoxyolean-12-ene-3β,24(4β)-diol, was provided by Meiji Seika Kaisha, Ltd. (Tokyo, Japan). ME3738 preparations were tested for endotoxin contamination by the LPS Test from Chromogenix (Chromogenix, Mölndal, Sweden). No endotoxin contamination was detected (data not shown). Concanavalin A, aflatoxin B_1 were purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan). Human α1-acid glycoprotein, recombinant human apo-serum amyloid A, and human α 1-antitrypsin were purchased from CosmoBio Co., Ltd. (Tokyo, Japan), Peprotech EC (London, UK), and Serva Electrophoresis (Heidelberg, Germany), respectively. Commercial kits for interleukin-6, tumour necrosis factor-α, were obtained from Endogen (MA, USA) and Pharmingen (CA, USA), respectively. Commercial kits for interleukin-1α, and interleukin-1β were obtained from Genzyme (Cambridge, MA, USA).

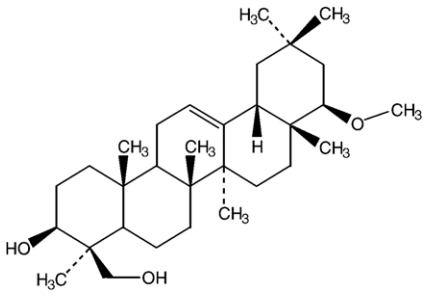

Fig. 1. Structure of ME3738.

Hep G2 cells were purchased from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan), and were cultured in Minimum Essential Medium (Invitrogen Corp., Carlsbad, CA, USA) containing non-essential amino acids, 10% fetal bovine serum (FBS; ICN Biomedicals, Inc., Aurora, IL, USA), 2200 mg/l $NaHCO_3$, 110 mg/l sodium pyruvate, and 292 mg/l L-glutamine in an atmosphere of 5% CO_2 in air at 37 °C.

2.3. ME3738 preparation and injection

Control mice were given 0.5% carboxymethyl cellulose sodium salt (CMC-Na) administered subcutaneously (s.c.) into the back (control group). ME3738 suspended in CMC-Na was injected s.c. at 80 mg/kg body weight. Animals were not starved overnight prior to sacrifice, and were decapitated under ether anesthesia between 0900 and 1000 h.

2.4. Treatment with α1-acid glycoprotein or serum amyloid A prior to concanavalin A injection

Control mice were given saline intraperitoneally (1 ml), then 2 h later were given an intravenous injection of sterilized phosphate-buffered saline. After an intraperitoneal injection of saline (1.0 ml), the concanavalin A group was intravenously injected with concanavalin A (20 mg/kg body weight). The concanavalin A/α1-acid glycoprotein group, the concanavalin A/serum amyloid A group, and the concanavalin A/albumin group were respectively given a solution (1.0 ml) containing 5 mg/ml of α1-acid glycoprotein, 0.03 or 0.1 mg/ml of serum amyloid A, or 5 mg/ml of albumin intraperitoneally at 2 h prior to an intravenous injection of concanavalin A. The animals were not starved overnight prior to sacrifice, and were decapitated under ether anesthesia between 0900 and 1000 h.

2.5. Plasma transferase assay

Plasma and liver samples were obtained at various intervals after concanavalin A injection. Alanine aminotransferase (ALT) levels were determined at 340 nm according to standard methods with an automatic analyzer (Kuzuhara et al., 2000).

2.6. Histological examination

Liver samples were fixed in 10% phosphate-buffered formalin, dehydrated in an alcohol series, and embedded in paraffin. Tissue sections (4 μm thick) were stained with hematoxylin–eosin.

2.7. Determination of α1-acid glycoprotein and serum amyloid A mRNA levels

Gene expression in liver was analyzed by cDNA microarrays (Reilly et al., 2001). RNA was converted to double-stranded cDNA using an oligo (dT) 24-mer containing a T7 promoter

sequence. The resulting double-stranded cDNA was transcribed into biotin-labeled cRNA using T7 RNA polymerase. This cRNA was hybridized to GeneChip Murine Genome U74A v2 arrays (Affymetrix, Inc., Santa Clara, CA, USA). Unbound RNA was washed from the arrays, the remaining biotinylated RNA was stained, and the arrays were scanned. Scanned array images were analyzed using Microarray Suite software (Affymetrix, Inc.). In each array, the hybridization intensity data of individual genes was normalized by the mean value of the intensity data of all measured genes to make experiments comparable.

2.8. Determination of cytokine and serum amyloid A levels

For cytokine determinations, blood was withdrawn from the right atrium. After a brief centrifugation, plasma was recovered and stored at −80 °C until used for measuring cytokine and ALT levels.

Levels of interleukin-6, tumour necrosis factor-α, interleukin-1α, and interleukin-1β in plasma were detected by ELISA as described previously (Kuzuhara et al., 2000). Serum amyloid A levels in plasma were determined with a mouse serum amyloid A ELISA (Zuckerman and Surprenant, 1986).

2.9. In vitro cytotoxicity assay

Cells were plated at 8×10^4 cells/well in 24-well plates and cultured for 2 days. Media were then replaced with fresh medium containing 6% fetal bovine serum and aflatoxin B_1, with or without α1-acid glycoprotein, serum amyloid A, or α1-antitrypsin. Cultures were incubated for 2 days, and cell morphology was examined under a phase-contrast microscope. Cells were fixed with 10% formalin and stained with 0.1% crystal violet (Kuzuhara et al., 2006). Cell viability was determined with a spectrophotometer (Biorad Benchmark Plus microplate spectrophotometer, Tokyo, Japan) at 590 nm after extracting crystal violet by sodium dodecyl sulfate (SDS) at final concentration of 0.2%.

Table 1
Effects of ME3738 on mRNA of serum amyloid A 2 and α1-acid glycoprotein in mouse livers

Gene symbol	Unigene no.	Ratio of expression signal (ME3738/control)	
		4 h	8 h
Serum amyroid A 2	Mm.200941	2.4	9.9
α1-acid glycoprotein 1	Mm.4777	1.1	1.4
α1-acid glycoprotein 2	Mm.14173	0.9	3.6

The data were determined by Affymetrix microarray, and the ratio of expression signal was calculated from the values normalized to the mean of all probe set data in each microarray. The ratio of serum amyloid A 2 to α1-acid glycoprotein 1 represents the mean of the data measured by two different probe sets.

2.10. Statistical analysis

Statistical analysis was performed with Bonferroni's test for the cytotoxicity assays and Student's *t*-test for the cytokine assay. The in vivo data were evaluated by the nonparametric Dunnett's multiple comparison.

3. Results

3.1. Plasma interleukin-6 levels increased in animals treated with ME3738 up to 8 h following injection, and returned to control levels by 12 h (Fig. 2). In contrast to the increase in plasma interleukin-6 levels, plasma levels of tumour necrosis factor-α, interleukin-1α and interleukin-1β in animals treated with ME3738 were within the same range as those of the control animals (Fig. 2).

3.2. Gene expression analysis using cDNA microarrays indicated that ME3738 treatment increased liver mRNA levels of α1-acid glycoprotein 2 and serum amyloid A 2 by 3.6-fold and 9.9-fold, respectively, at 8 h after administration (Table 1). α1-Acid glycoprotein 1 mRNA showed a time-dependent increase, although the maximum

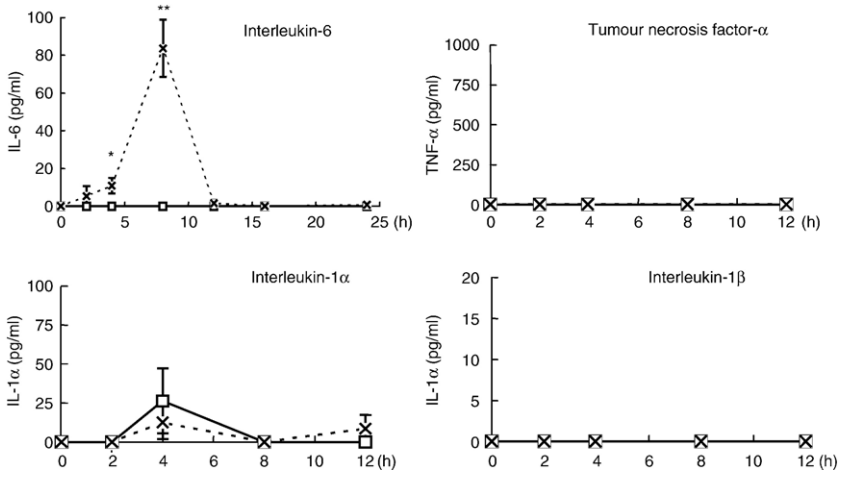

Fig. 2. Time course of interleukin-6, tumour necrosis factor-α, interleukin-1α, and interleukin-1β levels in the control and ME3738 groups. Data are expressed as the mean ± S.E.M. of five mice treated with 0.5% CMC-Na (control group: squares), or ME3738 (80 mg/kg body weight) (ME3738 group: crosses) at various times after administration. Values in no treatment animals and control animals were below detection limits for all cytokines, except for interleukin-1α in control group at 4 h (26.3 ± 20.9 pg/ml). Significantly different from the control group by Student's *t*-test at $P<0.05$ (*) or $P<0.01$ (**).

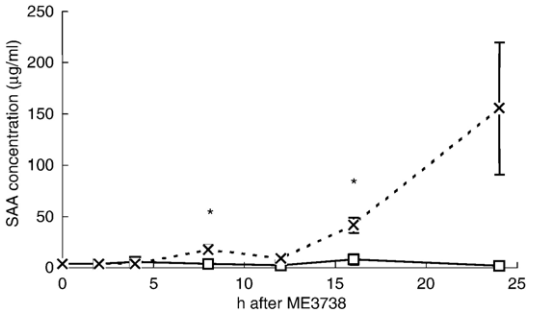

Fig. 3. Time course of serum amyloid A in plasma levels after ME3738 treatment. Serum amyloid A (SAA) levels are expressed as the mean±S.E.M. of five mice treated with 0.5% CMC-Na (squares) or ME3738 (80 mg/kg of body weight) (crosses) at each time point. Significantly different from the control group by Student's *t*-test at $P<0.05$ (*).

level was less than 1.4 times the control (Table 1). ME3738 significantly increased serum amyloid A levels at 8 and 16 h after treatment, especially serum amyloid A concentration at 24 h after treatment increased more than a 40-fold (Fig. 3). No increases in serum amyloid A levels were detected in control animals (Fig. 3).

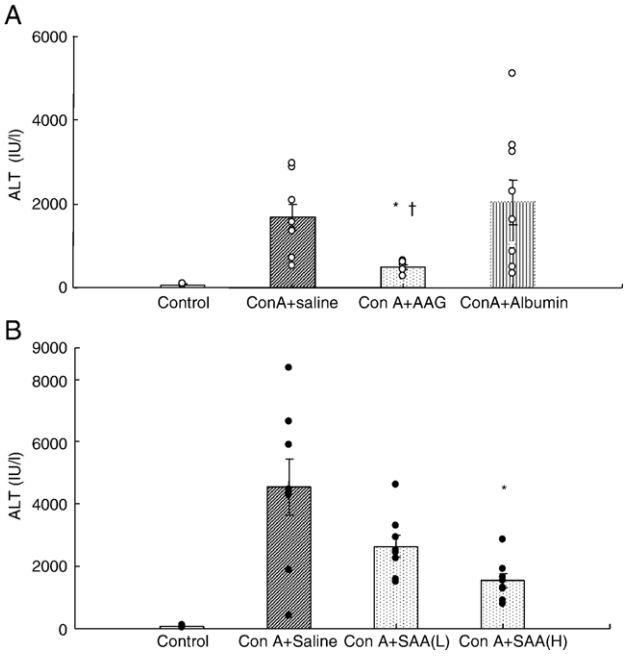

Fig. 4. Effect of α1-acid glycoprotein and serum amyloid A pretreatment on plasma ALT activity at 24 h after concanavalin A treatment. Data are mean±S.E.M. Control mice received a saline injection (i.p.) 2 h prior to an intravenous injection of concanavalin A (20 mg/kg). A pretreatment was injected 2 h prior to intravenous concanavalin A in the concanavalin A (ConA)/α1-acid glycoprotein (5 mg/animal; AAG) group, concanavalin A/albumin group, and concanavalin A/serum amyloid A (0.03 mg/mouse or 0.1 mg/mouse; SAA) group. Animals were sacrificed 24 h after treatment with concanavalin A. (A) shows plasma ALT levels in control: $n=4$; concanavalin A alone, $n=9$; concanavalin A/α1-acid glycoprotein, $n=9$; concanavalin A/albumin, $n=9$. (B) shows plasma ALT levels in control, $n=4$; concanavalin A alone, $n=8$; concanavalin A/serum amyloid A (low dose or high dose), $n=8$. Significantly different from the concanavalin A alone group at $P<0.01$ (*), or from the concanavalin A/albumin at $P<0.05$ (†), based on Dunnett's multiple comparison.

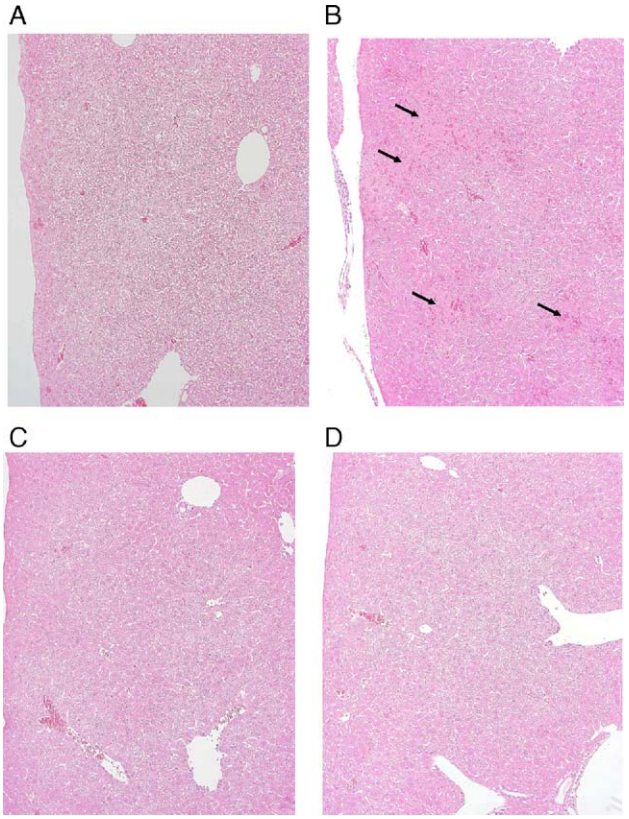

Fig. 5. Histopathological changes in the liver in the control, concanavalin A/α1-acid glycoprotein, and concanavalin A/serum amyloid A groups. A. Control group (H&E staining, ×400). B. Concanavalin A group (H&E staining, ×400). Scattered and massive necrosis with steatosis (arrows in figure) was observed. C and D. (H&E staining, ×400). The scattered, massive necrosis observed in the concanavalin A group was markedly reduced in the concanavalin A/α1-acid glycoprotein (C) and concanavalin A/serum amyloid A (high dose) groups (D).

3.3. Plasma ALT levels in the concanavalin A/α1-acid glycoprotein and concanavalin A/serum amyloid A (high dose) group were markedly lower than those in the concanavalin A alone or the concanavalin A/albumin groups (Fig. 4A–B), and marked alleviation of the multifocal necrosis of hepatocytes in the concanavalin A/α1-acid glycoprotein and concanavalin A/serum amyloid A group was observed at 24 h after concanavalin A treatment (Fig. 5A–D).

3.4. α1-Acid glycoprotein, serum amyloid A and α 1-antitrypsin at indicated concentrations were added to the culture medium with aflatoxin B_1. Under these conditions, α1-acid glycoprotein and serum amyloid A dose-dependently reduced the cell toxicity due to aflatoxin B_1 (Fig. 6A–B). In contrast to these results, α 1-antitrypsin did not reduce aflatoxin B_1-induced cytotoxicity even though at a concentration of 1000 μg/ml (Fig. 6C).

4. Discussion

ME3738 protects against liver injury in different models, including acetaminophen-induced liver injury, α-naphthylisothiocyanate-induced cholestasis, and carbon tetrachloride-induced chronic liver injury, as well as concanavalin A-induced

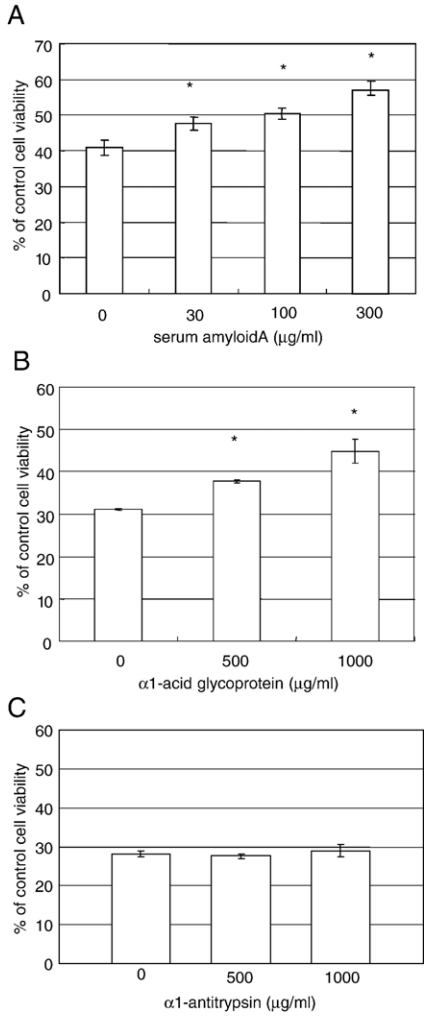

Fig. 6. Dose-dependent improvement in cell viability in Hep G2 cells treated with aflatoxin B_1 and serum amyloid A, α1-acid glycoprotein, or α 1-antitrypsin. Data represent the mean±standard deviation of percentage of control cell viability from six wells treated with fresh culture medium containing fetal bovine serum and aflatoxin B_1, with or without serum amyloid A (A), α1-acid glycoprotein (B), or α 1-antitrypsin (C). Significantly different from the control group at $P<0.05$ (*), based on Bonferroni's test.

hepatitis (Meiji Seika Kaisha, 2005). ME3738 also showed therapeutic potential in a Phase II trial in patients with chronic hepatitis C infection (Meiji Seika Kaisha, 2005). We sought to define how ME3738 protected against concanavalin A-induced liver injury, since concanavalin A activates immune cells in the liver – mainly CD4-positive T-cells and mononuclear cells – which participate in autoimmune or viral hepatitis (Tiegs et al., 1992; Gantner et al., 1995; Trautwein et al., 1998). Interleukin-6 plays an important role in liver regeneration and injury repair (Cressman et al., 1996; Kovalovich et al., 2000, 2001). ME3738-mediated protection against concanavalin A-induced damage involved a reduction in interleukin-6 serum levels and nuclear factor-κB (NF-κB) binding to DNA in liver nuclear extracts (Klein et al., 2003). However, ME3738 can induce STAT3 DNA binding prior to concanavalin A injection. ME3738 treatment induces interleukin-6 serum levels and activates STAT 3 DNA binding and target gene transcription (Klein et al., 2003).

In the present study, we investigated whether ME3738 treatment selectively induces plasma interleukin-6 levels, without an increase in the concentration of pro-inflammatory cytokines, such as tumour necrosis factor-α, interleukin-1α, and interleukin-1β. Plasma interleukin-6 levels increased in animals treated with ME3738 up to 8 h following injection, and returned to normal levels by 12 h. ME3738 treatment did not affect plasma levels of tumour necrosis factor-α, interleukin-1α, or interleukin-1β. Hepatocytes are the major source of acute phase proteins, which are regulated by cytokines such as interleukin-1, interleukin-6, and interleukin-10 (Mejdoubi et al., 1999). Interleukin-6 is well known to signal an increase in acute phase proteins, including α1-acid glycoprotein and serum amyloid A (Klein et al., 2003). Our gene expression analysis using cDNA microarrays indicated that ME3738 treatment increased liver mRNA levels of α1-acid glycoprotein and serum amyloid A at 8 h after administration and significantly increased serum amyloid A levels in the plasma after treatment. These observations suggest that ME3738 increases interleukin-6 levels, leading to increases in α1-acid glycoprotein and serum amyloid A mRNA in the liver, as well as serum amyloid A increases in plasma. However, the role of these acute phase proteins in inhibiting liver injury in the concanavalin A model is unclear. Bovine α1-acid glycoprotein and human α 1-antitrypsin can specifically inhibit the hepatocyte apoptosis induced by tumour necrosis factor-α/galactosamine, but not by anti-Fas in vivo (Libert et al., 1996; Van Molle et al., 1997, 1999). We therefore examined whether exogenously administered α1-acid glycoprotein and serum amyloid A could inhibit the induction of liver necrosis. α1-Acid glycoprotein and serum amyloid A markedly alleviated multifocal hepatocyte necrosis at 24 h after concanavalin A treatment. Thus, the ME3738-induced increases in plasma α1-acid glycoprotein and serum amyloid A concentrations via induction of interleukin-6 may prevent concanavalin A-induced liver failure. α1-Acid glycoprotein and α1-antitrypsin may protect indirectly, since these proteins did not inhibit tumour necrosis factor-α/actinomycin D-induced apoptosis in hepatoma cell lines (Libert et al., 1996; Van Molle et al., 1997, 1999). However, in the present study, α1-acid glycoprotein and serum amyloid A, but not α1-antitrypsin, protected against cellular lesions in Hep G2 cells (Fig. 6A–C). α1-Antitrypsin did not reduce cellular toxicity, even at a concentration of 1000 μg/ml (Fig. 6C). These results suggest that acute phase proteins have distinct mechanisms for protecting against liver injury.

Acknowledgements

We gratefully acknowledge Prof. Hiroshi Yamamoto of Osaka University for his critical review of this manuscript. We also acknowledge the assistance of Miss Nozomi Hoshino in preparation of the manuscript.

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