Glycyrrhizin Inhibits TNF-Induced, but Not Fas-Mediated, Apoptosis in the Human Hepatoblastoma Line HepG2

Masahide YOSHIKAWA,‡ Masahisa TOYOHARA,§ Sigehiko UEDA,§ Akira SHIROL,§ Hiroshi TAKEUCHI,‡
Toshimasa NISHIYAMA,‡ Takatsugu YAMADA,§ Hiroshi FUKUI,§ and Shigeaki ISHIZAKA

Department of Parasitology,‡ Third Department of Internal Medicine,§ Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634–8522, Japan. Received February 19, 1999; accepted June 11, 1999.

To determine the transaminase-lowering action of glycyrrhizin (GL) immunologically, the effect of GL on tumor necrosis factor (TNF)-α- and Fas-mediated apoptosis was assessed using a human hepatoblastoma line, HepG2 cells. The HepG2 cells were resistant to TNF-α and anti-Fas antibody, but were rendered susceptible to TNF-α and anti-Fas antibody in the presence of actinomycin D (Act D), an inhibitor of RNA synthesis. The cytotoxicity induced by TNF-α/Act D or anti-Fas/Act D was accompanied by DNA fragmentation, indicating apoptotic death of HepG2 cells. GL partially prevented the apoptosis of HepG2 cells induced by TNF-α/Act D in a GL-dose dependent fashion. However, this protective effect of GL was not observed in the cytotoxicity of HepG2 caused by anti-Fas/Act D. Although the protection mechanism of GL, observed in a limited fashion against TNF-α-mediated apoptosis, is unclear, the present results provide an immunological explanation for the transaminase-lowering action of GL in the GL treatment of chronic liver diseases involving apoptotic hepatocyte death in their pathogenesis.

Key words apoptosis; tumor necrosis factor-α; Fas; glycyrrhizin

Glycyrrhizin (GL), a saponin fraction of licorice with a defined chemical structure, is used intravenously for various liver diseases including chronic viral hepatitis, and its transaminase-lowering effect is clinically well-recognized in Japan.1–3 Recently, hepatocyte apoptotic cell death, mediated by tumor necrosis factor alpha (TNF-α) receptor 1 (TNF-R1) and Fas, has been believed to be involved in the pathogenesis of chronic viral hepatitis.4–6 Although various pharmacological actions of GL, such as an anti-inflammatory effect,7–10 antiviral effect,11,12 interferon-γ-inducing activity,13 and corticosteroid-enhancing activity,14,15 are considered as mechanisms by which GL lowers the transaminase levels, the effect of GL on hepatocyte apoptosis has not been investigated. In the present study, we investigated the effects of GL on TNF-α- and Fas-mediated apoptosis using a human hepatoblastoma line.

MATERIALS AND METHODS

Cell Lines HepG2 cells,16 a human hepatoblastoma cell line, were obtained from the Riken Cell Bank (Tsukuba, Japan) and were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Nissui Pharmaceutical Co., Tokyo) supplemented with 10% fetal bovine serum (FBS), 2 mmol/l glutamine, 100 IU/ml of penicillin, and 100 mg/ml of streptomycin. A20-HL cells,17 a murine B lymphoma cell line, were cultured in RPMI1640 medium supplemented with 10% FBS, 2 mmol/l glutamine, 100 IU/ml of penicillin, and 100 mg/ml of streptomycin. The penicillin–streptomycin–glutamine mixture and FBS were purchased from Life Technologies Ltd. (Tokyo) and used for the cell cultures.

Reagent Authentic plant test grade GL was obtained from Nacalai Tesque, Inc. (Kyoto). Recombinant human TNF-α was purchased from Genzyme Diagnostics (Cambridge, MA, U.S.A.) and actinomycin D (Act D) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). A murine anti-human Fas monoclonal antibody (CH-11)18 which induces apoptosis in Fas-expressing cells was purchased from MBL (Nagoya, Japan). An anti-murine Fas monoclonal antibody (Jo2)19 was also obtained from MBL.

TNF-α and Fas-Mediated Cytotoxicity HepG2 cells were seeded into 24-well culture plates at 2×10⁵ cells/1.0 ml per well and cultured for 24 h. They were washed with 2.0 ml of 0.01 mol/l phosphate buffered saline, pH 7.4 (PBS), replenished with fresh medium, treated with either TNF-α or anti-Fas in the presence of Act D, and then further incubated at 37°C in the presence or absence of GL. After a 24 h culture, the cell viability was quantitated using a tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.20 Briefly, MTT (obtained from Nacalai Tesque, Kyoto) was added to each well at a final concentration of 0.5 mg/ml. After 3 to 4 h of incubation at 37°C with MTT, the unreacted MTT and medium were removed, and 1.0 ml of dimethyl sulfoxide was added to solubilize the MTT formazan. After gentle agitation for 10 min, the optical density of each well was measured with a microplate reader (Microplate Reader MTP-32, Corona, Japan) equipped with a 540-nm filter. The spectrophotometer was calibrated at 0 absorbance using wells that contained only medium and MTT. The number of live cells was calculated using curve-fitting parameters based on the Marquardt method. The survival rate was determined by comparing the number of live cells in the wells containing TNF-α/Act D or anti-Fas/Act D with that in the well containing medium alone.

DNA Extraction and Agarose Gel Electrophoresis HepG2 cells were seeded in a 9.1 cm² dish at 5×10⁵ cells/3.0 ml and cultured for 24 h. They were washed with PBS, replenished with fresh medium, treated with either TNF-α or anti-Fas in the presence of Act D, and were further incubated at 37°C in the presence or absence of GL. After a 24 h culture, cells from each dish, including detached cells floating in the culture medium and cells that remained attached to the plastic surface, were combined and centrifuged at 800g for 5 min at 4°C. After washing with 3.0 ml of ice-cold PBS, the cell pellets were resuspended in 0.5 ml of a hypotonic lysing solution containing 0.1 mol/l Tris–HCl (pH

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* To whom correspondence should be addressed.
7.5), 10 mmol/l EDTA, and 0.2% Triton X-100, and were incubated on ice for 10 min. The lysate was centrifuged at 15000 g for 10 min at 4 °C. The resultant supernatant, which contained DNA of a small molecular weight, was sequentially treated with phenol and phenol/chloroform/isoamyl-alcohol. The aqueous phase was adjusted to contain 150 mmol/l NaCl and was precipitated with two volumes of ethanol and 1/10 volume of 3 M sodium acetate solution at −20 °C overnight. The pellets were dried and dissolved in 20 μl of 10 mmol/l Tris–HCl containing 1 mmol/l EDTA. The DNA solution was incubated with 20 μl of DNase-free RNase solution (Wako Pure Chemical) for 30 min at 37 °C and was mixed with 0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol. Electrophoresis was performed using 40 mmol/l Tris–acetate and 1 mmol/l EDTA (pH 8.0) in a 2% agarose gel. The gel was then stained with 0.5 μg/ml ethidium bromide for DNA visualization by UV light.

RESULTS

Apoptotic Cell Death of HepG2 Cells Induced by TNF-α and Anti-Fas Antibody Before investigating the effects of GL on HepG2 cell apoptosis induced by TNF-α and anti-Fas, we first examined whether the HepG2 cells in our test were sensitive to these agents. Treatment with TNF-α alone (up to 200 ng/ml) caused no cytotoxicity in HepG2 cells (Fig. 1, left). However, when these cells were cultured in the presence of Act D, the addition of TNF-α induced cytotoxicity in a dose-dependent fashion. The viability of the HepG2 cells was approximately 33% after a 24-h treatment with 1 ng/ml of TNF-α and 0.5 μg/ml of Act D. At concentrations greater than 10 ng/ml of TNF-α, almost all of the HepG2 cells were dead at the end of the 24-h culture.

Similarly, HepG2 cells were nearly resistant to treatment with anti-Fas alone (Fig. 1, right). The decrease in viability after a 24-h treatment with 100 ng/ml of anti-Fas was about 8%. However, the viability of the HepG2 cells decreased by a combination treatment with anti-Fas and Act D in an anti-Fas-dose dependent fashion. The viability of the Hep G2 cells treated with 10 ng/ml and 100 ng/ml of anti-Fas in the presence of 0.5 μg/ml of Act D was 43% and almost 0%, respectively. Internucleosomal DNA fragmentation was also observed in the cells treated with either TNF-α or anti-Fas in the presence of Act D (Fig. 2).

Effects of GL on TNF-α- and Fas-Mediated Apoptosis of HepG2 Cells We next investigated the effects of GL on TNF-α- and Fas-mediated apoptosis of HepG2 cells. When GL was added to the HepG2 cultures containing 1 ng/ml of TNF-α and 0.5 μg/ml of Act D, the viability of the HepG2 cells was increased in a GL-dose dependent fashion (Fig. 3, left). In addition, the formation of internucleosomal DNA fragmentation was partly inhibited by GL (Fig. 3, right). We then examined the effect of GL on Fas-mediated apoptosis of HepG2 cells. The addition of GL into the HepG2 cultures containing 10 ng/ml of anti-Fas and 0.5 μg/ml of Act D did not improve the cell viability (Fig. 4, left). The formation of internucleosomal DNA fragmentation was not inhibited by...
GL (Fig. 4, right). The anti-apoptotic action of GL was not detected in repeated experiments at various concentrations of anti-Fas and Act D, other than 10 ng/ml of anti-Fas and 0.5 µg/ml of Act D (data not shown).

DISCUSSION

GL, one of the aqueous extracts from licorice root (Glycyrrhiza glabra), is known to decrease elevated alanine aminotransferase (ALT) levels and is used as a therapeutic agent for chronic viral hepatitis. Some pharmacological actions, such as the inhibition of phospholipase A2 activity, the inhibition of complement activity, and the enhancement of endogenous glucocorticoid action by suppressing its metabolism, have been proposed as mechanisms by which GL elicits a transaminase-lowering action. Recently, hepatocyte apoptosis has been thought to play an important role in the pathogenesis of chronic viral hepatitis. However, the effect of GL on hepatocyte apoptosis has not been studied. Although we previously demonstrated an anti-apoptotic action of GL against TNF-α-mediated apoptosis using a murine fibroblast line, the effect of GL on hepatocyte apoptosis, especially Fas-mediated apoptosis, has not been investigated. In the present study, we sought to learn whether GL has the ability to prevent apoptosis induced by TNF-α or an agonistic anti-Fas antibody using human hepatoblastoma HepG2.
cells, because these cells are known to be sensitive to TNF-α in the presence of Act D and to express Fas antigen on their cell surface unlike other cell lines including Hep3B hepatoblastoma cells and PRF/PRC/5 hepatoma cells.

GL partially prevented the TNF-α-mediated apoptosis of HepG2 cells, as expected from our previous results regarding the protective effect of GL on TNF-α-mediated apoptosis in a murine fibroblast line. This protective effect was observed at GL concentrations of more than 20, but not at a concentration of 2 μg/ml, in the present in vitro experiments. According to studies on the pharmacokinetics of GL after intravenous (i.v.) administration of the usual clinical dose (80 mg/m in healthy and hepatitis subjects, 22–25) the serum concentrations of GL at 30 min, 4 h and 24 h after GL injection were reported to be approximately 20, 10 and <5 μg/ml, respectively. Therefore, it seems unlikely that serum GL concentrations rise and are retained in ranges high enough to elicit the protective effect on TNF-α-mediated apoptosis after intravenous administration of the usual clinical dose. However, GL has been shown to be distributed preferentially in liver and excreted in the bile, 26–27 suggesting the possibility that immunomodulation by GL could be elicited in liver. In addition, i.v. administration of a high GL dose, 200 mg/m, was reported to give serum levels of 30–60 μg/ml at 30 min and 10–30 μg/ml 6 h after injection. 26 Considering these pharmacokinetic behaviors of GL after i.v. administration, repeated GL administration of higher doses (>200 mg) is most useful to induce the protective effect of GL on TNF-α-mediated apoptosis of hepatocytes.

However, the protective effect of GL was not observed in the cytotoxicity of HepG2 caused by anti-Fas and Act D. GL failed to prevent the Fas-mediated apoptosis of HepG2. As the transcriptional block by Act D was known to potentiate the Fas-induced apoptosis, 26,27 we assessed the effect of GL on direct cytotoxicity of anti-Fas. For this purpose, we used a murine B lymphoma line, A20-HEL. A20-HEL cells were already known to express the Fas antigen on the cell surface and to fall in apoptosis by treatment with the anti-Fas antibody, Jo2, alone, 29 although they are resistant to TNF-α. As shown in Fig. 5, the anti-apoptotic effect of GL was not observed in the A20-HEL cells, suggesting that GL is unable to protect Fas-expressing cells from the apoptosis mediated by the Fas antigen, irrespective of the presence of Act D.

It is known that TNFR1 and Fas share a signal cascade in one apoptotic pathway, but the former is also known to activate additional signaling pathways, including one that activates a survival signal through the activation of the nuclear factor kapper B (NFκB). 31 In contrast, Fas is believed to deliver only a death signal. The protection mechanism of GL, observed in a limited fashion against TNF-α-mediated apoptosis, is unclear. Two possibilities can be considered. First, GL may preferentially activate some survival signaling pathway mediated by TNFR1. Second, GL may inhibit the death-delivering pathway, but the inhibitory effect may be extinguished by the high magnitude of Fas-induced apoptosis.

In previous reports regarding the effects of GL on immune responses, GL has been shown to augment major histocompatibility complex I expression on murine tumor cell lines and interleukin-2 production of murine splenocytes in vitro, 32,33 and has also been shown to enhance the in vivo production of interferon-γ in mice. 13 These immunological actions may potentiate cytotoxic T lymphocyte (CTL)-mediated cytotoxicity against hepatocytes and lead to a rise in serum transaminase levels, contrary to the clinical observations of a decrease in serum transaminase levels following intravenous administration of GL. Although precise mechanisms of GL in protecting hepatocytes from apoptosis remain to be investigated, the present result provides an immunological explanation for the transaminase-lowering effect of GL in the treatment of chronic viral hepatitis.

REFERENCES


