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Role of the Fas/Fas Ligand Death Receptor Pathway in Ginseng Saponin Metabolite-Induced Apoptosis in HepG2 Cells

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This research team found in previous studies, that the ginseng saponin metabolite IH901 induces apoptosis in HepG2 cells *via* a mitochondrial-mediated pathway, which resulted in the activation of caspase-9 and subsequently of caspase-3 and -8. Based on these results, the involvement of the Fas/Fas ligand (FasL) death-receptor pathway, in IH901-induced apoptosis in HepG2 cells, was investigated. Levels of Fas and the Fas ligand (FasL) mRNA or protein were not increased by IH901, rather they were decreased significantly at 18 h post treatment. Soluble FasL (sFasL) was detectable by immunoprecipitation analysis in the medium of HepG2 cells treated with IH901. Increased levels of sFasL were inversely correlated with the levels of FasL. Preincubation of HepG2 cells with antagonistic anti-Fas antibody showed little protective effect, if any, on IH901-induced cell death. At a 30 μM (24 and 48 h) and 40 μM (24 h) concentration of IH901, the cytotoxic effect of IH901 was less then 50%, anti-Fas antibody prevented IH901-induced cell death. However, at a 60 μM (24 and 48 h) and 40 μM (48 h) concentration of IH901, cell death rates were about 80% or more and most of the chemopreventive and chemotherapeutic effects of IH901 were manifested. Blocking the Fas receptor did not influence IH901-induced cell death. These results indicate that the Fas/FasL system is engaged, but not required for IH901-induced cell death, at pharmacologically significant concentrations.

Key words: Ginseng saponin metabolite, IH901, HepG2, Apoptosis, Fas/Fas ligand

INTRODUCTION

Fas is a 48-kD membrane protein which belongs to the tumor necrosis factor (TNF) receptor family of proteins. It has been found in several tissues, including thymus, liver, spleen, ovary and heart, and in a number of cells, such as activated T and B lymphocytes (Poulaki *et al.*, 2001). Ligation of Fas by the Fas ligand (FasL) or cross-linking antibodies, results in receptor trimerization followed by the binding of the adaptor molecule, Fas-associated death domain (FADD), to the cytoplasmic domain of the receptor. FADD, in turn, recruits and activates procaspase-8. The activation of the first step of the caspase cascade triggers the activation of several effector procaspases involving caspase-3 (Medema *et al.* 1997).

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FasL, a transmembrane protein belonging to the TNF family, has been implicated in the apoptosis of accumulated cytotoxic T cells (Kagi *et al.*, 1994; Braun *et al.*, 1996). FasL is expressed in many malignant tumors, and has therefore been suggested to participate in the mechanism underlying immune evasion by the malignancies (Connell *et al.*, 1996; Strand *et al.*, 1996; Niehans *et al.*, 1997). Membrane-bound FasL may be cleaved by a specific matrix metalloproteinase-like enzyme to a soluble form (sFasL), which consists of the largest part of the extracellular domain of the FasL molecule (Kayagaki *et al.*, 1995; Mitsiades *et al.*, 1998). Although the role of sFasL in apoptosis is still controversial, the capacity of naturally processed sFasL to induce apoptosis is thought to be much lower than that of cell-surface FasL, indicating that shedding FasL is a mechanism by which its proapoptotic potency is downregulated (Schneider *et al.* 1998; Tanaka *et al.*1998).

20-*O*-(β-D-Glucopyranosyl)-20(*S*)-protopanaxadiol (IH901), an intestinal-bacterial metabolite of ginseng saponins formed from the ginsenosides Rb_1, Rb_2 and

Rc, is suggested to be a potential chemopreventive agent. IH901 is non-toxic, inhibits glucose uptake by tumor cells (Hasegawa *et al.*, 1995a) and reverses multidrug resistance in tumor cells (Hasegawa *et al.*, 1995b). Moreover, it has previously been found that IH901 possesses chemopreventive-chemotherapeutic potential, as it demonstrated antigenotoxic and anticlastogenic activity induced by benzo[a]pyrene (Lee *et al.*, 1998), antitumor activity in cisplatin-resistant pulmonary adenocarcinoma cells (Lee *et al.*,1999) and apoptosis-inducing activity in HL-60 cells (Lee *et al.*, 2000). Recently it was demonstrated that IH901 induces apoptosis in HepG2 cells *via* a mitochondria-mediated pathway, which resulted in the activation of caspase-9 and subsequently of caspase-3 and -8. Caspase-8 cleaves Bid to form tBid, which then relocates to the mitochondria and amplifies the mitochondrial pathway (Oh & Lee, 2004). In the present study, the role of the Fas/FasL system in IH901-induced apoptosis of HepG2 cells was examined. It was found that sFasL is increased by treatment with IH901, with a concomitant decrease in the level of membrane-bound FasL. Antagonistic anti-Fas antibody showed only marginally protective effects, if any, on IH901-induced cell death. This data suggests that the Fas death receptor pathway is not required for IH901-induced apoptosis in HepG2 cells.

MATERIALS AND METHODS

Chemicals and cell culture

IH901 was biosynthesized according to the method of Hasegawa *et al.*, (1996). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of the highest purity grade available. HepG2 cells were maintained in the logarithmic phase of growth in RPMI 1640 medium (GIBCO BRL, Grand Island, NY) at 37°C in a 5% CO_2-95% air humidified incubator and supplemented with heat inactivated 10% fetal bovine serum (GIBCO BRL) and 2 mM L-glutamine (Sigma Chemical Co.).

Inhibition of the Fas/FasL interaction

Cells were preincubated for 1 h at 37°C with nonspecific mouse IgG or anti-Fas antibody (1 μg/mL; Santa Cruz) and IH901 was added. After 24 and 48 h, cells were analyzed for the percentage of death, by MTT assay.

Reverse transcriptase-polymerase chain reaction (RT-PCR) for Fas and FasL

The total cellular RNA was extracted using an RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturers instructions. First strand cDNA was synthesized from 5 μg of each RNA , using AMV reverse transcriptase XL and oligo dT-adaptor primers (Takara Biomedicals, Ohtsu, Japan). Amplification for Fas and FasL was performed using a premix Taq kit (Takara Biomedicals) as follows: an initial denaturation at 94°C for 2 min followed by 35 cycles of denaturation (94°C, 1 min), annealing (53°C, 1 min) and elongation (72°C, 1.5 min). The cycling conditions for GAPDH amplification were identical, except that the annealing temperature was set to 55°C and amplification was reduced to 30 cycles. Specific primer sets were as follows: Fas (sense, 5-GAC CCA GAA TAC CAA GTG CAG ATG TA-3; antisense, 5-CTG TTT CAG GAT TTA AGG TTG GAG ATT-3), FasL (sense, 5-TCT CAG ACG TTT TTC GGC TT-3; antisense, 5-CCT CTA GTC TTC CTT TTC CA-3) and GAPDH (sense, 5-CCC CTT CAT TGA CCT CAA CTA C-3; antisense, 5-CAT GGT GGT GAA GAC GCC AG-3. Twelve μL (GAPDH: 6 μL) of each PCR were separated by electrophoresis on 1.8% agarose gels and visualized by ethidium bromide staining.

Western blot analysis

Cells were washed with PBS and lysed (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 5 mM EGTA, 50 mM β-glycerophosphate, 20 mM NaF, 1 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin and 10 μg/mL aprotinin). Cell lysates were centrifuged and the protein content was determined. Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (12-15%), transferred to a nitrocellulose membrane and immunoblotted with antibodies as indicated. Detection was performed by using enhanced chemiluminescence Western Blotting Detection Reagents (Amersham, Piscataway, NJ). Monoclonal human anti-Fas and anti-FasL antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Transduction Laboratory (Lexington, KY), respectively. Monoclonal human anti-sFasL antibody was purchased from PharMingen (San Diego, CA).

Immunoprecipitation for sFasL

One mL of incubation medium was immunoprecipitated, with mouse antibody to human CD95L (NOK-1, PharMingen) and IMMUNOcatcher (CytoSignal Research Products, Irvine, CA), according to the suppliers instruction.

RESULTS AND DISCUSSION

The role of the Fas/FasL system in chemotherapy-induced apoptosis of tumor cells is well documented. To study the involvement of the Fas/FasL system in IH901-induced apoptosis, the effects of IH901 on cell death in HepG2 cells were tested first. At 60 μM, IH901 induced time-dependent cell death and DNA fragmentation in HepG2 cells (Fig. 1A, B). The effect of IH901 (60 μM) on

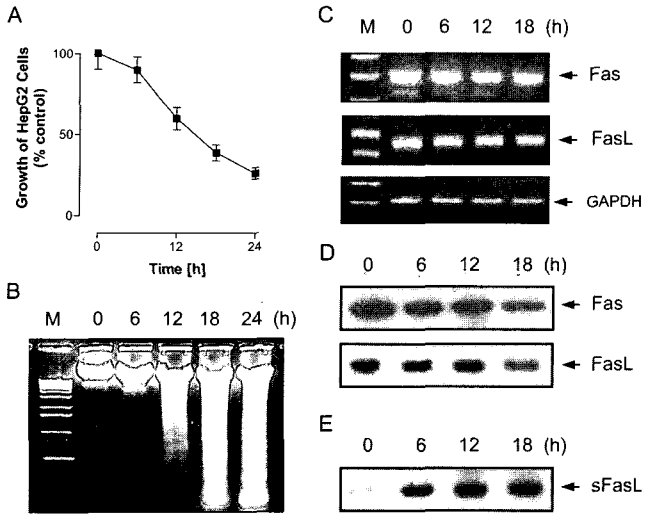

Fig. 1. Expression of the Fas/FasL transcript and protein in HepG2 cells treated with IH901. (A) Cells were incubated with IH901 (60 μM) and cell survival was determined by MTT assay. Data represents the mean ± SD of three independent experiments. (B) Genomic DNA was prepared and separated in 1.5% agarose gel then visualized by UV illumination after ethidium bromide staining. (C) Total cellular RNA and (D) protein was prepared from HepG2 cells treated with IH901 (60 μM) and RT-PCR and Western blot analysis was performed. (E) For the detection of sFasL, cells were treated with IH901 and the incubation media were immunoprecipitated with antibody to human CD95L and IMMUNOcatcher. The precipitate was separated on 12% SDS-polyacrylamide gel. Representative data from three independent experiments are presented.

Fas mRNA and protein expression was investigated by RTPCR and western blot analysis. IH901 treatment did not upregulate either Fas or FasL mRNA or protein expression. The protein levels were decreased at 18 h post treatment with IH901 (Fig. 1C, D). Metalloproteinase cleaves the 40-kD membrane-bound FasL to generate the 2629-kD soluble fragment (Kayagaki *et al.*, 1995; Mitsiades *et al.*1998). It was found that processed sFasL was detectable in the supernatants of HepG2 cells treated with IH901. Levels of sFasL were increased by treatment with IH901 and were inversely correlated with levels of FasL (Fig. 1E).

To further investigate the role of the Fas/FasL system in IH901-induced apoptosis, HepG2 cells were pretreated with nonspecific mouse IgG or ZB4, an antibody antagonistic to Fas that binds to the FasL binding site on a Fas receptor and blocks Fas activation. IH901-induced cell death was then examined by MTT assay. Pretreatment of cells with antagonistic antibody showed only marginally protective effects, if any, on IH901-induced cell death. At a 30 μM (24 and 48 h) and 40 μM (24 h) concentration of IH901, where the cytotoxic effect of IH901 was less then 50%, anti-Fas antibody prevented IH901-induced cell death. However, at a 60 μM (24 and 48 h) and 40 μM (48 h) concentration of IH901, where cell death rates were about 80% or more and most of the chemopreventive and chemotherapeutic effects of IH901 were manifested, blocking the Fas receptor did not influence IH901-induced cell death (Fig. 2). These results indicate that the Fas/FasL system is engaged but not required, for IH901-induced cell death at pharmacologically significant concentrations.

The Fas/FasL system is a key signal transduction pathway of apoptosis in cells and tissues. Ligation of Fas by FasL or cross-linking antibody, results in receptor trimerization followed by the binding of the adaptor molecule FADD to the cytoplasmic domain of the receptor. FADD, in turn, recruits and activates procaspase-8. This activation of the first step of the caspase cascade, triggers activation of several effector procaspases involving caspase 3 (Medema *et al.*, 1997). In contrast to the ubiquitous expression of Fas, the expression of FasL is limited to activated T lymphocytes and natural killer cells, as well as to some immunoprivileged tissues, such as, the testis, placenta, brain and eye [reviewed in 1]. FasL is also expressed in a wide variety of tumors which include various carcinomas, such as, hepatocellular carcinoma (Strand *et al.*, 1996; Lee *et al.*, 2001), lung carcinoma (Niehans *et al.*, 1997) and liver metastasis of colon carcinoma cells (Shiraki *et al.*, 1997). It helps them evade the immune system by killing Fas-expressing activated lymphocytes. Like TNF-α, membrane-bound FasL is processed to sFasL and shed from the surfaces of the cells by a metalloproteinase (Kayagaki *et al.*, 1995; Mitsiades *et al.*, 1998). Soluble TNF-α induces inflammation, whereas membrane TNF-α mediates cytotoxicity (Bazzoni & Beutler, 1996; Perez *et al.*, 1990). However, both membrane and soluble FasL are capable of inducing apoptosis and non-apoptotic responses, including inflammation, induction of transcription factors and cell proliferation (Wajant, 2002; Nelson *et al.*, 2000; Wollert *el al.*, 2000). Nonetheless, membrane FasL is more effective than sFasL in inducing cell apoptosis

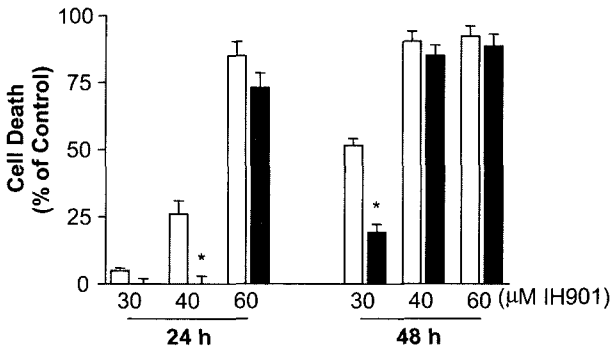

Fig. 2. HepG2 cells were preincubated with anti-Fas antibody (1 μg/mL) (■) or medium only (□), for 1 h and then treated with IH901. Cell death was monitored by MTT assay. Data represent mean ± SD of three independent experiments.

and tissue inflammation, suggesting that the shedding of membrane FasL provides a mechanism by which Fas/FasL signaling is down-regulated (Schneider *et al.*, 1998; Tanaka *et al.*, 1998; Nagata, 1999; Date *et al.*, 2003).

The role of sFasL in the induction of apoptosis is controversial. Lethal effects on Fas-bearing cells are exerted by sFasL (Aoki *et al.*, 2001; Frankel *et al.*, 2000). However, *in vitro* sFasL exerted an anti-apoptotic effect by competing with membrane-bound FasL for its binding to Fas (Oyaizu *et al.*, 1997). Tanaka *et al.* found that the release of sFasL down-regulated the expression of membrane-bound FasL (Tanaka *et al.*, 1998). Considering the hepatotoxicity of FasL, circulating sFasL, if active, would be pathological. Neither patients with tumors, nor individuals with high numbers of activated T cells suffer from hepatitis, implying that sFasL *in vivo* is at best poorly active. Schneider *et al.*, by injection of sFasL into mice, with no signs of detrimental effects, demonstrated that sFasL, without cross-linking antibody, is not active *in vivo* (Schneider *et al.*, 1998).

Recently, it was demonstrated that IH901 induces apoptosis in HepG2 cells *via* a mitochondrial-mediated pathway, this results in the activation of caspase-9 and subsequently of caspase-3 and -8. Caspase-8 cleaves Bid to tBid, which then relocates to the mitochondria and amplifies the mitochondrial pathway (von Haefen *et al.*, 2003). These results and the present data indicate clearly that the Fas/FasL death receptor pathway is not critical for IH901-induced apoptosis. However, the role of sFasL released by treatment with IH901 remains unanswered. This should be investigated thoroughly in the next set of experiments. Taken together, these findings suggest that the Fas/FasL system is engaged but not required for IH901-induced death of HepG2 cells.

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