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Induction of Fas receptor and Fas ligand by nodularin is mediated by NF-κB in HepG2 cells

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ABSTRACT

Nodularin is a natural toxin with multiple features, including inhibitor of protein phosphatases 1 and 2A as well as tumor initiator and promoter. One unique feature of nodularin is that this chemical is a hepatotoxin. It can accumulate into the liver after contact and lead to severe damage to hepatocyte, such as apoptosis. Fas receptor (Fas) and Fas ligand (FasL) system is a critical signaling network triggering apoptosis. In current study, we investigated whether nodularin can induce Fas and FasL expression in HepG2 cell, a well used *in vitro* model for the study of human hepatocytes. Our data showed nodularin induced Fas and FasL expression, at both mRNA and protein level, in a time- and dose-dependent manner. We also found nodularin induced apoptosis at the concentration and incubation time that Fas and FasL were significantly induced. Neutralizing antibody to FasL reduced nodularin-induced apoptosis. Further studies demonstrated that nodularin promoted nuclear translocation and activation of p65 subunit of NF-κB. By applying siRNA targeting p65, which knocked down p65 in HepG2 cells, we successfully impaired the activation of NF-κB by nodularin. In these p65 knockdown cells, we observed that Fas and FasL expression and apoptosis induced by nodularin were significantly reduced. These findings suggest the induction of Fas and FasL expression and thus cell apoptosis in HepG2 cells by nodularin is mediated through NF-κB pathway.

Introduction

Nodularin is a cyclic nonribosomal peptide produced by brackish-water cyanobacterium *Nodularia spumigena* (Carmichael, 1988; Rinehart et al., 1988). The cyanobacterium *N. spumigena* has been implicated in toxic bloom formation in brackish and estuarine environments, such as lakes, reservoirs and slow rivers; accumulates in mussels, flounder, and cod; and often contaminates the drinking water of rural communities (Sivonen and Jones, 1999). Nodularin has been identified as a potent inhibitor of protein phosphatases 1 and 2A, thereby resulting in hyperphosphorylation of a number of proteins, such as cytokeratins 8 and 18 (Yoshizawa et al., 1990; Honkanen et al., 1991; Ohta et al., 1994). It was noted that nodularin increases the formation of reactive oxygen species and induces oxidative stress and lipid peroxidation (Lankoff et al., 2002; Bouaicha and Maatouk, 2004). Nodularin is also identified as a carcinogen possessing both tumor initiator and promoter properties (Ohta et al., 1994; Higashi et al., 2004).

One of most unique feature of nodularin is its hepatotoxicity. After entering the body, nodularin accumulates into the liver through active uptaking of bile acid carrier transport system and causes serious damage to the liver, including cytoskeletal disorganization, loss of membrane integrity, DNA fragmentation, cell blebbing, and apoptosis (Carmichael, 1997; Dittmann and Wiegand, 2006). In severe cases, acute accumulation of nodularin liver will lead to necrosis, intrahepatic bleeding and hemorrhagic shock (Carmichael, 1997; Dittmann and Wiegand, 2006). Previous studies have shown that nodularin-induced apoptosis is related to protein phosphatase inhibition, oxidative stress and DNA damage (Fladmark et al., 1999; Lankoff et al., 2006; Solstad et al., 2008). However, the detailed mechanism on how nodularin induces apoptosis remains to be explored.

Apoptosis, also known as programmed cell death, is a complex process which plays a critical role during cell function, development and a wide variety of pathophysiological processes (Earnshaw et al., 1999). Fas receptor (Fas) and Fas ligand (FasL) signaling is one of the major mechanisms initiating apoptosis. FasL is a 40 kDa type-II transmembrane protein that belongs to tumor necrosis factor family of cytokines; and Fas is a 48 kDa type I transmembrane receptor that belongs to death receptor 2 (Maher et al., 2002). How Fas/FasL initiate apoptosis is well studied. The binding of FasL and Fas will result in the interaction between death domains (DD) in the cytoplasmic region of Fas and the C-terminal of Fas associated death domain (FADD); and therefore the recruitment of FADD, the cellular FLICE inhibitory protein, caspase-8 and caspase-10 to form death inducing signaling complex (DISC) (Chinnaiyan et al., 1995; Kischkel et al., 1995; Guicciardi and Gores, 2009). The DISCs are quickly formed after the engagement of Fas and subsequently activate caspase-8 and 10 to

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initiate apoptosis. There are 2 types of Fas-induced apoptosis. In type I, cells are characterized by high level of DISC formation and sufficient amount of activated caspase-8. In type II, there are lower levels of DISC formation and activated caspase-8. In this case, caspase-8 catalyzes the cleavage of BH3 interacting domain death agonist (Bid) into its truncated form, tBid; and tBid subsequently mediates the release of pro-apoptotic proteins, such as cytochrome c and Smac/DIABLO, from mitochondria. This leads to the activation of procaspase-9, which in turn cleaves downstream caspases (Guicciardi and Gores, 2009).

Many studies have reported that Fas/FasL system is a key signaling pathway in the regulation of apoptosis in different cell types, e.g., in the immune system and the progression of cancer cells (Guicciardi and Gores, 2009). It is also found that hepatocytes are very sensitive to Fas-induced-apoptosis (Ogasawara et al., 1993; Rouquet et al., 1996; Muller et al., 1997). However, it still remains largely unknown how FasL/Fas system is regulated to maintain the proliferation and apoptotic signals in response to extrinsic and intrinsic stimulants.

In this study, we investigated whether nodularin can induce Fas and FasL expression in HepG2 cell, a well used *in vitro* model for the study of human hepatocytes. Our data showed nodularin induced Fas and FasL expression as well as cell apoptosis. Further studies demonstrated that nodularin induced the activation of NF-κB. siRNA targeting p65 subunit of NF-κB inhibits nodularin-induced NF-κB activation, Fas and FasL expression and apoptosis. These findings suggest that NF-κB mediates the induction of Fas and FasL expression and cell apoptosis by nodularin in hepatocytes.

Materials and methods

Chemicals and reagents. Nodularin was obtained from Alexis Biochemicals and dissolved in water to 1.2 mM. Nodularin solution was stored at −80 °C until use. SignalSilence NF-κB p65 siRNA and scrambled siRNA were obtained from Cell Signaling. Neutralizing antibody to human FasL was obtained from R&D Systems, USA. Unless otherwise noted, all other materials were obtained from Sigma-Aldrich, USA.

Cells and culture conditions. HepG2 (Human hepatocellular carcinoma cell line) was obtained from ATCC and maintained in Dulbecco's modified Eagle's medium (Invitrogen, USA) with 10% fetal bovine serum (Invitrogen), 10% nonessential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen) and 1% antibiotics (Invitrogen) in 5% CO2 and 37 °C incubator. For most experiments, HepG2 cells were seeded at equal density on culture plates till they reach 80% confluence before any treatment. For transient transfection studies with scrambled or NF-κB siRNA, cells were transfected in suspension and then attaching condition for 24 h before any treatment.

RNA extraction, reverse transcription-PCR (RT-PCR) and quantitative real-time PCR. After treatment, total RNA was extracted with TRIzol reagent (Invitrogen) following the manufacturer's instruction. cDNA was synthesized with oligo-dT primer by using Sprint RT Complete kit (Clontech, USA).

For semi-quantitative RT-PCR, synthesized cDNA was subjected to a 20-cycle amplification with the following primers: Fas sense, 5′-GAC CCA GAA TAC CAA GTG CAG ATG TA-3′; Fas antisense, 5′-CTG TTT CAG GAT TTA AGG TTG GAG ATT-3′; FasL sense, 5′-ATG TTT CAG CTC TTC CAC CTA CAG AAG GA-3′; FasL antisense, 5′-CAG AGA GAG CTC AGA TAC GTT GAC-3′; Actin sense, 5′-TCG TGG GCC GCC CTA GGC A-3′; and Actin antisense, 5′-CGT GAG GGA GAG CAT AGC C-3′. PCR products were separated on 1% agarose gel, stained with ethidium bromide and photographed.

For real-time PCR, synthesized cDNA was amplified with iQ SYBR Green PCR Supermix (Bio-rad, USA). PCR products were subjected to melting curve analysis by using i-Cycler system (Bio-rad) to detect and quantify targeted genes. The levels of target genes were calculated after normalization with GAPDH levels. Sequences of the

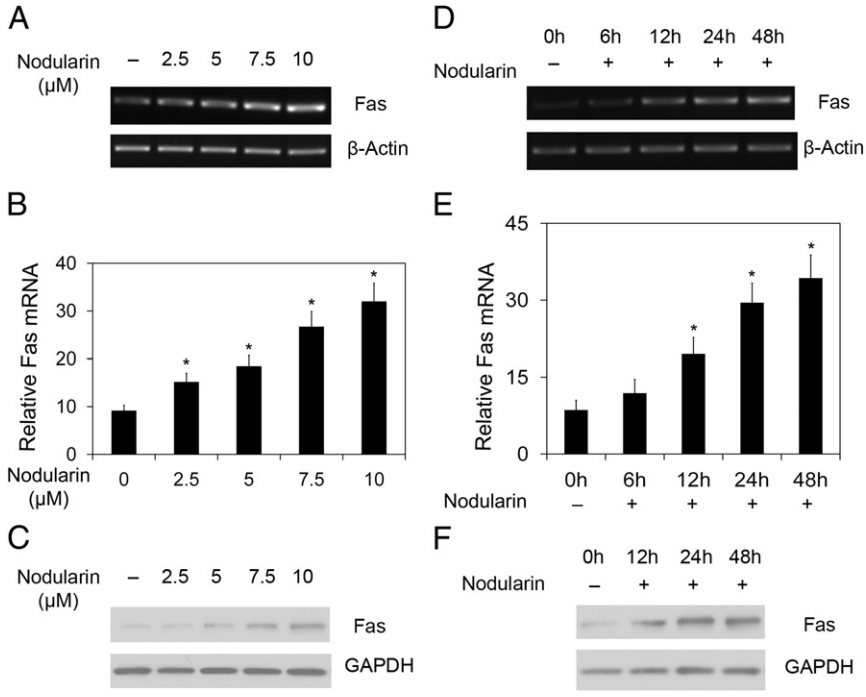

Fig. 1. Nodularin induces Fas expression in dose- and time-dependant manner. For dose–response studies, HepG2 cells were treated with nodularin at a concentration of 2.5, 5, 7.5 or 10 μM for 24 h. mRNA or protein was then extracted and the expression of Fas was evaluated by (A) semi-quantitative RT-PCR, (B) quantitative real-time PCR and (C) immunoblots. Expression of Fas was normalized by expressions of β-actin mRNA, GAPDH mRNA and GAPDH protein, which were used as internal control in panels A–C, respectively. For time course studies, HepG2 cells were treated with 7.5 μM nodularin 6, 12, 24 and 48 h for Fas mRNA and 12, 24 and 48 h for Fas protein. Expression of Fas was evaluated by (D) semi-quantitative RT-PCR, (E) quantitative real-time PCR and (F) immunoblots. β-actin mRNA, GAPDH mRNA and GAPDH protein were used as internal control in panels D–F, respectively. Data are representative from three independent experiments. For real-time PCR results (B and E), each bar represents mean ± SD. *, $P<0.05$ compared to non-treatment control.