Sensitization of Human Hepatic Stellate Cells to Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand-Induced Apoptosis by Leflunomide

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During the resolution phase of hepatic fibrosis, a crucial mechanism is the apoptosis of activated hepatic stellate cells (HSCs). It is necessary to find more anti-fibrosis drugs that would modulate HSCs to be more susceptible to apoptotic stimuli. Here we showed that A771726, the active metabolite of leflunomide, markedly enhanced tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in the human hepatic stellate cell line LX-2. A771726 could increase caspase activity in LX-2 cells in a dose-dependent manner. A771726 did not increase the expression of TRAIL receptors in LX-2 cells but could inhibit activation of the c-Jun NH2-terminal kinase (JNK) pathway through decreasing TRAIL-induced JNK and c-Jun phosphorylation. Moreover, A771726 could accelerate TRAIL-induced apoptosis via inhibiting nuclear factor-kappaB (NF-κB) activation in LX-2 cells. In conclusion, our results indicated leflunomide could enhance the sensitivity of LX-2 cells to TRAIL-induced apoptosis via inhibiting the survival pathways and provided a promising approach to anti-fibrotic therapy with leflunomide.

Key words hepatic fibrosis; leflunomide; hepatic stellate cell; tumor necrosis factor-related apoptosis-inducing ligand; apoptosis

Hepatic fibrosis is a common consequence of chronic hepatitis and chronic liver injury of any etiology leads to liver cirrhosis.1) Hepatic fibrosis is characterized by the excessive accumulation of extracellular matrix (ECM) proteins including collagens that collectively form hepatic scars. If the hepatic injury persists, hepatic stellate cells (HSCs) proliferate and undergo dramatic transdifferentiation from quiescent vitamin A-storing cells to activated myofibroblast-like cells.2) The activated HSCs express collagen I and other extracellular matrix genes and are the major fibrogenic cell type that contributes to collagen accumulation during fibrosis.

Current evidence indicates that hepatic fibrosis is reversible as liver injury subsides.3) Spontaneous recovery from hepatic fibrosis includes degradation of ECM proteins, apoptosis of activated HSCs and regeneration of hepatocytes.4) During spontaneous recovery from liver fibrosis, a vital mechanism is the apoptosis of activated HSCs. Apoptosis has emerged as an important mechanism to reduce numbers of activated HSCs during the resolution phase of hepatic fibrosis.5) For the development of anti-fibrosis therapy, it is important to make HSCs more susceptible to apoptotic stimuli. Previous study showed that HSCs exhibited an increased susceptibility to soluble Fas ligand (sFasL)-mediated apoptosis in combination with cycloheximide.6) It is necessary to find more compounds that would enhance the apoptosis sensitivity of HSCs.7)

Leflunomide is a novel immunosuppressive and anti-inflammatory agent. Several drug-related side events are known to be associated with leflunomide therapy, such as diarrhoea, increase in liver enzymes, pancypotenopia, rash or even severe interstitial pneumonitis. Nevertheless, clinical studies have evidently shown that leflunomide has a positive effect on the treatment of rheumatoid arthritis (RA) patients.8) Leflunomide is a prodrug, which is rapidly converted to the active metabolite A771726 in the gut, plasma and liver. A771726 exerts the immunosuppressive activity.9) Recent evidence suggested that A771726 showed inhibitory effect on carbon tetrachloride (CCL4)-induced hepatic fibrosis in rats.10) Leflunomide pretreatment significantly inhibited the deposition of type I collagen in rat HSCs.11) Based on these results, in this study, we tried to elucidate the effect of leflunomide on HSCs apoptosis.

MATERIALS AND METHODS

Cells and Reagents The human stellate cell line LX-2 was used for these studies. LX-2 cells are an immortalized cell line derived from human HSCs. LX-2 cells were seeded onto plastic tissue culture flasks at 0.9×10^6 cells/ml in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 5% fetal calf serum (FCS), 2 mM l-glutamine, 100 mg/ml streptomycin, and 100 IU penicillin and incubated at 37 °C in a 5% CO2 humidified atmosphere. Recombinant human tumor necrosis factor-related apoptosis-inducing ligand (rhTRAIL) was purchased from R&D Systems (Minneapolis, MN, U.S.A.). Antibodies against α-smooth muscle actin (α-SMA), Poly(ADP-ribose) polymerase (PARP), β-actin and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, goat anti-rabbit IgG antibody were purchased from Cell Signaling Technology. Fluorescein isothiocyanate (FITC)-labelled annexin V-FITC was purchased from BD Pharmingen. Special chemical inhibitors Z-VAD-FMK, Z-DEVD-FMK, SP600125 and BAY 11-7082 were from Calbiochem (La Jolla, CA, U.S.A.). Leflunomide and its active metabolite, A771726, were kindly donated by Cinkate Co. (Shanghai, China).

Apoptosis Assay Briefly, HSCs were seeded in triplicate 6-well plates. LX-2 cells were pretreated with A771726 (0.1, 1.0, 10 μM) for 2 h and stimulated with rhTRAIL for 24 h. Apoptosis was analyzed by staining with annexin V-FITC. FITC-labelled cells were analysed in a BD flow cytometer. All experiments were repeated at least three times.

Western Blotting Analysis LX-2 cells were collected
and washed in cold PBS, and lysed in ice-cold RIPA buffer (10 μM Tris–HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 10 μg/ml protease inhibitor cocktail) for 30 min on ice. The extracted cell protein of each sample was applied to a 7—12% SDS-polyacrylamide gel and transblotted to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Immunodetected proteins were visualized using ECL assay kit (Amersham Biosciences, Buckinghamshire, U.K.).

Quantitative Real-Time Polymerase Chain Reaction (Q-PCR) Total RNA was extracted using Trizol reagent (Invitrogen) and digested with RNase-free DNaseI (Qiagen Ltd.). First-strand cDNA synthesis was performed using a RT-PCR kit with oligo (dT) primers (Promega, Madison, WI, U.S.A.). Q-PCR was performed using the SYBR® Green I Q-PCR MasterMix (Bio-RED) and a Bio-RED IQ5 (Bio-RED) according to the manufacturer’s instructions. The mRNA expression levels of death receptor 4 (DR4) and death receptor 5 (DR5) were calculated using the threshold cycle (Ct) values. All Ct values were normalized to the housekeeping gene β-actin. Specific primers used in the Q-PCRs were as follows: DR4 (sense: 5'-AGAGAGAAATCCCTGCACCA-3', antisense: 5'-GTCACCTCACGGCGTACAAT-3'), DR5 (sense: 5'-CACCGGTTGTTATCGTG-3', antisense: 5'-CCACGGTGTCCTTCATCGC-3'), β-actin (sense: 5'-CATGTTGCATTACCGGCC-3', antisense: 5'-CTCCTTAAATGTCACGGCAGAT-3'). All experiments were repeated at least three times.

NF-κB Luciferase Reporter Gene Assay LX-2 cells were transfected with pNFκB-Luc (Clontech) and pRL-CMV (Promega, Madison, WI, U.S.A.) plasmids using FuGENE 6 (Boehringer Mannheim) and incubated for 18 h at 37 °C. Cells were pretreated with A771726 (0.1, 1.0, 10 μM) for 2 h followed by the treatment with rhTRAIL (300 ng/ml). After 24 h, LX-2 cells were incubated with annexin V-FITC and examined by flow cytometry. Results showed that A771726 could enhance TRAIL-induced caspase-dependent apoptosis. It is not clear whether pretreatment with A771726 could affect the expression of DR4 and DR5 in LX-2 cells, which could be enhanced by pretreatment with A771726 (Fig. 2A). PARP was cleaved to its signature peptide in LX-2 cells (Fig. 2B). Furthermore, prior treatment with pancaspase inhibitor Z-VAD-FMK or caspase-3 inhibitor Z-DEVD-FMK could inhibit TRAIL-induced LX-2 cells apoptosis obviously (Fig. 2C). The results showed that A771726 could enhance TRAIL-induced caspase activation in LX-2 cells.

A771726 did not increase the expression of DR4 and DR5 in LX-2 cells On binding the tumor necrosis factor (TNF) receptor family members DR4 or DR5, TRAIL induces cell death via caspase-dependent apoptosis. It is not clear whether pretreatment with A771726 could affect the expression of DR4 and DR5 in LX-2 cells, which may be a molecular mechanism of sensitization activity of A771726 on TRAIL-induced apoptosis in LX-2 cells. The Q-PCR assay was used to detect the expression of DR4 and DR5 in LX-2 cells. The results showed that the expression of DR4 and DR5 did not change obviously in LX-2 cells when pretreated with A771726 (Fig. 3). The results suggested that A771726 could accelerate TRAIL-induced apoptosis in LX-2 cells via other mechanisms.

A771726 Inhibited JNK Pathway Activation in LX-2 Cells The activation of JNK pathway could promote the proliferation and activation of HSCs. Activated JNK translocates to the nucleus, phosphorylates, and activates the
AP-1 component c-Jun. We detected the activation of JNK pathway in LX-2 cells. The results showed that the phosphorylation of JNK is increased in response to TRAIL stimuli, which could be inhibited by SP600125. Pretreatment with A771726 in LX-2 cells could significantly inhibit TRAIL-induced JNK activation. To show the inhibitory effect of A771726 on JNK activity, phosphorylation of c-Jun, a down-stream target of JNK, was assessed by Western blotting. c-Jun activation has been implicated in a wide range of cellular events. In HSCs, JNK-c-Jun pathway activation induced fibrogenesis and increased proliferation. In agreement with the result of JNK phosphorylation, TRAIL-induced c-Jun phosphorylation was inhibited by A771726 in a dose-dependent manner in LX-2 cells (Fig. 4A). Inhibition of JNK phosphorylation by SP600125, as well as A771726, was effective in enhancing TRAIL-induced apoptosis in LX-2 cells (Fig. 4B). The results suggested that A771726 could accelerate TRAIL-induced apoptosis via inhibiting the activation of JNK pathway in LX-2 cells.

A771726 Inhibited NF-κB Activation in LX-2 Cells

Cytokines of the TNF superfamily, upon receptor ligation, induce a survival signal mediated by activation of NF-κB. We detected the activation of NF-κB in LX-2 cells. As expected, TRAIL stimulation led to a rapid phosphorylation of IκBα in LX-2 cells. Pretreatment of the cells with A771726, as well as BAY 11-7082, inhibited the phosphorylation of IκBα induced by TRAIL (Fig. 5A). The cytoplasmic levels of IκBα protein were examined by Western blot analysis. TRAIL caused a decrease in IκBα protein levels. Pretreatment with either A771726 or BAY 11-7082 inhibited the degradation of IκBα (Fig. 5B). Because NF-κB activation also requires nuclear translocation of the p65 subunit of NF-κB, we measured the level of p65 in the cytoplasm and in the nucleus by immunofluorescence staining.
nucleus. A771726 inhibited nuclear translocation of p65 induced by TRAIL (Fig. 5C). Furthermore, luciferase reporter gene assay showed that pretreatment with A771726 could significantly inhibit TRAIL-induced NF-κB activation in a dose-dependent manner in LX-2 cells (Fig. 5D). Inhibition of NF-κB activation by BAY 11-7082 or A771726 increased TRAIL-induced apoptosis of LX-2 cells (Fig. 5E). NF-κB inhibitor decreased the number of activated HSCs, induces apoptosis of activated HSCs and attenuates the extent of liver fibrosis in animal model. It is an important proof that fibrosis could be treated by inducing apoptosis of activated HSCs.

Fig. 5. A771726 Inhibited TRAIL-Induced NF-κB Activation in LX-2 Cells

Two hours before rhTRAIL (300 ng/ml) stimulation, LX-2 cells (at 12 d of culture) were treated with A771726 (0.1, 1.0, 10 μM) or BAY 11-7082 (5 μM). The cell lysates were collected 30 min later. (A) Effect of A771726 on phosphorylation of IκBα induced by TRAIL. (B) Effect of A771726 on degradation of IκBα induced by TRAIL. (C) Effect of A771726 on translocation of p65. The levels of p65 in the cytoplasm extracts (CE) and nuclear extracts (NE) were detected by Western blotting analysis. (D) NF-κB reporter gene assays. NF-κB-luc-transfected LX-2 cells were pretreated with A771726 or BAY 11-7082 for 2 h followed by the treatment with rhTRAIL. Cell lysates were collected to detect NF-κB-luc reporter activity. Fold induction of NF-κB-luciferase for each treatment was based on untreated values normalized to the fold induction of pRL-CMV reporter values. (E) Effect of NF-κB inhibitor on TRAIL-induced apoptosis of LX-2 cells. Results are the mean±S.D. from three independent experiments. *p<0.05 versus control values; **p<0.01 versus rhTRAIL values.

DISCUSSION

Hepatic fibrosis is commonly observed in chronic liver disease. Hepatic fibrosis implies an imbalance between the deposition and the degradation of connective tissue. Activated HSCs, as a source of fibrillar collagens that characterise fibrosis, are central to the pathogenesis of liver fibrosis. Hepatic fibrosis is in principle a reversible process in which the activated HSCs have been identified as the central fibrogenic cells. Lots of attention has focused on the process of activated HSC apoptosis because stimulation of this process in vivo promotes accelerated rates of recovery from rat liver fibrosis. Recent researches indicate that during recovery from liver fibrosis in the rat carbon tetrachloride model of fibrosis, there is a decrease in the number of HSCs mediated by apoptosis. In the process of recovery from fibrosis, cytokines may also be important in determining apoptosis of activated HSCs. HSCs can indeed undergo apoptosis induced by FasL, nerve growth factor (NGF), or TRAIL. Gliotoxin, an inhibitor of NF-κB, significantly decreases the number of activated HSCs, induces apoptosis of activated HSCs and attenuates the extent of liver fibrosis in animal model. It is an important proof that fibrosis could be treated by inducing apoptosis of activated HSCs.

TRAIL has been recently demonstrated as a critical factor in hepatic disorders. Normal liver is resistant to TRAIL cytotoxicity. TRAIL-mediated apoptosis of hepatocytes in vivo is triggered by viral infection, such as HCV infection.
TRAIL receptor deficiency in mice promotes susceptibility to chronic inflammation. An increase in TRAIL receptor 1 and TRAIL receptor 2 protein expressions in human HSCs was observed during activation by culture. Glatiramer acetate treatment increased the expression of TRAIL and the rate of HSCs apoptosis in mice hepatic fibrosis model. It is necessary to find more drugs that would make HSCs more susceptible to apoptotic stimuli.

Here we showed that pretreatment with leflunomide could significantly increase the susceptibility of LX-2 cells to TRAIL-mediated apoptosis. Leflunomide enhanced the activation of caspase in LX-2 cells. Furthermore, leflunomide inhibited TRAIL-induced JNK pathway activation in LX-2 cells. JNK pathway activation has been implicated in a wide range of cellular events, including apoptosis or proliferation. Previous studies showed that JNK cascades regulated collagen expression in HSCs. JNK and c-Jun were positive regulators of cell proliferation in HSCs. Blocking JNK activity with a dominant-negative form of JNK prevented cell proliferation in quiescent or culture-activated HSCs.

Here, our results showed that the negative effect of leflunomide on JNK activation enhanced TRAIL-mediated apoptosis in LX-2 cells. It suggested a potential application of JNK inhibitors for the treatment of hepatic fibrosis.

Our results indicated that leflunomide could inhibit the phosphorylation and the degradation of IκBα and nuclear translocation of p65 in LX-2 cells. It has been confirmed that the apoptotic response depends on the balance of pro-apoptotic signals as mediated by caspases and anti-apoptotic pathway controlled by NF-κB. It is also noteworthy that the ability of TRAIL to induce NF-κB activation is mediated by TRAIL receptors. Recent research showed that the key role of NF-κB in mediating the biological effects of TRAIL was demonstrated by the ability of pharmacological inhibitors of the NF-κB pathway. Nitrosylcobalamin suppressed NF-κB survival signaling and sensitized neoplasms to the anti-tumor effects of TRAIL.

Pyrrolidine dithiocarbamate and bortezomib had represented novel therapeutic strategies for the treatment of diseases. However, the sensitization to TRAIL-induced apoptosis by inhibition of NF-κB seems to be cell type specific. Previous work in our laboratory indicated that A771726 had the preventive and therapeutic effect on animal models of liver fibrosis. Here we showed that leflunomide could inhibit TRAIL-induced NF-κB activation and accelerate TRAIL-induced apoptosis in LX-2 cells. Low-activated HSCs may be more resistant to apoptotic stimuli. For the development of anti-fibrotic therapy, it is necessary to find more drugs that would modulate HSCs to be more susceptible to apoptotic stimuli. Leflunomide may be useful in reducing fibrosis by inducing stellate cell apoptosis. However, it should be draw attention to the adverse side effects of leflunomide on liver function that have been reported in 10% of patients using it for the treatment of RA. Therefore, it must be cautious when considering leflunomide for treating liver diseases.

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REFERENCES