Effect of ZVAD-fmk on hepatocyte apoptosis after bile duct ligation in rat

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Abstract

AIM: Retention and accumulation of toxic hydrophobic bile salts within hepatocyte may cause hepatocyte toxicity by inducing apoptosis. Apoptosis is a pathway of cell death orchestrated by a family of proteases called caspases. Z-Val-Ala-Asp (OMe)-fluoromethyl ketone (ZVAD-fmk) is a cell-permeable irreversible inhibitor of caspase. The purpose of this study was to evaluate the possible effect of ZVAD-fmk on hepatocyte apoptosis after bile duct ligation in the rat.

METHODS: Male Sprague-Dawley rats, weighing 250-300 g, were randomized to five groups of five rats each. Group 1 underwent common bile duct ligation and simultaneous treatment with ZVAD-fmk (dissolved in dimethylsulfoxide (DMSO)). Group 2 underwent common bile duct ligation and simultaneous treatment with Z-Phe-Ala-fluoromethyl ketone (ZFA-fmk, dissolved in DMSO). Group 3 underwent sham operation and simultaneous treatment with the same amount of DMSO. Group 4 underwent sham operation and simultaneous treatment with the same amount of ZFA-fmk. Group 5 underwent common bile duct ligation without other manipulation. After three days, liver tissue was harvested for histopathologic analysis and measurements of apoptosis.

RESULTS: When compared with sham operation, common bile duct ligation significantly increased hepatocyte apoptosis ($P = 0.008$) and ductular proliferation ($P = 0.007$). ZVAD-fmk significantly diminished the increased hepatocyte apoptosis and ductular proliferation after common bile duct ligation ($P = 0.008$ and $P = 0.007$, respectively). ZFA-fmk did not show the same effects.

CONCLUSION: Hepatocyte apoptosis and ductular proliferation significantly increased after common bile duct ligation. ZVAD-fmk effectively diminished the increased hepatocyte apoptosis and ductular proliferation after common bile duct ligation, whereas ZFA-fmk did not.

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Key words: Apoptosis; Obstructive jaundice; ZVAD-fmk; ZFA

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INTRODUCTION

Apoptosis is an important process in a wide variety of biological functions, including normal cell turnover, immune responses, embryonic development, metamorphosis and hormone-dependent atrophy, and in chemical-induced cell death[4,5]. Inappropriate apoptosis is implicated in many human diseases[4,5]. Cholestasis, an impairment in bile formation, occurs in many human liver disease[6]. Although the pathogenic events culminating in cholestasis differ in each disease, hepatocellular injury is a consistent feature of cholestasis that causes liver dysfunction, promoting fibrogenesis, and ultimately leads to liver failure[7].

Retention and accumulation of toxic hydrophobic bile salts within hepatocyte may cause hepatocyte toxicity by inducing apoptosis[8-13]. Apoptosis is a pathway of cell death orchestrated by a family of proteases called caspases[14-17]. Z-Val-Ala-Asp (OMe)-fluoromethyl ketone (ZVAD-fmk) is a cell-permeable irreversible inhibitor of caspase and recent data suggest that it might block the processing of many caspases[3,18-20]. The purpose of our study was to evaluate the possible effect of ZVAD-fmk on hepatocyte apoptosis after bile duct ligation in the rat.

MATERIALS AND METHODS

Animal and experimental design

Male Sprague-Dawley rats, weighing 250-300 g, were housed under controlled temperature, humidity and 12-h dark/light cycles living in stainless-steel cages and were allowed free access to water and rat chow before and after operation. The animals were randomized to five groups of five rats each.

Group 1 underwent common bile duct ligation and simultaneous treatment with ZVAD-fmk (dissolved in dimethylsulfoxide (DMSO)).
dimethylsulfoxide (DMSO), Enzyme Systems Products, Dublin, CA). The first dose of ZVAD-fmk (0.5 mg) was injected into the inferior vena cava immediately after bile duct ligation. Subsequent doses of ZVAD-fmk (0.5 mg twice daily) were given intraperitoneally on the first and second postoperative days. The last dose (0.5 mg) was given on the morning of the third postoperative day.

Group 2 underwent common bile duct ligation and simultaneous treatment with Z-Phe-Ala-fluoromethyl ketone (ZFA-fmk, dissolved in DMSO, Enzyme Systems Products). The first dose of ZFA-fmk (0.5 mg) was injected into the inferior vena cava immediately after sham operation. Subsequent doses of ZFA-fmk (0.5 mg twice daily) were given intraperitoneally on the first and second postoperative days. The last dose (0.5 mg) was given on the morning of the third postoperative day.

Group 3 underwent sham operation and simultaneous treatment with the same amount of DMSO. The first dose of DMSO was injected into the inferior vena cava immediately after sham operation. Subsequent doses of DMSO (the same amount twice daily) were given intraperitoneally on the first and second postoperative days. The last dose was given on the morning of the third postoperative day.

Group 4 underwent sham operation and simultaneous treatment with the same amount of normal saline. The first dose of normal saline was injected into the inferior vena cava immediately after sham operation. Subsequent doses of normal (the same amount twice daily) were given intraperitoneally on the first and second postoperative days. The last dose was given on the morning of the third postoperative day.

Operative procedures
By using sterile techniques, a mid-line incision was made, the common bile duct was identified, double ligated with 5-0 silk and divided between the two ligatures. In sham-operated animals, the common bile duct was freed from the surrounding soft tissue without ligation and transection. The operation was performed by using intraperitoneal anesthesia induced with ketamine 80 mg/kg plus xylazine 10 mg/kg.

Harvest of tissues
After three days, the animals were anesthetized, and laparotomy was repeated. Liver tissue was harvested, embedded in optimal cutting temperature compound (Sakura Finetechnical, Tokyo, Japan) and immediately snap-frozen in liquid nitrogen for histopathologic analysis and measurements of apoptosis.

TUNEL assay
Hepatocyte apoptosis was quantitated by using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay. This specific assay uses terminal deoxynucleotidyl transferase to attach biotinylated deoxyuridine triphosphate to free 3'-OH DNA ends. All liver tissue specimens about 5 mm in size were fixed in freshly prepared 4% paraformaldehyde in PBS. The tissue blocks were embedded in Enclosed Processing System (Sakura, Tokyo). Tissue sections (5 µm) were prepared using a microtome and placed on glass slides. The sections were deparaffinized in xylene and dehydrated in ethanol. The sections were incubated with 20 µg/mL proteinase K in PBS for 20 min at room temperature. After rinsing the specimen twice with PBS, the sections were processed following the instruction of a commercial kit (DeadEnd Colorimetric Apoptosis Detection System, Promega, Madison, WI). Sections were stained by streptavidin-horseradish peroxidase conjugate, then counterstained with hematoxylin. The peroxidase-positive cells were identified morphometrically by brown staining nuclei. The number of TUNEL-positive cells was counted in 10 random microscopic fields (400×). Each microscopic field contained approximately 913±59 hepatocytes.

Histopathology
H&E and trichrome-stained liver specimens from the rats undergoing bile duct ligation were evaluated by light microscopy for ductular proliferation. The specimens were scored by an experienced hepatopathologist. Ductal proliferation was scored using the following grading system: 0, <10% of portal areas involved; 1, 10-50% of portal areas involved; 2, >50% of portal areas involved; 3, circumferential involvement of at least 50% of the portal area without significant expansion of portal tract; 4, circumferential involvement of at least 50% of the portal area with significant expansion of portal tract; 5, same as 4 plus bridging of the portal tracts in <20% of instances; and 6, same as 4 plus >20% of the portal tracts showing bridging involvement.

Statistical analysis
All the results were analyzed and given as mean±SD. Differences with a P value of less than 0.05 were considered statistically significant.

RESULTS
Tables 1 and 2 and Figures 1 and 2 show the results of TUNEL-positive cells/field and ductular proliferation (grade). Compared with sham operation groups, common bile duct ligation significantly increased hepatocyte apoptosis (P = 0.008) and ductular proliferation (P = 0.007). After the administration of ZVAD-fmk, the increased hepatocyte apoptosis and ductular proliferation after ligation were significantly diminished (P = 0.008 and P = 0.007

| Table 1 | TUNEL-positive cells/field |
|---------|---------------------------|
| OBZVAD  | OBZFA | SDMSO | SNS | OB |
| (n = 5) | (n = 5) | (n = 5) | (n = 5) | (n = 5) |
| 2.40±0.55 | 9.00±1.58 | 1.40±0.89 | 1.40±0.89 | 9.60±1.14 |

OBZVAD: obstructive jaundice with ZVAD. OBZFA: obstructive jaundice with ZFA. SDMSO: sham operation with DMSO. SNS: sham operation with normal saline. OB: obstructive jaundice. P = 0.008 (OBZVAD vs OBZFA). P = 0.008 (OBZVAD vs OB). P = 0.522 (OBZFA vs OB). P = 1.000 (SDMSO vs SNS). P = 0.008 (SDMSO vs OB). P = 0.008 (SNS vs OB).
of cholestasis causing liver dysfunction, promoting fibrogenesis, and its regulation is not completely understood, it has become clear that a family of cysteine endoproteases called caspases and their processing of many caspases have a toxic effect associated with high morbidity and mortality rates.

Apoptotic cell death has been recently shown to have a central role in many physiologic and pathophysiologic processes. Although the apoptotic cascade is complex and its regulation is not completely understood, it has become clear that a family of cysteine endoproteases called caspases plays a critical role in the execution of apoptotic cell death. ZVAD-fmk is a cell-permeable irreversible inhibitor of caspase and recent data suggest that it might block the processing of many caspases. In this study, ZVAD-fmk effectively diminished hepatocyte apoptosis and ductular proliferation after common bile duct ligation, whereas ZFA-fmk, a structurally similar molecule with no anticaspase activity, did not show the same effect.

**DISCUSSION**

Cholestasis, an impairment in bile formation, occurs in a wide variety of human liver diseases. Retention and accumulation of toxic hydrophobic bile salts within hepatocyte may cause hepatocyte toxicity by inducing apoptosis. Our study using the bile duct ligation rat as a model of extrahepatic cholestasis demonstrated that increased hepatocyte apoptosis and ductular proliferation were significantly enhanced after bile duct ligation (obstructive jaundice) and the administration of ZVAD-fmk could effectively attenuate this phenomenon. If confirmed in clinical trial, such manipulation may provide a rational adjuvant strategy for the treatment of patients with obstructive jaundice and is expected to reduce the incidence of perioperative mortality and morbidity in obstructive jaundice.

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