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# Methotrexate-induced apoptosis in hepatocytes after partial hepatectomy

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**Abstract**

To investigate apoptosis induced by methotrexate in hepatocytes in vivo, rats received a single injection of methotrexate immediately after partial hepatectomy and apoptosis was assessed by the terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL) and gel electrophoresis of DNA. Characteristic DNA fragmentation was obvious at 2 h and peaked at 4 h after partial hepatectomy with methotrexate injection. TUNEL-positive staining was observed in nuclei and nuclear fragments of hepatocytes in the methotrexate-injected liver (partial hepatectomy with methotrexate), with negligible background staining in the control (partial hepatectomy only) and in the methotrexate-injected normal (normal with methotrexate) rat liver. The involvement of the c-Jun N-terminal kinase (JNK) activator protein 1 (AP-1) pathway and p53 in apoptosis was also examined. The activity of JNK increased at 15 min and peaked at 1 h after partial hepatectomy. This increase was repressed by methotrexate injection. Western blot analysis showed that the levels of c-Fos and c-Jun protein expression, which increased at 1 h after partial hepatectomy, were also reduced by methotrexate. The levels of p53 protein were markedly increased after partial hepatectomy with methotrexate injection. The increase in p53 protein was followed by an up-regulation of $p21^{WAF1/CIP1}$ protein at 2 h after partial hepatectomy. These results suggested that the inhibition of the JNK-AP-1 pathway and concurrent up-regulation of p53 and $p21^{WAF1/CIP1}$ were involved in hepatocyte apoptosis induced by partial hepatectomy with methotrexate. 

*Keywords:* Apoptosis; JNK (c-Jun N-terminal kinase); c-Fos; c-Jun; p53; $p21^{WAF1/CIP1}$

## 1. Introduction

Methotrexate is an antifolate compound widely used in chemotherapy. Chemotherapeutic agents can elicit a number of cellular responses including growth arrest and activation of apoptosis or programmed cell death. It has been suggested that apoptosis is an important and ubiquitous mode of death for cells treated with chemotherapeutic drugs (Barry et al., 1990; Dive and Hickman, 1991; Hickman, 1992). Methotrexate was reported to stop the cell cycle almost immediately and induced apoptosis after a delay of 2 days in K562 cells (Walker et al., 1997). Methotrexate inhibits dihydrofolate reductase, preventing the regeneration of tetrahydrofolate, and bringing the folate cycle to a halt. The resulting lack of dTTP causes a nucleotide imbalance, which leads to a miscorporation of nucleotides into the DNA and eventually to the death of the cell by apoptosis. The folate cycle is considered a good target for therapeutic strategies, and a folate deficiency was reported to induce apoptosis in chinese hamster ovary cells (James et al., 1994). However, it is not known whether methotrexate induces apoptosis in vivo. In this study, we investigated whether methotrexate induced apoptosis in hepatocytes in vivo, using regenerating liver.

The regenerating liver following two-thirds partial hepatectomy in the rat is considered one of the best models in which to study the cellular changes occurring in vivo in different phases of the cell cycle during cell proliferation because of the relevant synchronism of the first cell cycle, the timing of which has been well established (Rabes, 1978). After partial hepatectomy, most remaining hepatocytes, which are normally quiescent, rapidly reenter the cell cycle accompanied by the induced expression of a number of growth responsive genes. There is a sequential and regulated induction of gene expression, including the induction of immediate early genes such as c-*fos* and c-*jun* (Fausto, 1990). The products of the jun and fos family of genes are components of the transcription factor activator protein 1 (AP-1). Jun family proteins bind to the AP-1 site as homodimers or heterodimers of Fos or activating transcription factor (Karin et al., 1997). The transcriptional

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activity of the c-Jun protein increases through its phosphorylation at Ser63 and Ser73 within the N-terminal transactivation domain (Pulverer et al., 1991; Smeal et al., 1991; Franklin et al., 1992; Adler et al., 1992; Hibi et al., 1993), which is catalyzed by c-Jun N-terminal kinase (JNK), also known as stress-activated protein kinase (SAPK) (Derijard et al., 1994). JNK is a member of the mitogen-activated protein (MAP) kinase family and controlled by an upstream dual specificity MAP kinase kinase (MKK4) that requires initial activation by an upstream MAP kinase kinase kinase (MEKK1) (Sanchez et al., 1994; Derijard et al., 1995; Lin et al., 1995). Recently, the JNK and AP-1 pathway was suggested to be involved in apoptosis (Colotta et al., 1992; Verheij et al., 1996; Jochum et al., 2001). However, the induction of JNK activity is one of the earliest events during liver regeneration after partial hepatectomy (Westwick et al., 1995). The activation of JNK and the resulting enhanced phosphorylation of c-Jun and AP-1 activity are essential for DNA synthesis during liver regeneration (Riabowol et al., 1992; Westwick et al., 1995).

Apoptosis is regulated by a network of genes the connections of which to cell cycle genes have not yet been fully elucidated. Among these, the tumor suppressor gene p53 is now widely recognized as a transducer of genome damage into growth arrest and/or apoptosis (Hartwell and Kastan, 1994; Ko and Prives, 1996). p53 is thought to exert its function by a p53-dependent transcriptional activation of $p21^{WAF1/CIP1}$ (EL-Deiry et al., 1993). p21 protein is an inhibitor of cyclin-dependent kinase (CDK) and plays an important role in regulating CDK activity and cell cycle progression in response to a wide variety of stimuli (Harper et al., 1993). In addition to normal cell cycle progression, p21 has been postulated to participate in growth suppression and apoptosis through a p53-dependent or -independent pathway (EL-Deiry, 1998).

In the present study, we examined whether methotrexate induced apoptosis in the hepatocytes of regenerating liver after partial hepatectomy and investigated the possible roles of the JNK-AP-1 pathway and p53 in this phenomenon. Our results showed that methotrexate induced apoptosis in hepatocytes at an early stage of liver regeneration and the methotrexate-induced apoptosis was associated with repression of the induction of JNK activity and up-regulation of p53 and p21.

## 2. Materials and methods

### 2.1. Materials

The reagents were purchased from the following sources: Methotrexate, Sigma; In Situ Cell Death Detection Kit, Boehringer-Mannheim, Germany; Bicinchoninic acid (BCA) protein assay kit, Pierce Chemicals; SAPK/JNK Assay kit, New England Biolabs; Immobilon transfer membranes, Millipore; Antibodies to c-Jun, p-c-Jun (Ser63), c-Fos, p53 and p21, Santa Cruz Biotechnology. All other reagents were of analytical grade.

### 2.2. Animals

Male Wistar rats weighing 180–200 g were used for all experiments. The animals were kept in a temperature-controlled room with a 12-h light–dark cycle and given a commercial laboratory chow (MF, Oriental Yeast, Osaka, Japan) and water ad libitum. A two-thirds partial hepatectomy was performed by the procedure of Higgins and Anderson (1931). Methotrexate dissolved in 0.1 M $NaHCO_3$ (1 mg/kg body weight) was injected intraperitoneally immediately after the operation to the partially hepatectomized or normal (without partial hepatectomy) rats. Control rats were partially hepatectomized and received the same quantity of the vehicle alone. The rats were killed under diethyl ether anesthesia and their livers were excised at various times. Animal experiments were performed in accordance with international criteria for the use and care of experimental animals as outlined in the ''Guide for the Care and Use of Laboratory Animals'' published by the National Institutes of Health.

### 2.3. In situ end-labeling of 3′ -OH ends of DNA fragments

Paraformaldehyde-fixed paraffin-embedded liver sections, obtained from methotrexate-injected normal (normal with methotrexate) rats and the regenerating liver of the control (partial hepatectomy only) and methotrexate-injected (partial hepatectomy with methotrexate) rats at 4 h after partial hepatectomy, were processed for in situ detection of DNA fragmentation by the terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL) technique (Gavrieli et al., 1992) using an In Situ Cell Death Detection Kit (Boehringer-Mannheim). Briefly, deparaffinized tissue sections were enzymatically labeled with fluorescein-nucleotide via terminal deoxynucleotidyl transferase and subsequently exposed to horseradish peroxidase-conjugated anti-fluorescein antibody. Staining was developed in diaminobenzidine and sections were counterstained with Mayer's hematoxylin.

### 2.4. Isolation and gel electrophoresis of DNA

The liver was homogenized in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 10 mM EDTA and 0.5% sodium dodecyl sulfate (SDS), and incubated overnight with proteinase K (200 μg/ml) at 50 °C. After RNase digestion, DNA was extracted and electrophoresed on 2% agarose gels as previously described (Ozeki and Tsukamoto, 1999).

### 2.5. Determination of JNK activity

The activity of JNK was measured using a SAPK/JNK assay kit (New England Biolabs) according to the protocol

provided by the manufacturer. Briefly, 50 mg of the liver tissue was homogenized in 1 ml of ice-cold lysis buffer. After centrifugation at 14,000 × *g* for 10 min, the supernatant was collected and used for the determination of protein concentration and JNK activity. Protein was measured by the BCA protein assay. The supernatant (250 μg of protein) was then incubated with glutathione *S*-transferase (GST)-c-Jun (1–89) coupled to GSH-Sepharose beads overnight at 4 °C. The beads were washed and the solid-phase kinase reaction was carried out at 30 °C for 30 min. Phosphorylation of GST-c-Jun at Ser-63 was analyzed after immunoblotting with phospho-specific c-Jun (Ser-63) antibody.

### 2.6. Western blotting analysis

Nuclear proteins were prepared from the normal and regenerating liver at each time point as previously described (Iwao and Tsukamoto, 1999). The protein concentration of the nuclear sample was determined by BCA protein assay. For immunoblotting analysis, equal amounts of nuclear proteins were electrophoresed on SDS-polyacrylamide gels and transferred to membranes. The membranes were blocked in 10 mM Tris–HCl buffer (pH 7.2) containing 0.15 M NaCl, 0.05% Tween 20 and 10% nonfat dry milk overnight and incubated with a specific antibody to p-c-Jun (Ser63), c-Jun, c-Fos, p53 or p21. After incubation with secondary antibody conjugated to horseradish peroxidase, immunoreactive proteins were detected by the enhanced chemiluminescense system (ECL, Amersham). The equal loading of protein samples was confirmed by BCA protein assay and staining of the gel with coomassie brilliant blue.

## 3. Results

### 3.1. Apoptosis induced by methotrexate

A characteristic ladder pattern of DNA was observed in the methotrexate-injected rat liver on agarose gel electrophoresis (Fig. 1). DNA isolated from the control rat liver yielded bands only in the high molecular weight region. A time course study of DNA fragmentation showed that significant DNA cleavage occurred as early as 2 h and peaked at 4 h after partial hepatectomy in the methotrexate-injected rat liver. Fig. 2 shows a representative example of in situ labeling of apoptotic cells in liver sections from the control and methotrexate-injected rats at 4 h after partial hepatectomy. TUNEL-positive staining was observed in nuclei and nuclear fragments with the morphological characteristics of apoptosis in the methotrexate-injected rat liver (partial hepatectomy with methotrexate), with negligible background staining in the control (partial hepatectomy only) and also in the methotrexate-injected normal rat liver (normal with methotrexate). The identification of stained apoptotic bodies was confirmed by specific morphological

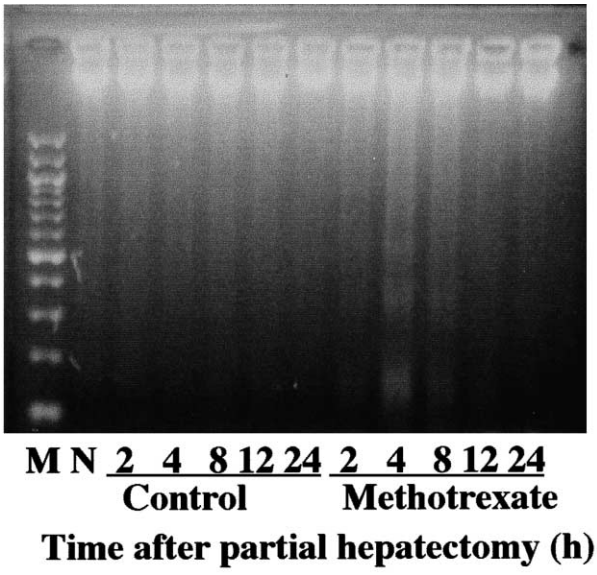


Fig. 1. Analysis of DNA fragmentation by agarose gel electrophoresis. Methotrexate (1 mg/kg body weight) was intraperitoneally injected immediately after partial hepatectomy. Genomic DNA was isolated from the livers of the normal (no partial hepatectomy without methotrexate injection), control (partial hepatectomy only) and methotrexate-injected (partial hepatectomy with methotrexate-injection) rats at 2, 4, 8, 12 and 24 h after partial hepatectomy. Lane M contained a 100-bp DNA ladder marker. Lane N was DNA from the normal rat liver. The results presented here are typical of four separate experiments.

criteria including nuclear condensation, cytoplasmic compaction and detachment from neighboring cells (Kerr et al., 1994). In the present evaluation, hepatocytes with necrotic morphology were absent under light microscopy after hematoxylin and eosin staining.

### 3.2. Effects of methotrexate on JNK activity and phosphorylated c-Jun protein levels

JNK activity was induced to a detectable level at 15 min, peaked at 1 h and then decreased after 2 h following partial hepatectomy (Fig. 3A). Methotrexate markedly reduced the JNK activity to 30% and 2% of the corresponding control values at 30 min and 1 h after partial hepatectomy, respectively.

Endogenous JNK activity was also evaluated by measuring the phosphorylation status of c-Jun in the liver nuclear fraction. As shown in Fig. 3B phosphorylated c-Jun was not detectable in methotrexate-injected rats during 4 h after partial hepatectomy, while it increased and peaked at 1 h after partial hepatectomy in the controls.

### 3.3. Effects of methotrexate on c-Jun, c-Fos, p53 and p21 protein expression

The level of c-Jun protein reached a maximum at 1 h and remained at this level until 4 h after partial hepatectomy as

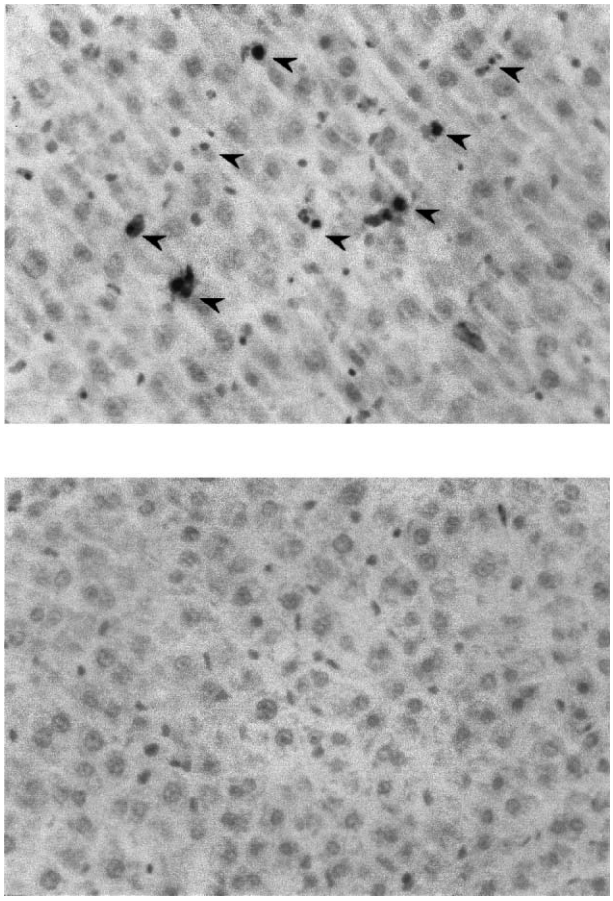

Fig. 2. In situ end-labeling of the apoptotic bodies in liver sections of methotrexate-injected (upper) and control (lower) rats at 4 h after partial hepatectomy. Methotrexate (1 mg/kg body weight) was intraperitoneally injected immediately after partial hepatectomy. Paraformaldehyde-fixed paraffin-embedded liver sections, obtained from the regenerating liver of methotrexate-injected and control rats at 4 h after partial hepatectomy, were processed for in situ detection of DNA fragmentation by TUNEL as described in Materials and methods. TUNEL-stained nuclei are marked by arrows. Hematoxylin counterstaining. The results presented here are typical of four separate experiments (original magnification $\times 200$).

shown in Fig. 3C. In methotrexate-treated rats, the level of c-Jun protein was markedly decreased to about 20% of the corresponding control value at 1 and 2 h, and further reduced to an undetectable level at 4 h after partial hepatectomy. The c-Fos protein level increased and peaked at 1 h after partial hepatectomy in the control liver. Methotrexate also decreased c-Fos protein to less than 10% of the corresponding control level during 4 h after partial hepatectomy (Fig. 3C).

The levels of p53 protein were not significantly changed for 4 h after partial hepatectomy in the control (Fig. 3C). Injection of methotrexate increased the p53 protein level to about fourfold the control value at 30 min. The increased level of p53 was maintained until 4 h after partial hepatectomy in the methotrexate-injected rats.

The p21 protein was barely detectable during 4 h after partial hepatectomy in the control as shown in Fig. 3C. In

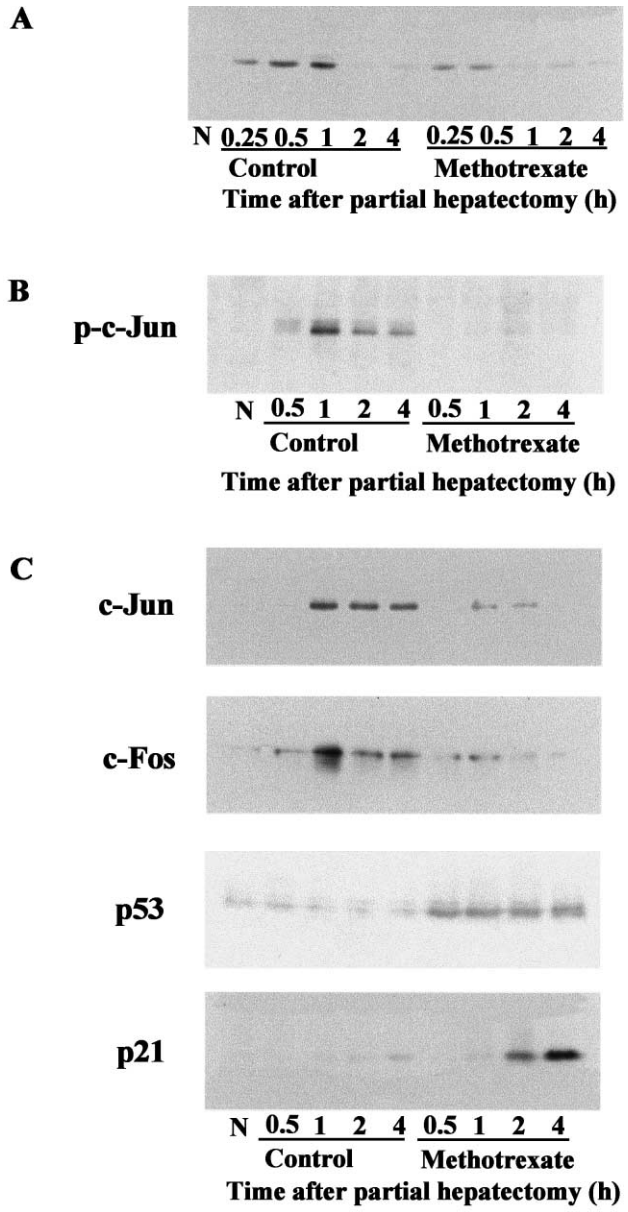


Fig. 3. Effects of methotrexate on JNK activity and the protein levels of phosphorylated c-Jun, c-Jun, c-Fos, p53 and p21 during liver regeneration. (A) JNK activity. Solid-phase in vitro JNK assays were performed using the liver lysate and SAPK/JNK assay kit as described in Materials and methods. The liver samples from normal (no partial hepatectomy without methotrexate-injection), control (partial hepatectomy only) and methotrexate-injected (partial hepatectomy with methotrexate-injection) rats at 15 min, 0.5 h, 1 h, 2 h and 4 h after partial hepatectomy were used. (B) (C) Western blotting analysis of phosphorylated c-Jun (p-c-Jun), c-Jun, c-Fos, p53 and p21 protein. The nuclear proteins (6, 6, 6, 60 and 40 μg for p-c-Jun, c-Jun, c-Fos, p53 and p21, respectively) of normal, control and methotrexate-injected rats at 0.5, 1, 2 and 4 h after partial hepatectomy were resolved by SDS-polyacrylamide gel electrophoresis (10%, 10%, 8%, 8% and 12.5% polyacrylamide gel for p-c-Jun, c-Jun, c-Fos, p53 and p21, respectively). After transfer, the blots were probed with specific antibodies and detected by ECL as described in Materials and methods. The results presented are typical of six separate experiments.

methotrexate-injected rats, the p21 protein band appeared at 2 h and increased until 4 h after partial hepatectomy.

## 4. Discussion

This study clearly demonstrated that methotrexate induced apoptosis in hepatocytes at an early phase of liver regeneration. Inhibition of the folate cycle has been considered responsible for the cytotoxicity and chemotherapeutic activity of methotrexate. In fact, methotrexate caused the death of K562 cells by apoptosis through folate depletion and a resulting lack of dTTP (Walker et al., 1997). However, 2 days were necessary for the cells to undergo apoptosis. In the hepatocytes of regenerating liver, DNA fragmentation was observed as early as 2 h after partial hepatectomy with methotrexate injection. There was a delay of only 2 h before the onset of apoptosis. This delay appears to be too short for folate depletion and a deoxynucleotide imbalance in hepatocytes (James et al., 1994). Indeed, DNA synthesis of hepatocytes begins at about 12–18 h after partial hepatectomy. It seemed unlikely that inhibition of the folate cycle caused the hepatocyte apoptosis observed after partial hepatectomy with methotrexate. Apoptosis was scarcely observed at all in normal quiescent hepatocytes after methotrexate injection. This result suggested that 'priming' was required for methotrexate-induced apoptosis of hepatocytes in vivo. The 'priming' step, which causes quiescent hepatocytes to enter a state of replicative competence before they can fully respond to growth factors, is an initiating event in liver regeneration (Fausto et al., 1995). Tumor necrosis facror-α (TNF-α) primes hepatocytes in liver regeneration after partial hepatectomy (Webber et al., 1998). Athough TNF-α by itself is not generally apoptotic, it can cause apoptosis when given in conjunction with drugs that block transcription and translation (Flick and Gifford, 1985; Sugarman et al., 1985; Leist et al., 1994; Nagata, 1997). These findings suggested a possible role for TNF-α signaling in the apoptosis of hepatocytes in regenerating liver after partial hepatectomy with methotrexate.

Methotrexate-induced DNA fragmentation was preceded by a marked decrease in the induction of JNK activity (Fig. 3A and B). JNK, a member of the MAP kinase family, is activated through phosphorylation by the MAP kinase cascades involving a sequential protein kinase reaction, MEKK1-MKK4-JNK (Fanger et al., 1997; Ip and Davis, 1998). The activated JNK in turn phosphorylates c-Jun at Ser63 and Ser73, increasing its transcriptional activity. The decreases in phosphorylated c-Jun protein in the methotrexate-injected liver as well as in the in vitro JNK activity suggested the repression of these kinase cascades and their downstream target, the transcription factor AP-1. Further, c-Jun and c-Fos protein levels were also reduced by methotrexate-injection (Fig. 3C), suggesting that methotrexate decreased AP-1 activity. This is ample evidence that AP-1 proteins, mostly those that belong to the Jun group, control cell life and death through their ability to regulate the expression and function of cell cycle regulators such as p53 and p21 (Shaulian and Karin, 2001). The role of c-Jun in hepatic proliferation is reflected in the failure of liver development in a c-Jun knock-out mouse (Hilberg et al., 1993). In the regenerating liver, c-Jun and AP-1 transcriptional activity are required for DNA synthesis (Riabowol et al., 1992; Westwick et al., 1995). Taken together, the results suggested that the repression of the JNK/AP-1 pathway was involved in the inhibition of proliferation and the induction of apoptosis in hepatocytes after partial hepatectomy with methotrexate.

The increase in p53 protein also preceded DNA fragmentation. The increase in p53 induced by methotrexate-injection was followed by an up-regulation of p21 protein (Fig. 3C). Overexpression of p21 has been shown to induce apoptosis (Gorospe et al., 1996; Wang and Walsh, 1996; Waldman et al., 1996; Polyak et al., 1996), although the role of p21 in apoptosis remains somewhat controversial (EL-Deiry, 1998). In quercetin-induced apoptosis in the regenerating liver, up-regulation of p21 was also observed (Iwao and Tsukamoto, 1999). In hepatocytes after partial hepatectomy, p21 overexpression may be a key factor in apoptosis.

In conclusion, the present study showed that methotrexate induced apoptosis associated with repression of the induction of JNK activity, c-Jun expression and c-Fos expression and the up-regulation of p53 and p21 protein in the hepatocytes of regenerating liver. Further studies are needed, however, to clarify the relationships between TNF-α, JNK signaling and the p53 pathway in hepatocyte apoptosis induced by partial hepatectomy with methotrexate.

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