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Apoptosis induced by 5-(*N*,*N*-hexamethylene)-amiloride in regenerating liver after partial hepatectomy

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Abstract

The effects of a specific inhibitor of the Na^+/H^+ exchanger, 5-(*N*,*N*-hexamethylene)-amiloride (HMA), on liver regeneration after partial hepatectomy were investigated. A single injection of HMA inhibited DNA synthesis and caused apoptosis in regenerating liver. Characteristic DNA fragmentation was observed at 4 h after partial hepatectomy with HMA-injection. The activity of Jun N-terminal kinase (JNK) increased to a maximal level at 15 min after partial hepatectomy in HMA-injected rats, while it was not detected until 30 min in the control. Western blot analysis revealed that the injection of HMA markedly increased c-Jun and phosphorylated c-Jun protein levels at 30 min after partial hepatectomy. An increase in p53 was also observed at 30 min after the HMA-injection and was followed by the upregulation of $p21^{WAF1/CIP1}$ protein expression at 1 h after partial hepatectomy. These results suggested that HMA induced apoptosis accompanied by the activation of JNK and the upregulation of c-Jun, p53 and $p21^{WAF1/CIP1}$ expression at an early stage of liver regeneration.

Keywords: HMA (5-(*N*,*N*-hexamethylene)-amiloride); Apoptosis; JNK; c-Jun; p53; $p21^{WAF1/CIP1}$

1. Introduction

A small but significant cytoplasmic alkalinization, via the Na^+/H^+ antiport, is observed upon stimulation of cell proliferation with growth factors in many cell types (Moolenaar, 1986). In contrast to activation of the Na^+/H^+ exchanger and the concomitant increase in intracellular pH (pHi) induced by various stimuli, a decrease in pHi can result in apoptosis (Li and Eastman, 1995). The inhibition of the Na^+/H^+ exchanger was reported to cause the acidification of cells with a concomitant induction of apoptosis (Rich et al., 2000; Coakley et al., 2002; Altairac et al., 2003). Amiloride has been reported to inhibit growth factor-induced Na^+ influx and DNA synthesis in hepatocytes in vitro and liver regeneration in vivo (Koch and Leffert, 1979; Leffert et al., 1988). However, amiloride has been reported to have nonspecific effects including the inhibition of Na^+/K^+ ATPase (Renner et al., 1988), protein kinase C (Bestermann et al., 1985) and protein synthesis (Leffert et al., 1982; Allemain et al., 1984). The amiloride analog 5-(*N*,*N*-hexamethylene)-amiloride (HMA) is more than 500 times more potent than amiloride as an inhibitor of Na^+/H^+ exchange (Kleyman and Cragoe, 1988). We examined whether HMA, a specific inhibitor of the Na^+/H^+ exchanger, inhibited DNA synthesis and the inhibition was accompanied by apoptosis in hepatocytes in vivo using regenerating liver after partial hepatectomy.

The hepatocyte is a highly differentiated cell that rarely divides in the normal adult liver. After two-thirds partial hepatectomy, however, most remaining hepatocytes promptly enter the cell cycle in a synchronous manner (Rabes, 1978). The first 'priming' step, which makes quiescent hepatocytes enter a state of replicative competence before they can fully respond to growth factors, encompasses the first 4–6 h after partial hepatectomy and a second 'progression' stage follows (Fausto et al., 1995). After a prereplicative phase of the priming and progression

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stage lasting 12–16 h, the expression of thymidylate synthase (TS; EC 2.1.1.45) and thymidine kinase (TK; EC 2.7.1.21) which are rate-determining enzymes of DNA synthesis (Komatsu and Tsukamoto, 1998), is induced at the G1/S boundary (Wakabayashi et al., 1994; Tsukamoto et al., 1996). DNA synthesis starts at about 18 h and peaks at 24 h after partial hepatectomy.

An increase in the expression of the immediate early genes, c-*fos* and c-*jun*, occurred within 30–60 min after partial hepatectomy (Fausto and Webber, 1994). 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 factors (Karin et al., 1997). The transcriptional 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; Adler et al., 1992; Franklin 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). The induction of JNK activity is also 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 (Westwick et al., 1995; Riabowol et al., 1992). However, the JNK and AP-1 pathway was also suggested to be involved in apoptosis of hepatocytes after partial hepatectomy (Kobayashi and Tsukamoto, 2001; Nango et al., 2003) as well as cell lines (Colotta et al., 1992; Verheiji et al., 1996).

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).

Apoptosis is regulated by a network of genes whose connection to the cell cycle or proliferation has not yet been fully elucidated. In this study, we examined whether the inhibition of proliferation by HMA was accompanied by 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 HMA induced apoptosis in hepatocytes at an early stage of liver regeneration and HMA-induced apoptosis was associated with the activation of JNK and the upregulation of c-Jun, p53 and $p21^{WAF1/CIP1}$ expression.

2. Materials and methods

2.1. Materials

5-(*N*,*N*-hexamethylene)-amiloride (HMA) was purchased from Sigma, the SAPK/JNK Assay kit from New England Biolabs, and the Immobilon PVDF transfer membrane from Millipore. The antibodies to c-Jun, p53 and p21 were obtained from 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 temperature-controlled rooms with 12-h alternating light and dark cycles and given commercial laboratory chow (MF, Oriental Yeast, Osaka, Japan) and water ad libitum. A two-thirds partial hepatectomy was performed according to the procedure of Higgins and Anderson (1931). HMA suspended in saline was intraperitoneally injected immediately after the partial hepatectomy or to normal (without partial hepatectomy) rats. Control rats were partially hepatectomized and received the same quantity of the vehicle as the experimental animals. The rats were killed under diethyl ether anesthesia and their livers were excised at the indicated times.

2.3. Determination of the activities of TS and TK and amounts of DNA, RNA and protein

The excised liver was homogenized with 5 volumes of 50 mM Tris–HCl buffer (pH 7.3) containing 0.25 M sucrose, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride and 1 mM EDTA. TS and TK activities of the supernatant fraction of a 20% liver homogenate were determined and expressed as pmols of product formed/min per mg protein at 37 °C as described previously (Komatsu and Tsukamoto, 1998). Protein was measured by the method of Lowry et al. (1951) with bovine serum albumin as the standard. The DNA and RNA contents of the liver were measured using the diphenylamine (Burton, 1956) and orcin (Schneider, 1957) reactions, respectively, after extraction with trichloroacetic acid according to the procedure of Schneider (1957). Statistical analyses of data were done with a one-way analysis of variance with pairwise comparison by the Bonferroni method.

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

Paraformaldehyde-fixed paraffin-embedded liver sections, obtained from HMA-injected normal (no partial hepatectomy) rats and the regenerating liver of control or HMA-injected rats at 4 h after partial hepatectomy and the injection were processed for in situ detection of DNA fragmentation by the terminal deoxynucleotidyl transferase

(TdT)-mediated nick-end-labeling technique (TUNEL) (Gavrieli et al., 1992) using the In Situ Cell Death Detection Kit. 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. The number of apoptotic hepatocytes was counted in 8–10 fields (×200 magnification) and expressed as a percentage of total cells.

2.5. Isolation and gel electrophoresis of DNA

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

2.6. Determination of JNK activity

The activity of JNK was measured using the 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 the protein concentration and JNK activity. Protein was measured using the bicinchoninic acid protein assay kit (Pierce). The supernatant was then incubated with c-Jun (1–89) fusion protein coupled to glutathione sepharose beads overnight at 4 °C. After the beads were washed, a solid-phase kinase reaction was carried out at 30 °C for 30 min. Phosphorylation of c-Jun fusion protein at Ser-63 was analyzed after immunoblotting with phospho-specific c-Jun (Ser-63) antibody.

2.7. Western blot 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 resultant nuclear sample was determined with the bicinchoninic acid protein assay. For immunoblot 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 either c-Jun, p53 or p21. After incubation with secondary antibody conjugated to horseradish peroxidase, immunoreactive proteins were detected with the enhanced chemiluminescense system (ECL; Amersham). The equal loading of protein samples was confirmed by the bicinchoninic acid protein assay and staining of the gel with coomassie brilliant blue.

3. Results

3.1. Effects of HMA on the activities of TS and TK, the DNA, RNA and protein content, and the weight of regenerating liver

The activities of TS and TK increased to about five and nine times the normal value (resting in Go state; just after partial hepatectomy) at 24 h after partial hepatectomy, respectively, as shown in Table 1. When HMA was injected immediately after partial hepatectomy at a dose of 10 mg/kg body weight, TS and TK activity decreased to 30% and 12% of the corresponding control value, respectively. The decreases in TS and TK activity were followed by a significant reduction of the increase in liver DNA content. The RNA content and gross parameters such as protein content and liver weight were also significantly reduced. When the dose of HMA was reduced to 1 mg/kg body weight, no inhibitory effect on liver regeneration was observed. These results indicated that HMA inhibited liver regeneration after partial hepatectomy at a dose of 10 mg/kg body weight.

3.2. Apoptosis induced by HMA

HMA induced DNA fragmentation as shown in Fig. 1. A characteristic ladder pattern of DNA was detected in the HMA-injected rat liver (partial hepatectomy with HMA) on

Table 1
The effects of HMA on liver regeneration after partial hepatectomy

Treatment	Enzymatic activity (pmol/min/mg of protein)		Total liver content (mg)			Liver weight (g)
	TS	TK	DNA	RNA	Protein	
Control	54.8±7.6[a]	278.6±35.8[a]	6.63±0.18[a]	38.2±1.5[a]	545.1±21.3[a]	3.06±0.09[a]
HMA (10 mg/kg)	16.4±2.1[b]	32.4±4.8[b]	4.93±0.16[b]	31.2±1.4[b]	477.8±15.6[b]	2.60±0.10[b]
(10 mg/kg)	53.2±5.4[a]	248.0±28.9[a]	6.39±0.18[a]	41.1±1.4[a]	543.6±23.4[a]	2.98±0.08[a]
Normal	10.5±1.7[b]	31.3±6.6[b]	4.83±0.10[b]	27.4±0.6[b]	464.6±13.2[b]	2.56±0.14[b]

Values are means±S.E. of five to eight rats.
[a] Significant difference from the normal ($P<0.05$).
[b] Significant difference from the control ($P<0.05$).

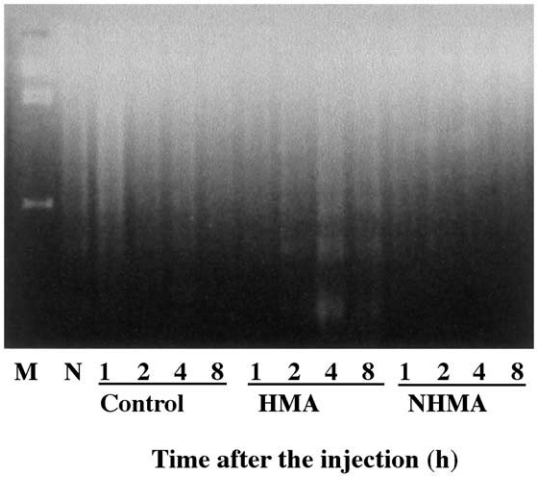

Fig. 1. Analysis of DNA fragmentation by agarose gel electrophoresis. HMA (10 mg/kg body weight) was intraperitoneally injected immediately after partial hepatectomy into the partially hepatectomized or normal (without partial hepatectomy) rats. Genomic DNA was isolated from liver of normal (N), control (partial hepatectomy with saline), HMA-injected (HMA: partial hepatectomy with HMA), and HMA-injected normal (NHMA) rats at 1, 2, 4 and 8 h after the injection. Lane M contained HindIII-digested λDNA as a molecular size marker. The result presented here is typical of four separate experiments.

agarose gel electrophoresis (Fig. 1). DNA isolated from the liver of normal, control (partial hepatectomy with saline) and HMA-injected normal (no partial hepatectomy with HMA) rats yielded bands only in the high-molecular-weight region. A time course study of DNA fragmentation showed that significant DNA cleavage occurred at 4 h after partial hepatectomy in HMA-injected rat liver. Fig. 2 is a representative example of the in situ labeling of apoptotic cells in a liver section from the control and the HMA-injected rat at 4 h after partial hepatectomy. TUNEL-positive staining was observed in nuclei and nuclear fragments with the morphological characteristics of apoptosis in the HMA-injected rat liver (partial hepatectomy with HMA), with negligible background staining in the control (partial hepatectomy with saline) and also in the HMA-injected normal (no partial hepatectomy with HMA) rats. The extent of apoptosis was assessed to be $11 \pm 2.6\%$ at 4 h after partial hepatectomy in the HMA-injected rat liver. The identification of stained apoptotic bodies was confirmed with specific morphological criteria including nuclear condensation, cytoplasmic compaction and detachment from neighboring cells (Kerr et al., 1994). In the present evaluation, hepatocytes with a necrotic morphology were a rare occurrence and foci of inflammatory cells were absent under light microscopy after hematoxylin and eosin staining.

3.3. Effects of HMA on JNK activity

JNK activity was barely detectable at 15 min, increased at 30 min, peaked at 1 h and then decreased after 2 h following partial hepatectomy in the control (Fig. 3A). The injection of HMA induced the JNK activity to a maximal level at 15 and 30 min after partial hepatectomy. The activation of JNK was not observed in HMA-injected normal liver during 4 h after the injection.

3.4. Effects of HMA on c-Jun, p53 and p21 protein levels

As shown in Fig. 3B, anti-c-Jun antibody detected the c-Jun and the phosphorylated c-Jun with a slower migration consistent with previous reports (Guo et al., 1998; Luo et al., 1999; Kobayashi and Tsukamoto, 2001; Nango et al., 2003). The c-Jun increased after 1 h following partial hepatectomy in the control, but the phosphorylated form of c-Jun was not detected during 4 h. In HMA-treated rats, c-Jun with the phosphorylated c-Jun remarkably increased at 30 min and 1 h after partial hepatectomy. The total c-Jun levels of the HMA-injected rats increased to about threefold the corresponding control level at 1 and 2 h after partial hepatectomy.

The protein levels of p53 did not significantly change during 4 h after partial hepatectomy in the control (Fig. 3B). In HMA-injected rats, the levels increased at 30 min and the increase was maintained until 4 h after partial hepatectomy.

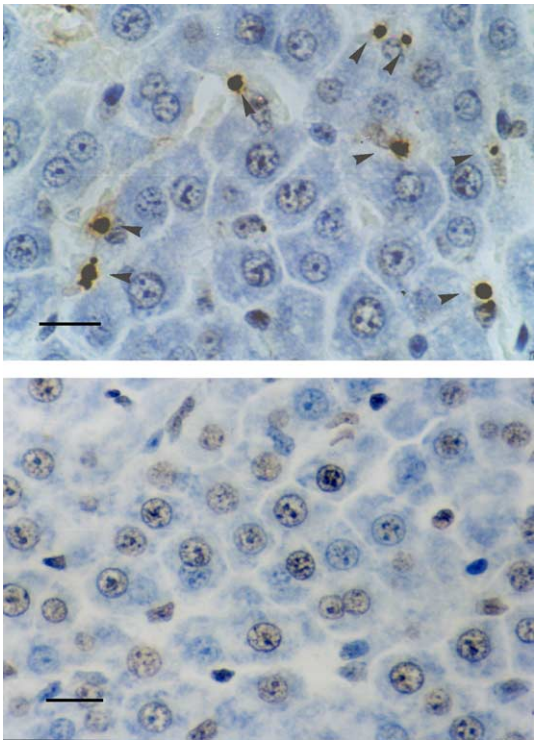

Fig. 2. In situ end-labeling of the apoptotic bodies in a liver section of a HMA-injected (upper) and control (lower) rat at 4 h after partial hepatectomy. HMA (10 mg/kg body weight) was intraperitoneally injected immediately after partial hepatectomy. Paraformaldehyde-fixed paraffin-embedded liver sections, obtained from the regenerating liver of HMA-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. Bars, 25 μm.

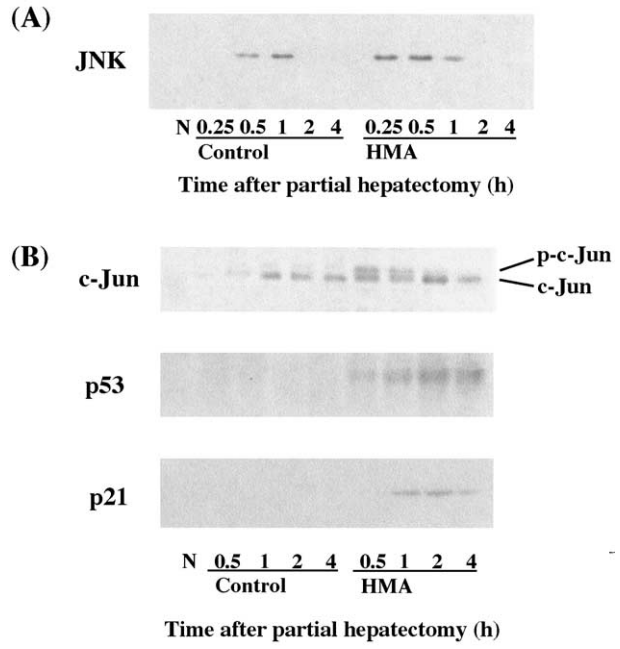

Fig. 3. Effects of HMA on JNK activity and the protein levels of c-Jun, p53 and p21 during liver regeneration. HMA (10 mg/kg body weight) was intraperitoneally injected immediately after partial hepatectomy. (A) JNK activity. Solid-phase in vitro JNK assays were performed using a SAPK/JNK assay kit and the liver lysate as described in Materials and methods. The liver lysates were prepared from normal liver and the regenerating liver of control and HMA-injected rats at 0.25, 0.5, 1, 2 and 4 h after partial hepatectomy. (B) Western blot analysis of c-Jun, p53 and p21 protein. The nuclear proteins (6, 60, and 40 μg for c-Jun, p53 and p21, respectively) of the liver in normal, control and HMA-injected rats at 0.5, 1, 2, and 4 h after partial hepatectomy were resolved by SDS-polyacrylamide gel electrophoresis (10%, 8% and 12.5% polyacrylamide gel for c-Jun, p53 and p21, respectively). After the transfer, the blot was probed with antibody and detected by ECL as described in Materials and methods. p-c-Jun: phosphorylated c-Jun. The results presented are typical of six separate experiments.

The $p21^{WAF1/CIP1}$ protein band appeared after 1 h following partial hepatectomy in HMA-injected rats, while the band was barely detectable during 4 h after partial hepatectomy in the control as shown in Fig. 3B. No change in the levels of c-Jun, p53 and p21 in HMA-injected normal rats was observed during 4 h after the injection.

4. Discussion

This study demonstrated that a specific inhibitor of the Na^+/H^+ exchanger, HMA, inhibited DNA synthesis in regenerating liver after partial hepatectomy. Hepatocytes express the growth factor-inducible form of the Na^+/H^+ exchanger, i.e. NHE-1 (Orlowsky et al., 1992). Activation of NHE-1 resulted in a 1:1 efflux of H^+ and influx of Na^+ ions, with a concomitant increase in intracellular pH (pHi). The hepatocellular Na^+/H^+ exchanger was activated transiently and pHi increased 2 h after partial hepatectomy (Dallenbach et al., 1994). These results suggested that HMA inhibited the liver regeneration by inhibiting the Na^+/H^+ exchanger and the increase in pHi after partial hepatectomy. Furthermore, this study clearly showed that the injection of HMA indeed caused apoptosis in vivo as previously reported in in vitro experiments (Rich et al., 2000; Coakley et al., 2002; Altairac et al., 2003). Characteristic DNA fragmentation was observed at 4 h after partial hepatectomy with HMA (Fig. 1). The results of in situ end-labeling showed the appearance of apoptotic cells with specific morphological criteria in hepatocytes after partial hepatectomy with HMA (Fig. 2). Apoptosis as well as characteristic DNA fragmentation was not observed in the HMA-injected normal liver. These results indicated that the HMA-injection induced apoptosis in hepatocytes in the primed, not quiescent, state. This result was consistent with the report that the addition of HMA to normal peripheral blood mononuclear cells resulted in no effect in contrast to leukemic cell lines which were continuously proliferating (Rich et al., 2000). Amiloride and its analogs have been also reported to inhibit tumor growth in vivo (Sparks et al., 1983; Hasuda et al., 1994; Tatsuta et al., 1995). These chemopreventive activities may be explained by apoptosis.

HMA-induced DNA fragmentation was preceded by an increase in JNK activity. The activation of JNK was barely detected until 30 min after partial hepatectomy in the control (Fig. 3A). The injection of HMA increased the JNK activity within 15 min. The JNK activation in vivo was confirmed by an increase in phosphorylated c-Jun protein in the liver of HMA-injected rats. The phoshorylated c-Jun was detected at 30 min after partial hepatectomy in the HMA-injected rats, although it was scarcely observed during 4 h after partial hepatectomy in the control (Fig. 3B). The induction of JNK activity is one of the early events after partial hepatectomy (Westwick et al., 1995). However, a prolonged activation of JNK was also observed in cisplatin-induced apoptosis in hepatocytes after partial hepatectomy (Kobayashi and Tsukamoto, 2001) as well as radiation-induced apoptosis in T cells (Chen et al., 1996) and tumor necrosis factor-alpha-induced apoptosis in rat mesangial cells (Guo et al., 1998). These results suggest that the timing and duration of the JNK activation are critical in determining the cell fate, proliferation or apoptosis. The transcriptional activity of the c-Jun protein increases through its phosphorylation which is catalyzed by JNK. The increase in the phosphorylated c-Jun and c-Jun protein levels of HMA-injected rats suggests the involvement of the JNK and AP-1 pathway in HMA-induced apoptosis, although a role for JNK signaling in protection from cell death cannot be excluded (Potapova et al., 1997).

After the induction of JNK activation, the increase in p53 protein also preceded the DNA fragmentation. The increase in p53 protein was observed after 30 min following partial hepatectomy with HMA (Fig. 3B). This increase was followed by the upregulation of p21 protein expression (Fig. 3B). These results suggested that HMA activated the

p21 gene expression through a p53-dependent pathway. The upregulation of p21 accompanied by an increase in p53 protein levels was also observed in quercetin- and cisplatin-induced apoptosis in regenerating liver (Iwao and Tsukamoto, 1999; Kobayashi and Tsukamoto, 2001). Although the role of p21 in apoptosis remains somewhat controversial (EL-Deiry, 1998), p21 may lie downstream of p53 in apoptosis in regenerating liver.

This paper has provided evidence that HMA induced apoptosis associated with the activation of JNK and the upregulation of c-Jun, p53 and p21 expression at an early stage of liver regeneration. Further study is needed, however, to clarify the relation between JNK signaling and the p53 pathway in HMA-induced apoptosis.

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