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Manganese-induced apoptosis in hepatocytes after partial hepatectomy

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Abstract

To investigate the apoptosis induced by manganese (Mn) in hepatocytes in vivo, rats received a single injection of manganese chloride immediately after partial hepatectomy. Characteristic DNA fragmentation was observed at 4 h after partial hepatectomy with Mn-injection. The activation of caspase-3 by Mn-injection was detected as early as 30 min and peaked at 1 h after partial hepatectomy. The activity of Jun N-terminal kinase (JNK) increased to a maximal level, which was about 10-fold the maximal level of the control, at 15 min after partial hepatectomy and this increase was maintained for 4 h in Mn-injected rats, while a transient increase was observed at 1 h in the control. No effect of the Mn-injection on the activation of p38 mitogen-activated protein kinase (MAPK) was observed. Western blot analysis revealed that the injection of Mn markedly increased c-Jun and phosphorylated c-Jun protein levels at 1 h after partial hepatectomy. An increase in p53 was also observed at 30 min after the Mn-injection and followed by the upregulation of $p21^{WAF1/CIP1}$ protein expression at 2 h after partial hepatectomy. These results suggested that the activation of JNK and the upregulation of c-Jun, p53 and $p21^{WAF1/CIP1}$ were involved in the apoptosis of hepatocytes induced by partial hepatectomy with manganese.

Keywords: Manganese(II); Apoptosis; JNK (Jun N-terminal kinase); p38 MAPK (mitogen-activated protein kinase); c-Jun; p53; $p21^{WAF1/CIP1}$

1. Introduction

Manganese exhibits diverse effects in cellular physiology. It is an essential nutrient and in trace amounts serves as a cofactor for the activation of several enzymes. However, long-term exposure to relatively high concentrations of Mn leads to the intracellular accumulation (Lander et al., 1999; Bader et al., 1999) that is toxic to a number of cell types, including neuronal cells where such an accumulation can lead to the development of Parkinson-like syndromes (Brouillet et al., 1993). Mn also has been reported to induce the apoptosis of PC12, B cells and Hela cells (Hirata et al., 1998; Schrantz et al., 1999; Migheli et al., 1999; Oubrahim et al., 2001). However, the effect of Mn on hepatocytes in vivo is not known. In this study, we investigated whether Mn induces 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). 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; Fausto et al., 1995). 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), a member of the mitogen-activated protein kinase (MAPK) family. The MAPK cascade is considered to be a major signaling pathway which links signals from the cell surface to the nuclear events in cell cycle progression, apoptosis, and carcinogenesis. The induction of JNK and p38 MAPK activity is indeed one of the earliest

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events during liver regeneration after partial hepatectomy (Riabowol et al., 1992; Westwick et al., 1995; Chen et al., 1998). However, recent studies have implicated JNK/stress-activated protein kinase (SAPK) and p38 MAPK pathways as key regulators of apoptosis (Estus et al., 1994; Ham et al., 1995; Mesner et al., 1995; Namgung and Xia, 2000; Kondoh et al., 2002). The JNK and AP-1 pathway was 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). A critical role for the p38 MAPK cascade in apoptosis has been also described in many cell types (Xia et al., 1995; De Zutter and Davis, 2001; Murillo et al., 2001; Tanaka et al., 2003).

The tumor suppressor gene p53 is now widely recognized as a transducer of genomic damage into growth arrest and/or apoptosis (Hartwell and Kastan, 1994; Ko and Prives, 1996). p53 is thought to exert its function via 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 Mn induced apoptosis in the hepatocytes of regenerating liver after partial hepatectomy and investigated the possible roles of the MAPK pathway and p53 in this phenomenon. Our results showed that Mn induced apoptosis in hepatocytes at an early stage of liver regeneration and Mn-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

The reagents were purchased from the following sources: Manganese (II) chloride ($MnCl_2 \cdot 4H_2O$), Sigma; In Situ Cell Death Detection Kit, Boehringer-Mannheim; Bicinchoninic acid (BCA) protein assay kit, Piece Chemicals; SAPK/JNK and p38 MAPK Assay kit, New England Biolabs; Immobilon polyvinylidene difluoride (PVDF) transfer membrane, Millipore; The antibodies to c-Jun, p53 and p21, Santa Cruz Biotechnology. All other reagents were of analytical grade.

2.2. Animals

Male Wistar rats weighing 180 to 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 Co., Osaka, Japan) and water ad libitum. A two-thirds partial hepatectomy was performed according to the procedure of Higgins and Anderson (1931). Manganese chloride tetrahydrate (3mg/kg body weight) dissolved in saline was intraperitoneally injected immediately after the partial hepatectomy or into normal (without partial hepatectomy) rats. The dose level was determined based on our preliminary experiment. 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. Animal experiments were performed in accordance with the "Guidelines for the Care and Use of Laboratory Animals" of Nara Women's University.

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

Paraformaldehyde-fixed paraffin-embedded liver sections, obtained from Mn-injected normal (no partial hepatectomy) rats and the regenerating liver of control or Mn-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.

2.4. 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).

2.5. Determination of the enzyme activities

2.5.1. Caspase-3 activity

Liver was homogenized in 25 mM Tris–HCl buffer (pH 7.5) containing 1 mM EGTA, 5 mM $MgCl_2$ and 0.5% Triton X-100. After centrifugation at 14,000 ×*g*, the diluted supernatant was assayed for caspase-3 activity using 50 μM of synthetic fluorogenic substrate, Ac-DEVD-α-4-methyl-coumaryl-7-amide (Peptide Institute, Inc., Osaka, Japan). The fluorescent intensity was calibrated with standard concentrations of 7-amino-4-methyl-coumarin (AMC) and the caspase-3 activity was expressed in picomols of AMC released per minute per milligram of protein. Protein concentrations of the supernatant were assayed using the bicinchonic acid (BCA) protein assay kit (Pierce).

2.5.2. Glutamate–oxaloacetate transaminase activity

The activity of glutamate–oxaloacetate transaminase in serum was measured spectrophotometrically utilizing diagnostic kits (Wako Pure Chem. Co. Ltd., Osaka, Japan) and expressed as IU at 35 °C.

2.5.3. JNK and p38 MAPK activity

JNK and p38 MAPK assays were performed according to the manufacturer's protocols (New England Biolabs) using the supernatant of the liver tissues. The liver tissue (50 mg) 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 the kinase assays. Protein was measured using the bicinchoninic acid protein assay kit (Pierce). JNK was precipitated from the supernatant of liver lysates using the c-Jun (1–89) fusion protein coupled to glutathione sepharose beads. The precipitated JNK-cJun fusion protein complex was incubated with ATP. For the p38 MAPK, the supernatant of the liver lysate was reacted with the immobilized phospho-p38 MAPK antibody and the resulting immunoprecipitated phospho-p38 MAPK was incubated with ATF-2 fusion protein and ATP. All of the kinase reactions were performed at 30 °C for 30 min. The phosphorylation of c-Jun fusion protein at Ser-63 or ATF-2 fusion protein at Thr-71 was analyzed after immunoblotting with phospho-c-Jun (Ser-63) antibody or phospho-ATF-2 (Thr-71) antibody for the JNK or p38 MAPK assay, respectively.

2.6. 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 nuclear sample was determined with the bicinchoninic acid 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 either c-Jun, p53 or p21 (dilution of 1 : 100). After incubation with secondary antibody conjugated to horseradish peroxidase (dilution of 1 : 5000), 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. Apoptosis induced by manganese

Mn induced DNA fragmentation as shown in Fig. 1. A characteristic ladder pattern of DNA was detected in the Mn-injected rat liver (partial hepatectomy with Mn) on agarose gel electrophoresis (Fig. 1). DNA isolated from the liver of normal and control (partial hepatectomy with saline) 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 Mn-injected rats. Fig. 2 is representative of the in situ labeling of apoptotic cells in liver sections from control and Mn-injected rats at 4 h after partial

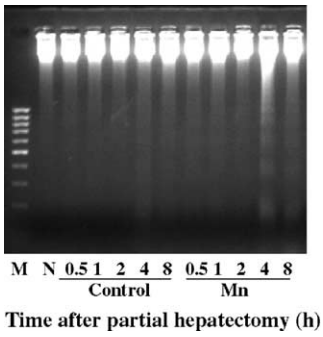

Fig. 1. Analysis of DNA fragmentation by agarose gel electrophoresis. Manganese chloride (3 mg/kg body weight) was intraperitoneally injected immediately after partial hepatectomy into the partially hepatectomized rats. Genomic DNA was isolated from liver of normal (N), control (partial hepatectomy with saline) and Mn-injected (Mn: partial hepatectomy with Mn) rats at 0.5, 1, 2, 4 and 8 h after the injection. Lane M contained a 100-bp ladder marker. The result presented here is typical of four separate experiments.

hepatectomy. TUNEL-positive staining was observed in nuclei and nuclear fragments with the morphological characteristics of apoptosis in the Mn-injected rat liver (partial hepatectomy with Mn), with negligible background staining in the control (partial hepatectomy with saline) and also in the Mn-injected normal (no partial hepatectomy with Mn) rats. 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 the light microscope after hematoxylin and eosin staining.

The activity of caspase-3, one of the important effector caspases, remarkably increased after 30 min and peaked at 1 h at about 3-fold the control level after partial hepatectomy in Mn-injected rats, while the activation was not observed in the control as shown in Table 1.

As a functional assay substantiating that the effect of Mn is apoptotic and not necrotic, the activity of glutamate–oxaloacetate transaminase released was determined as a marker of membrane integrity. The administration of Mn had no significant effect on serum glutamate–oxaloacetate transaminase activity as shown in Table 1, indicating that necrosis was not caused by Mn at the dose level used.

3.2. Effects of manganese on the activities of JNK and p38 MAPK

JNK activity was barely detectable at 15 and 30 min, increased at 1 h and then decreased from 2 h following partial hepatectomy in the control (Fig. 3A). The injection of Mn induced JNK activity to a maximal level, which was about 10-fold the maximal level of the control, at 15 min after partial hepatectomy. The activation of JNK was maintained for 4 h after partial hepatectomy with a gradual decrease in Mn-injected liver.

The p38 MAPK activity increased at 15 and 30 min after partial hepatectomy in the control and also in the Mn-injected

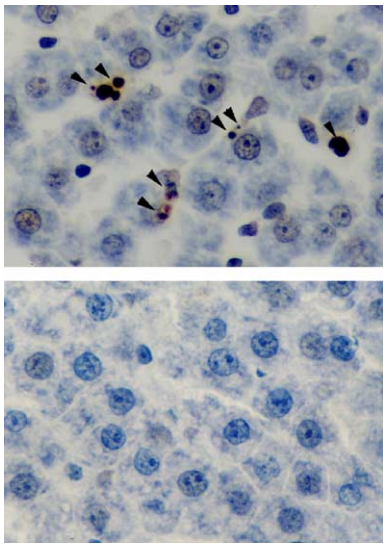

Fig. 2. In situ end-labeling of the apoptotic bodies in a liver section of a Mn-injected (upper) and control (lower) rat at 4 h after partial hepatectomy. Manganese chloride (3 mg/kg body weight) was intraperitoneally injected immediately after partial hepatectomy. Paraformaldehyde-fixed paraffin-embedded liver sections, obtained from the regenerating liver of Mn-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 arrowheads. Hematoxylin counter-staining. The results presented here are typical of four separate experiments.

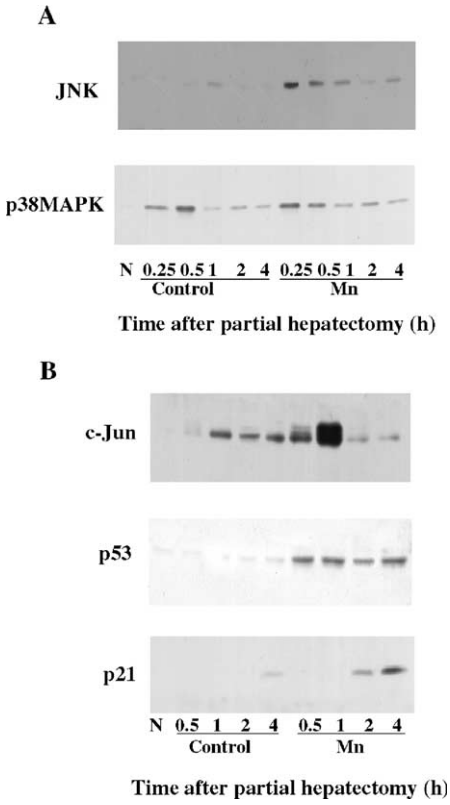

Fig. 3. Effects of manganese on JNK and p38 MAPK activity and the protein levels of c-Jun, p53 and p21 during liver regeneration. (A) JNK and p38 MAPK activity. The liver lysates were prepared from normal liver and the regenerating liver of control and Mn-injected rats at 0.25, 0.5, 1, 2 and 4 h after partial hepatectomy. Using the supernatant of the liver lysate, solid-phase in vitro JNK and p38 MAPK assays were performed as described in Materials and methods. (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 Mn-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 the specific antibody and detected by ECL as described in Materials and methods. The results presented are typical of six separate experiments.

liver. (Fig. 3A). No difference was observed between the two groups.

3.3. Effects of manganese 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 1 h after partial hepatectomy in the control, but the phosphorylated form of c-Jun was not detected during 4 h. In Mn-treated rats, c-Jun together with the phosphorylated c-Jun appeared at 30 min and remarkably increased at 1 h after partial hepatectomy. The total c-Jun levels of the Mn-injected rats increased to about 10-fold the corresponding control level at 1 h after partial hepatectomy.

The protein level of p53 was barely detectable during 4 h after partial hepatectomy in the control (Fig. 3B). In Mn-injected rats, the level increased after 30 min and remained increased for 4 h after partial hepatectomy.

The $p21^{WAF1/CIP}$ protein band increased at 4 h after partial hepatectomy in the control. The protein band increased at 2 and 4 h to about 2- and 5-fold the control level at 4 h, respectively, after partial hepatectomy in Mn-injected rats.

Table 1
Effects of manganese on the activities of liver caspase-3 and serum glutamate–oxaloacetate transaminase

	Time after partial hepatectomy (h)	Caspase-3 (pmol/min per mg protein)	Glutamate–oxaloacetate transaminase (IU)
Normal		52.1±7.7	49.6±5.5
Control	0.5	53.7±3.4	51.5±10.1
	1	57.8±13.5	58.7±7.9
	2	64.8±9.6	69.1±18.9
	4	58.8±9.8	114.9±18.0
	8	58.6±14.9	124.2±19.5
Mn-injected	0.5	114.5±16.8[a]	57.7±4.9
	1	190.8±26.1[a]	54.3±2.4
	2	124.6±17.4[a]	65.8±3.7
	4	101.2±4.3[a]	120.4±14.9
	8	94.9±9.7[a]	131.2±18.6

Manganese chloride (3 mg/kg body weight) was intraperitoneally injected immediately after partial hepatectomy. The activities of liver caspase-3 and serum glutamate–oxaloacetate transaminase were determined as described in Materials and methods. Values are means±S.D. of 5–8 rats.

[a] Significant difference from the corresponding control ($p<0.05$).

4. Discussion

This study clearly demonstrated that manganese induced apoptosis in hepatocytes at an early phase of liver regeneration. The results of in situ end-labeling showed the appearance of apoptotic cells with specific morphological criteria among hepatocytes after partial hepatectomy with Mn (Fig. 2). The activation of caspase-3 was observed 30 min following partial hepatectomy (Table 1). The serum level of glutamate–oxaloacetate transaminase activity excluded the possibility of necrosis (Table 1). Consistent with these results, earlier in vitro studies reported Mn-induced DNA fragmentation in PC12 cells, B cells, Hela cells and NIH3T3 cells (Hirata et al., 1998; Schrantz et al., 1999; Migheli et al., 1999; Oubrahim et al., 2001, 2002). This is the first evidence that Mn induces apoptosis in hepatocytes in vivo. However, apoptosis was scarcely observed in the Mn-injected normal liver. The Mn-induced apoptosis was observed only in replicative competent hepatocytes after partial hepatectomy, not in normal quiescent cells. Anticancer drugs inhibit proliferation and one of the major modes of their action may be via induction of apoptosis in tumor cells, namely replicative competent, proliferating cells. Many chemotherapeutic drugs have been found to induce apoptosis in cancer cells (Makin and Hickman, 2000; Herr and Debatin, 2001). These findings suggest the possibility that Mn can be used to prevent or cure cancer as either a chemopreventive or a chemotherapeutic agent.

Mn-induced DNA fragmentation was preceded by an increase in JNK activity. The activation of JNK was observed as early as 15 min and continued during 4 h after partial hepatectomy in Mn-injected liver, while it transiently increased at 1 h after partial hepatectomy in the control (Fig. 3A). The JNK activation in vivo was confirmed by an increase in phosphorylated c-Jun protein in the liver of Mn-injected rats. The phoshorylated c-Jun was detected at 30 min and 1 h after partial hepatectomy in the Mn-injected rats, although it was scarcely observed during 4 h after partial hepatectomy in the control (Fig. 3B). Activation of JNK was also reported in the induction of apoptosis by manganese in PC12 cells (Hirata et al., 1998). The induction of JNK activity is one of the earliest events after partial hepatectomy (Westwick et al., 1995). However, a prolonged activation of JNK was reported 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 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 Mn-injected rats suggests the involvement of the JNK and AP-1 pathway in Mn-induced apoptosis, although a role for JNK signaling in protection from cell death cannot be excluded (Potapova et al., 1997). The other MAPK, the p38 MAPK which responds to cellular stress signals as JNK does, was suggested to participate in Mn-induced apoptosis in NIH3T3 cells (Oubrahim, et al., 2002). However, the involvement of the p38 MAPK signaling pathway was not suggested in Mn-induced apoptosis of hepatocytes after partial hepatectomy. This discrepancy may be explained by the cell type difference.

After the activation of JNK, the increase in p53 protein preceded the fragmentation of DNA. The increase in p53 protein was observed 30 min after partial hepatectomy with Mn-injection (Fig. 3B). This increase was followed by the upregulation of p21 protein expression (Fig. 3B). The upregulation of p21 accompanied by an increase in p53 protein levels was also reported 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 the pathway of apoptosis in hepatocytes after partial hepatectomy.

This paper has provided evidence that manganese induced apoptosis associated with the activation of caspase-3 and JNK and the upregulation of c-Jun, p53 and p21 expression in hepatocytes at an early stage of liver regeneration. Further study is needed, however, to clarify the relation between JNK signaling and the p53 pathway in Mn-induced apoptosis.

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