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Jun N-terminal kinase activation and upregulation of p53 and p21$^{WAF1/CIP1}$ in selenite-induced apoptosis of regenerating liver

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Abstract

To investigate apoptosis induced by selenite in hepatocytes in vivo, rats received a single injection of sodium selenite immediately after partial hepatectomy. Characteristic DNA fragmentation in gel electrophoresis and in situ end-labeling and the increase in caspase-3 activity were observed at 4 h after partial hepatectomy with selenite injection. The activation of Jun N-terminal kinase (JNK) was observed as early as 15 min and increased to about 10-fold the maximal level of the control at 1 and 2 h after partial hepatectomy in selenite-injected rats, while a transient increase was observed at 1 h in the control. Western blot analysis revealed that the c-Jun and the phosphorylated c-Jun protein markedly increased after 30 min and reached a maximal level at 1 and 2 h after partial hepatectomy with selenite injection, although c-Jun and a faint band of the phosphorylated c-Jun were observed after 1 h in the control. The levels of c-jun mRNA and c-Fos protein and mRNA in selenite-injected rats also increased more than in the control. The rise in the p53 protein level after partial hepatectomy with selenite injection was followed by the upregulation of p21$^{WAF1/CIP1}$ mRNA and protein expression. These results suggested that selenite induced apoptosis accompanied by the activation of caspase-3 and JNK and the upregulation of c-jun, c-fos, p53 and p21$^{WAF1/CIP1}$ at the early stage of liver regeneration.

Keywords: Selenite; Apoptosis; JNK; c-Jun; p53; p21$^{WAF1/CIP1}$

1. Introduction

Selenium is an essential trace element with several important biological functions and has received considerable attention for its possible role as an effective, naturally occurring, anticarcinogenic agent. Epidemiological studies suggest an inverse relationship between Se intake (and tissue levels of Se) and incidence and mortality rates for several forms of cancer (Combs and Gray, 1998; Clark et al., 1996; Kneckt et al., 1990). Selenium supplementation in laboratory animals decreases tumorigenesis in several tumor models including skin, liver, colon and pancreas (El-Bayoumy, 2001). The chemopreventive activity of Se has been suggested to be related to the role of Se in inducing DNA strand breaks (Lu et al., 1994; Garberg et al., 1988; Wilson et al., 1992) and catalytic properties of Se with glutathione which result in the generation of reactive oxygen species (Shen et al., 1999; Zhong and Oberley, 2001; Davis and Spallholz, 1996). Several groups have shown that selenocompounds induce apoptosis in cell culture systems (Lu et al., 1994; Garberg et al., 1988; Wilson et al., 1992; Shen et al., 1999; Zhong and Oberley, 2001; Lanfear et al., 1994; Kim et al., 2001; Jiang et al., 2001). Selenite-induced apoptosis was observed in the absence of the activation of caspases in DU-145 prostate cancer cells (Zhong and Oberley, 2001). In HL-60 cells, selenite-induced cell death was suggested to be derived from necrosis rather than apoptosis (Kim et al., 2001). Thus, significant differences exist in the selenite-induced cell death between cell types. It is not known whether selenite induces apoptosis in vivo. In this study, we investigated the action of selenite on hepatocytes in vivo, using regenerating liver after partial hepatectomy.

The hepatocyte is a highly differentiated cell that rarely divides in the normal adult liver. However, after two-thirds partial hepatectomy, most remaining hepatocytes promptly

Abbreviations: JNK, Jun N-terminal kinase; CDK, cyclin-dependent kinase; GOT, glutamate–oxaloacetate transaminase; PH, partial hepatectomy; DIG, digoxigenin.

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enter the cell cycle in a synchronous manner (Rabes, 1978). 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 activity of the c-Jun protein increases through its phosphorylation at Ser^{63} and Ser^{73} 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 PH (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). Recently, however, the JNK and AP-1 pathway was suggested to be involved in apoptosis (Colotta et al., 1992; Verheiji et al., 1996; Chen et al., 1996; Guo et al., 1998; Kobayashi and Tsukamoto, 2001).

Apoptosis is regulated by a network of genes whose connection to cell cycle genes has 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, the possible roles of the JNK–AP-1 pathway, p53 and p21 in the effect of selenite were also examined.

The results showed that selenite induced apoptosis in hepatocytes at an early stage of liver regeneration and the selenite-induced apoptosis was preceded by the activation of JNK and upregulation of c-jun, c-fos, p53 and p21.

2. Materials and methods

2.1. Materials

The reagents were purchased from the following sources: sodium selenite, Nacalai tesque; SAPK/JNK Assay kit, New England Biolabs; digoxigenin (DIG) RNA labeling kit and DIG luminescent detection kit, Boehringer Mannheim Biochemica; oligo(dT)cellulose (type 3), Collaborative Research; gene screen nylon membrane, NEN Research Products; immobilon polyvinylidene difluoride (PVDF) transfer membrane, Millipore; the antibodies to c-Jun, phospho-specific c-Jun (Ser^{63}), 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 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. Two-thirds partial hepatectomy was performed by the procedure of Higgins and Anderson (Higgins and Anderson, 1931). Sodium selenite (1.5 mg/kg body weight), suspended in saline, was intraperitoneally injected immediately after partial hepatectomy to the partially hepatectomized or 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 indicated times.

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

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

Regenerating 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) (MCA) (Peptide Institute, Osaka, Japan). The fluorescence intensity was calibrated with standard concentrations of 7-amino-4-methyl-coumarin (AMC) and the caspase-3 activity was expressed in picomoles per minute per milligram of protein. Protein concentrations in the supernatant were assayed using the bicinchoninic acid (BCA) protein assay kit (Pierce). For the inhibitor study, Ac-DEVD-CHO was added to the supernatant 15 min before the substrate.

2.5.2. Glutamate–oxaloacetate transaminase activity

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

2.5.3. JNK activity assay

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 protein concentration and JNK activity. Protein was measured with the BCA protein assay. The supernatant was then incubated with GST-c-Jun (1–89) coupled to GSH-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 GST-c-Jun at Ser^{63} was analyzed after immunoblotting with phospho-specific c-Jun (Ser^{63}) antibody.

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 resultant nuclear sample was determined using the BCA 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 phospho-specific c-Jun (p-c-Jun), 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).

2.7. Isolation and Northern blot analysis of RNA

Total RNA was extracted from the liver in 4 M guanidium isothiocyanate (Chomczynski and Sacchi, 1987) and fractionated by affinity chromatography on an oligo(dT)-cellulose column to obtain poly(A)-rich RNA. The concentrations of RNA samples were measured by absorbance at 260 nm. The purities of RNA samples were determined from the ratio of $A_{260\ nm}/A_{280\ nm}$ (>1.8) and by electrophoresis in formaldehyde–agarose gels stained with ethidium bromide. The RNA preparations were denatured and electrophoresed on 1.2% agarose/2.2 M formaldehyde gels. After separation, the RNA was transferred to Gene screen membranes by capillary blotting. Hybridization was carried out using digoxigenin-labeled RNA probes and the chemiluminescent signals were quantitated by a densitometer as described previously (Ozeki and Tsukamoto, 1999). Equal lane loading and the transfer efficiency of RNA samples were verified based on the intensity of ethidium bromide fluorescence of the rRNA on the gel and the filter. Comparable levels of albumin mRNA, which do not vary during 24 h after partial hepatectomy (Thompson et al., 1986), were confirmed in the RNA samples used for blotting.

2.8. Preparation of RNA probe

The *Eco*RI/*Pst*I fragment (1.8 kb) of c-jun (ATCC 63026), the *Pst*I fragment (1.0 kb) of c-fos (ATCC 41040), the *Eco*RI fragment (0.85 kb) of a mouse p21 cDNA clone, p21-9C, the *Hin*dIII/*AccI* fragment (1.3 kb) of mouse p53 cDNA (RDB 1284), or the *Pst*I fragment (0.5 kb) of rat albumin cDNA was subcloned into the plasmid Bluescript. After linearization of the plasmid, T7 RNA polymerase was employed to obtain runoff transcripts of the antisense strands. Transcription and labeling were per-

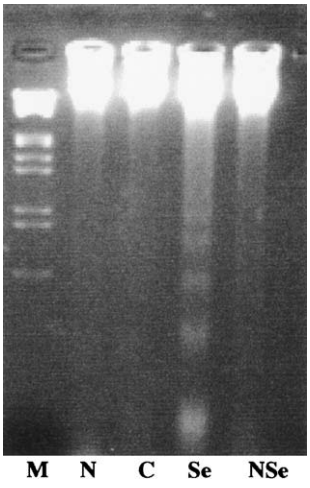

Fig. 1. Analysis of DNA fragmentation by agarose gel electrophoresis. Sodium selenite (1.5 mg/kg body weight) was intraperitoneally injected immediately after partial hepatectomy. Genomic DNA was isolated from liver of normal (N), control (partial hepatectomy only; C), selenite-injected (partial hepatectomy with selenite; Se) and selenite-injected normal (no partial hepatectomy with selenite; NSe) rats at 4 h after partial hepatectomy and injection. Lane M contained *Hin*dIII-digested λ DNA as a molecular size marker. The results presented here are typical of four separate experiments.

formed utilizing the commercial DIG RNA labeling system according to the manufacturer's instructions.

3. Results

3.1. Apoptosis induced by selenite

A characteristic ladder pattern of DNA was shown in the selenite-injected rat liver at 4 h after partial hepatectomy by agarose gel electrophoresis (Fig. 1). DNA isolated from the liver of normal, control and selenite-injected normal (no partial hepatectomy with selenite) rats yielded bands only in the high-molecular-weight region. Fig. 2 is a representative example of the in situ end-labeling of apoptotic cells in a liver section from the control and the selenite-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 selenite-injected rat liver (partial hepatectomy with selenite), with negligible background staining in the control (partial hepatectomy only) and also in the selenite-injected normal (no partial hepatectomy with selenite) rats. Apoptotic hepatocytes were rarely observed in selenite-injected normal animals, suggesting that entering to the cell cycle was required for selenite-induced apoptosis in hepatocytes. The identification of stained apoptotic bodies was confirmed by 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.

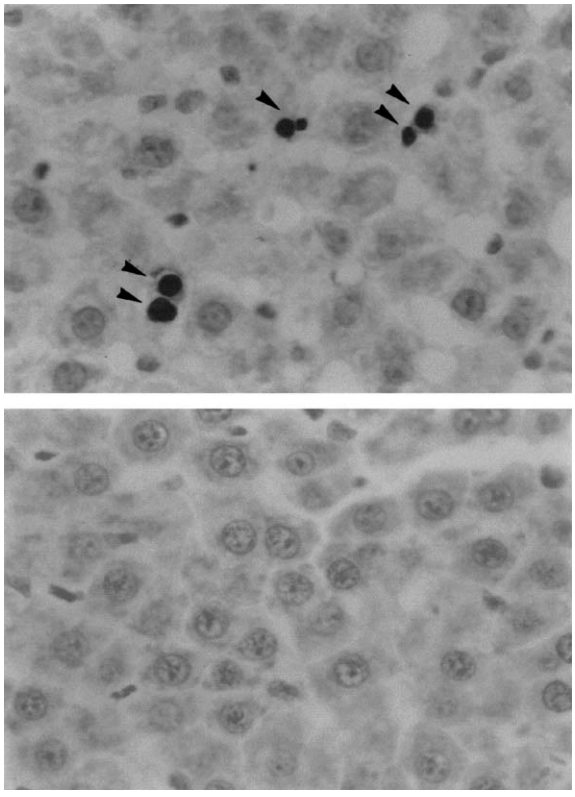

Fig. 2. In situ end-labeling of the apoptotic bodies in a liver section of selenite-injected (upper) and control rat (lower) at 4 h after partial hepatectomy. Sodium selenite (1.5 mg/kg body weight) was intraperitoneally injected immediately after partial hepatectomy. Paraformaldehyde-fixed paraffin-embedded liver sections, obtained from the regenerating liver of selenite-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 × 400).

Table 1
The effects of selenite on the activities of liver caspase-3 and serum glutamate–oxaloacetate transaminase

	Caspase-3 (pmol/min/mg protein)	Glutamate–oxaloacetate transaminase (IU)
Control	47.3 ± 3.8	158.1 ± 32.6
Se	199.9 ± 15.1[a]	135.2 ± 14.8
NSe	54.4 ± 5.3	48.2 ± 4.3[a]
Normal	52.1 ± 7.7	51.2 ± 5.5[a]

Sodium selenite (1.5 mg/kg body weight) was intraperitoneally injected immediately after partial hepatectomy to the partially hepatectomized (Se) or normal (without partial hepatectomy; NSe) rats. The activities of liver caspase-3 and serum glutamate–oxaloacetate transaminase were determined at 4 h after partial hepatectomy as described in Materials and methods. Values are means ± S.D. of three to five rats.

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

Caspases are known to execute apoptosis in a variety of systems (Cohen, 1997). The activity of caspase-3, one of the important effector caspases, remarkably increased to about fourfold the normal level in selenite-injected rats, while the activation was not observed in the control and the selenite-injected normal animals as shown in Table 1.

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

3.2. Effects of selenite on JNK activity

JNK activity was transiently induced to a barely detectable level at 1 h and then decreased after 2 h following partial hepatectomy in the control (Fig. 3A). In the selenite-injected rats, the activation of JNK similar to the control at 1 h was observed as early as 15 min and remarkably increased to about 10-fold the corresponding control level at 1 h after partial hepatectomy with selenite. The increased level was maintained until 2 h after partial hepatectomy with selenite.

To determine the JNK activation in vivo, the levels of phosphorylated c-Jun protein in the liver nuclear fraction were measured by Western blotting analysis. As shown in

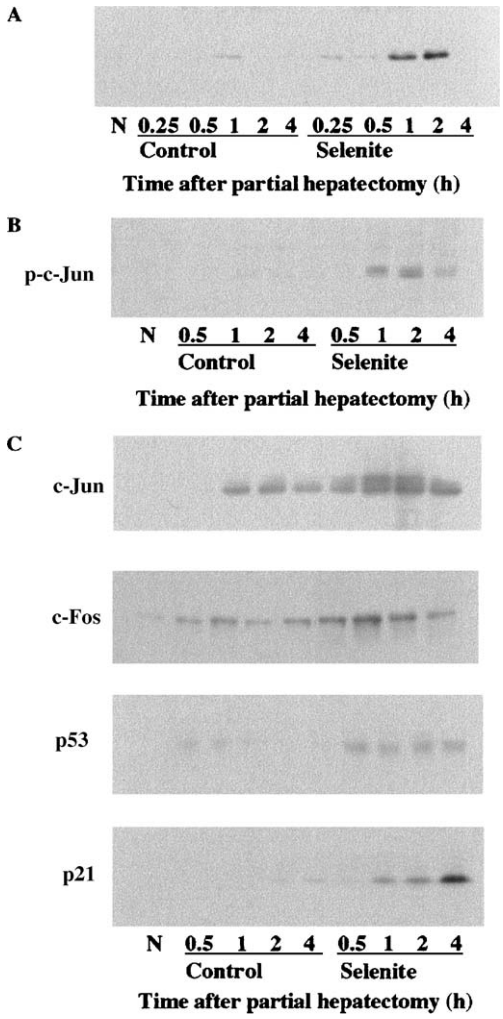

Fig. 3. Effects of selenite on JNK activation and the protein levels of phosphorylated c-Jun, c-Jun, p53 and p21 during liver regeneration. (A) JNK activity. Solid-phase in vitro JNK assays were performed using a SAPK/JNK assay kit and the liver lysate as described in Materials and methods. The liver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

Fig. 3B, the anti-p-c-Jun antibody detected two bands. This result was in agreement with the reports that phosphorylated (p-c-jun) and more highly phosphorylated c-Jun (pp-c-Jun) were observed in Western blot analysis (Guo et al., 1998; Luo et al., 1999; Kobayashi and Tsukamoto, 2001). In selenite-treated rats, p-c-Jun and pp-c-Jun protein remarkably increased at 30 min and reached a maximal level at 1 and 2 h after partial hepatectomy, although a faint band of the p-c-Jun was observed after 1 h in the control.

3.3. Effects of selenite on c-Jun, c-Fos, p53 and p21 protein level

As shown in Fig. 3C, c-Jun was recognized as a triplet, indicating that the two bands with slower migrations correspond to the phophorylated forms of c-Jun. The major band of c-Jun protein and a faint band of p-c-Jun were observed after 1 h following partial hepatectomy in the control. In selenite-treated rats, c-Jun with p-c-Jun and pp-c-Jun protein remarkably increased at 30 min and reached a maximal level at 1 and 2 h after partial hepatectomy. The c-Jun, p-c-Jun and pp-c-Jun levels of selenite-injected rats increased to about two-, five- and fivefold the corresponding control level at 1 and 2 h after partial hepatectomy, respectively.

The c-Fos protein level increased and peaked at 1 h after partial hepatectomy in the control. Selenite injection increased c-Fos protein expression to about threefold the corresponding control level at 30 min, 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. 3C). In selenite-injected rats, the p53 increased at 30 min and the increased level was maintained until 4 h after partial hepatectomy.

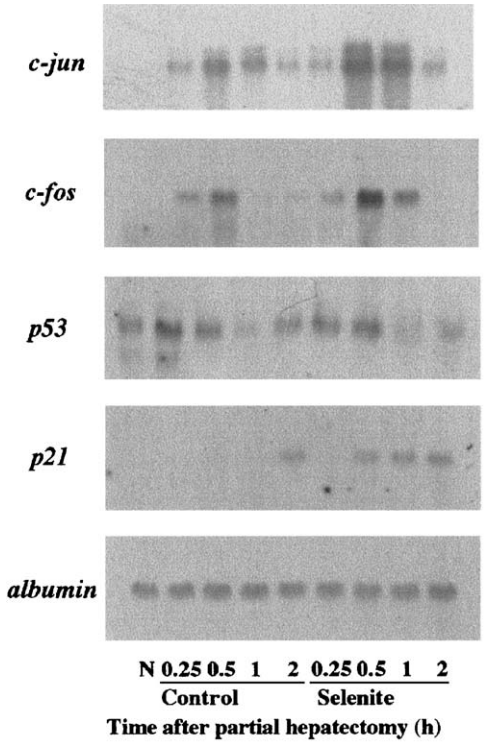

Fig. 4. Effects of selenite on the mRNA levels of c-jun, c-fos, p53 and p21 during liver regeneration after partial hepatectomy. Poly(A)$^+$-rich RNA (5 μg) from the liver of normal (lane N), control or selenite-injected rats at 0.25, 0.5, 1 and 2 h after partial hepatectomy was analyzed by Northern blotting using Dig-labeled RNA probes as described in Materials and methods. The comparable levels of albumin mRNA in the RNA samples used for the blotting are shown by the blot hybridized with albumin probe. The results presented here are typical of four separate experiments.

The $p21^{WAF1/CIP1}$ protein band was barely detectable during 4 h after partial hepatectomy in the control as shown in Fig. 3C. In selenite-injected rats, the protein band appeared at 1 h and increased up to 4 h after partial hepatectomy.

3.4. Effects of selenite on c-jun, c-fos, p53 and p21 mRNA levels

The level of c-jun transcripts was increased to about threefold the corresponding control at 30 min and 1 h after partial hepatectomy by selenite injection as shown in Fig. 4. A higher level of c-fos mRNA was also observed at 30 min and 1 h after partial hepatectomy in selenite-treated rats compared with the control.

The p53 mRNA levels were not significantly changed during 2 h after partial hepatectomy in both the control and selenite-injected rats. This result indicated that the increase in p53 protein levels caused by the selenite injection (Fig. 3C) was likely brought about by a post-transcriptional mechanism.

The p21 mRNA was barely detectable in the normal liver but increased at 2 h after partial hepatectomy. The mRNA level of p21 in the liver of selenite-injected rats increased after 30 min following partial hepatectomy.

4. Discussion

This study clearly demonstrated that selenite induced apoptosis at an early phase in the regenerating liver. The activation of caspase-3 and the characteristic DNA fragmentation were observed as early as 4 h after partial hepatectomy (Fig. 1). The role of caspases in selenium-induced apoptosis has not been established. In the selenite-induced cell death of HL-60 and prostate cancer cells, the activation of caspase-3 was not observed (Kim et al., 2001; Jiang et al., 2001). The cell death in HL-60 was suggested to be derived from necrosis rather than apoptosis (Kim et al., 2001). However, the results of in situ end-labeling showed the appearance of apoptotic cells with specific morphological criteria in hepatocytes after partial hepatectomy with selenite (Fig. 2). The serum level of glutamate–oxaloacetate transaminase activity also excluded the possibility of necrosis. Apoptosis was scarcely observed in the selenite-injected normal liver. The selenite-induced apoptosis was observed only in replicative competent hepatocytes after partial hepatectomy, not in normal quiescent cells. This result supports the possibility that selenite can be used to prevent or cure cancer as either a chemopreventive or a chemotherapeutic agent.

Selenite-induced DNA fragmentation was preceded by a marked increase in JNK activity. The activation of JNK associated with apoptosis was also observed in DU-145 prostate cancer cells exposed to selenite (Jiang et al., 2001). It was reported that selenite suppressed the UV-stimulated activity of JNK in 293T human embryonic kidney cells (Park et al., 2000). However, this suppression was due to the inhibition of JNK activity by the oxidation of the critical cysteine residue of JNK by selenite. On partial hepatectomy with selenite, not pretreatment with selenite, the activation of JNK was remarkably stimulated. The induction of JNK activity is one of the early events after partial hepatectomy (Westwick et al., 1995). The activation of JNK was observed transiently at 1 h after partial hepatectomy in the control (Fig. 3A). However, this activation was much less extensive than that in the selenite-injected rats. The activation in the selenite-injected rats was observed as early as 15 min and further increased at 1 h to about 10-fold the corresponding control. The increased level was maintained until 2 h after partial hepatectomy. The sustained JNK activation 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-α (TNF-α)-induced apoptosis in rat mesengial cells (Guo et al., 1998). These results suggest that the extent and duration of the JNK activation is critical in determining whether a cell will undergo apoptosis or progress through the cell cycle in regenerating liver consistent with our previous report (Kobayashi and Tsukamoto, 2001). The JNK activation in vivo was confirmed by the increase in phosphorylated c-Jun protein in the liver of selenite-injected rats. The two forms of phoshorylated c-Jun (p-c-Jun and pp-c-Jun) were detected as early as 30 min after PH with selenite, although they were scarcely observed in the control during 4 h after partial hepatectomy (Fig. 3B and C). The phosphorylation of c-Jun may be required to mediate a response to selenite. The c-*jun* gene is regulated by autostimulation of its AP-1 or c-Jun-ATF2 heterodimer binding site by c-Jun protein (Angel et al., 1988; Van Dam et al., 1993). In this experiment, the c-*jun* mRNA levels increased at 30 min and 1 h after partial hepatectomy with selenite in concert with the increase in the phosphorylated c-Jun protein level (Fig. 4). The increase in the mRNA levels was followed by the increase in c-Jun protein. In addition to the increase in c-jun expression, the levels of c-Fos protein and mRNA also increased in a similar manner to those of c-jun in the selenite-injected rats. The increase in Fos and Jun proteins, the principal AP-1 components, suggests that JNK and AP-1 may be involved in selenite-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. It is also reported that selenoglutathione, the direct metabolite of selenite, induced p53 expression and apoptosis in an ovarian cell line (Lanfear et al., 1994). The increase in p53 protein was observed after 30 min following partial hepatectomy with selenite (Fig. 3C). This increase was achieved with no induction of p53 mRNA (Fig. 4). One mechanism to rapidly and effectively regulate p53 is by post-translational modification including phosphorylation (Kastan et al., 1991; Zhang et al., 1994; Meek, 1998; Giaccia and Kastan, 1998; Appella

and Anderson, 2001; Pluquet and Hainaut, 2001). In non-stressed cells, p53 is in a latent form and is constitutively repressed by the binding of two proteins, Mdm2 and the inactive form of JNK, which mediate the degradation of p53 by the proteasome (Fuchs et al., 1998a,b; Haupt et al., 1997; Kubbutat et al., 1997). JNK association and targeting of p53 for ubiquitination take place during the G0 phase of the cell cycle, whereas Mdm2 targeting of p53 ubiquitination primarily occurs in the G2/M phase (Fuchs et al., 1998a). In response to stress, p53 phosphorylation coincides with its dissociation from both Mdm2 and JNK (Fuchs et al., 1998a; Shieh et al., 2000). p53 has been shown to be phosphorylated in vitro by JNK (Fuchs et al., 1998b; Lakin and Jackson, 1999). Further, the activated JNK phosphorylated p53 on Thr-81 in 293T cells (Buschmann et al., 2001). The JNK activation by WR1065 decreased complex formation between p53 and inactive JNK, and phosphorylated p53 at Thr-81 (Pluquet et al., 2003). These results suggest that the increase in p53 protein levels in the liver of selenite-treated rats might be due to stabilization through the phosphorylation, possibly by JNK, which has been activated. The increase in p53 protein levels after 30 min following partial hepatectomy with selenite was followed by the upregulation of p21 mRNA and protein expression (Figs. 3C and 4). The increase in p21 mRNA was detected after 30 min and accompanied by the appearance of p21 protein at 1 h following partial hepatectomy. These results suggest that selenite activates the p21 gene expression through a p53-dependent pathway. The upregulation of p21 mRNA and protein expression accompanied by an increase in p53 protein levels was also observed in cisplatin- and quercetin-induced apoptosis in regenerating liver (Kobayashi and Tsukamoto, 2001; Iwao and Tsukamoto, 1999). Although the role of p21 in apoptosis remains somewhat controversial (EL-Deiry, 1998), p21 overexpression seems to be a key factor involved in apoptosis in regenerating liver.

In conclusion, our paper has provided evidence that selenite induced apoptosis with the activation of caspase-3 at the early stage of liver regeneration and the selenite-induced apoptosis was preceded by the activation of JNK and the upregulation of c-jun, c-fos, p53 and p21. Further study is needed, however, to clarify the relation between JNK signaling, p53 pathway and caspase 3 activation in selenite-induced apoptosis.

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