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Lipid peroxidation and cell death mechanisms in rats and human cells induced by chloral hydrate

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Abstract

Chloral hydrate (CH) is widely used as a sedative and hypnotic in pediatric medicine. It is also a by-product of water chlorination and a metabolite of trichloroethylene. We examined the toxicological effects and cell death mechanisms of CH in rats and human Chang liver cells and lymphocytes. Monitoring of urinary 8-*epi*-PGF2α and serum levels of TNF-α served as index of lipid peroxidation and cytokine stimulation. The results indicated that a single intraperitoneal injection of 100 mg/kg CH in rats led to a nearly five-fold increase in urinary 8-*epi*-PGF2α on day 1, and a mild decrease on day 2 and day 3. The same treatment also induced significantly higher amounts of serum TNF-α on day 2 (about seven-fold). When the rats were treated with CH and vitamin E simultaneously, the amount of urinary 8-*epi*-PGF2α and serum TNF- were significantly lower than that in the rats treated with CH alone. CH caused a greater cytotoxic effect in human Chang liver cells than in comparison with lymphocytes. After treatment with CH, apoptosis features were observed in human lymphocytes, but not Chang liver cells. CH-induced cell damage in lymphocytes may offer signals for the induction of caspases activation. Further studies are needed to evaluate the relationship between caspases activation and the cleavage of other death substrates during postmitotic apoptosis in human lymphocytes.

Keywords: Chloral hydrate; Lipid peroxidation; 8-Epi-prostaglandin F2α; Tumor necrosis factor-α; Cell death mechanisms

1. Introduction

Chloral hydrate (CH) is a commonly found disinfection by-product in water purification and a sedative/hypnotic drug. It is also a reactive metabolite of trichloroethylene (TCE), a known carcinogen, and is structurally similar to other carcinogenic intermediates (Salmon et al., 1995). CH has been shown to be genotoxic in numerous prokaryotic and eukaryotic assay systems including human lymphocytes (reviewed in Beland, 1999). In vitro metabolism of CH by liver microsomes generated free radical intermediates that results in endogenous lipid peroxidation (Ni and Wong, 1994; Ni et al., 1996).

It has been proposed that tissue injuries catalysed by free radicals play a fundamental role in human disease (Cross et al., 1987). Particular constraints in addressing this hypothesis include the inability to assess free radical generation in vivo and the lack of information on drugs or vitamins that act as effective antioxidants in vivo. Isoprostanes are a family of prostaglandin isomers that are produced from oxidative modification of arachidonic acids through a mechanism catalyzed by free radicals (Pratico, 1999). One of the compounds that can be produced in abundance by such a mechanism is 8-*epi*-prostaglandin F2α (8-*epi*-PGF2α), a potent vasoconstrictor

Abbreviations: CH; chloral hydrate; 8-*epi*-PGF2α; 8-*epi*-prostaglandin F2α; MCA; methyl coumaryl-7-amine; MTT; 3; 4; 5-dimethylthiazol-2-yl-2; 5-diphenyl tetrazolium bromide; PBS; phosphate buffered saline; TCA; trichloroacetic acid; TCE; trichloroethylene; TCOH; trichloroethanol; TNF-α; tumor necrosis factor-α.

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and chemically stable end-product of lipid peroxidation. This compound has been shown to be a useful indicator of in vivo lipid peroxidation (Pratico, 1999; Wang et al., 2001).

Tumor necrosis factor-α (TNF-α) is secreted by macrophage, mononuclear phagocyte, lymphocyte and natural killer cells (Liu and Han, 2001). At low concentration, TNF-α is important in the homeostatic functions such as initiating tissue repair, whereas overproduction of TNF-α may cause damage to the endothelium, microthrombosis, and tissue damage (Old, 1985). Recent studies also provided evidence that pro-inflammatory cytokine was required for de novo carcinogenesis and that TNF-α was important to the early stages of tumor promotion. It was suggested to be the key cytokine for tumor promotion in mouse and probably for carcinogenesis in humans as well (Moore et al., 1999; Sugamuna et al., 1999). The induction of oxidative stress caused by CH is known for its cytotoxic and genotoxic effects (Ni et al., 1995). Tissue injuries caused by CH may be closely linked to oxidative reactions, and cytokines are mediators of the tissue with physiological and pathological functions. However, little is known about the relationship between oxidative stress and levels of cytokine TNF-α for predicting the outcome after acute CH poisoning.

Injuries to cells caused by chemicals can lead to a complex sequence of events that may result in cell death. Toxic cell damage can lead to necrosis when endogenous systems fail to compensate. Alternatively, cell death may be initiated by a programmed process called apoptosis. Recently, the critical role of the family of cysteine proteases (caspases) in mediating the apoptotic pathway has been demonstrated (Shi, 2002). Activated caspase can lead to the cleavage of other death substrates, to cellular and nuclear morphologic changes and, ultimately, to cell death (Zou et al., 1997; Abu-Oare and Abou-Donia, 2001). For instance, in the presence of dATP, procaspase 9 is converted to activate caspase 9, which then causes cleavage of procaspase 3 to caspase 3. Mammalian caspase 3 was shown to play a critical role in the apoptotic process both in vitro and in vivo (Porter and Janicke, 1999).

In the present study, we examined the effects of CH on the induction of lipid peroxidation and cytokine (TNF-α) production in rats. Monitoring of 8-*epi*-PGF2α in urine served as an indicator of oxidant stress in vivo. We also examined the protective effect of vitamin E, a chain-breaking antioxidant with the particular function of preventing lipid peroxidation in membrane systems against oxidative damage induced by CH. In addition, mechanisms of cell death induced by CH and the cytotoxic dosage were determined for human Chang liver cells and lymphocytes. The results indicated that apoptotic cell death can be induced by CH in human lymphocytes but not Chang liver cells. How cell injury can trigger the sequence of events that results in apoptosis is not well understood. Finally, this study further examined the caspases activities during the process of apoptosis induced by CH.

2. Materials and methods

2.1. Materials

CH and (+)α-tocopherol acetate (vitamin E acetate) were purchased from Sigma Chemical Co (St Louis, MO, USA). Dulbecco's modified Eagle's medium, cell culture supplements and fetal calf serum were ordered from Life Technologies (Gibco BRL, NY, USA). All other chemicals were commercially available products of analytical grade

2.2. Animals and treatment

Male Sprague–Dawley rats (250–350 g), purchased from the Animal Center of the National Cheng Kung University, were housed in metabolic cages in a room with temperature and humidity controls and 12-h light/dark cycles. Feed and tap water were provided ad lib. Five rats per group was used, rats were given a single ip injection of 100 mg/kg CH dissolved in 0.5 ml corn oil. For vitamin E pretreatment, 100 mg/kg (+)α-tocopherol acetate was injected 2 h prior to the CH treatment. The control group received the same amount of corn oil alone or 100 mg/kg of vitamin E. Urine samples were collected from the metabolic cage at 24, 48 and 72 h after treatment. Blood samples were collected from the heart and abdominal aorta at 24, 48 and 72 h after treatment. Serum samples were obtained from the blood samples. Both serum and urine samples were stored immediately at −70 °C until analysis.

2.3. Determination of urinary 8-epi-PGF2α and serum TNF-α

Urine samples were thawed and centrifuged at 1500 *g* for 15 min. Aliquot of 0.5 ml supernatant was applied to the 8-Isoprostane Affinity Column (Cayman Chemical Company) for purification of samples. Each purified sample was assayed in a 96-well plate coated with mouse anti-rabbit IgG monoclonal antibody to 8-*epi*-PGF2α (Cayman Chemical Company. 8-Isoprostane EIA Kit). The % cross-reactivity of the 8-*epi*-PGF2α antibody to 8-*epi*-PGF3 is about 20%, to 8-*epi*-PGFE1 and E2 is about 2%, to other isoprostanes is less than 1%. Urine creatinine was used as a denominator for urine 8-*epi*-PGF2α levels and was measured in each sample.

Serum TNF-α was determined by commercially available ELISA kits. Each serum sample was assayed in a 96-well plate coated with mouse anti-rabbit IgG monoclonal antibody to TNF-α in rat serum (R&D Chemical Company; TNF-α EIA Kit). Each sample underwent

assay in duplicates. The difference between the duplicated measurements of each sample was less than 15%.

2.4. Lymphocyte isolation and cell culture

Blood samples were collected from healthy donors, and mixed with an equal volume of RPMI 1640 medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum. Diluted blood was layered over a Histopaque 1077 density gradient (Sigma-Aldrich) and centrifuged at 800 *g* for 20 min at room temperature. The interface layer consisting of mononuclear cells was collected and washed three times with culture medium. Cells were counted, and the viability was assessed using the trypan blue exclusion method. The Chang liver cell line, which was derived from normal human liver tissue (with HeLa marker), was obtained from American Type Culture Collection (ATCC) and maintained in minimum essential medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum at 37 °C in a 5% carbon dioxide atmosphere. For exposure to CH, reagents were added in concentrated form to the culture medium and mixed gently. The cultures were then incubated for the times indicated in the figures. Cell viability was determined at the indicated times on the basis of MTT assay.

2.5. Trypan blue exclusion method and MTT assay

Cytotoxicity of human lymphocytes treated with different concentrations of CH was determined at indicated times based on the trypan blue exclusion method. Cells were seeded at a density of 5×10^4/well onto 24-well culture plates. The treated cells were centrifuged, and resuspented with 0.1 ml phosphate buffered saline (PBS). Each cell suspension (0.02 ml) was mixed well with 0.02 ml trypan blue solution (0.2% in PBS). Cytotoxicity of human Chang liver cells was determined by the MTT assay. Cells were also seeded at a density of 5×10^4/well onto 24-well culture plates. After treated with CH, 350 μl of 3,4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) solution (0.5 μg/ml in PBS) was added to each well, and incubated for 4 h. 10% SDS was then added for another 12 h to dissolve the dark blue crystals thoroughly. The absorbance at 570 nm was measured by an Emax precision microplate reader (Molecular Devices Instruments, USA).

2.6. DNA fragmentation assay

The control and treated cells were grown in 10-cm petri dishes. Both attached and unattached cells were harvested, washed twice with ice-cold PBS, suspended in TNE (10 mM Tris–HCl, pH 7.6; 140 mM sodium chloride; 1 mM EDTA) and lysed at 37 °C in 4 ml of extraction buffer (10 mM Tris–HCl, pH 8.0; 0.1 M EDTA, pH 8.0; 20 μg/ml pancreatic RNase; and 0.5% sodium dodecyl sulfate). The DNA was extracted by phenol/chloroform/isoamyl (25:24:1, by vol) before loading and analyzed by 1.5% agarose gel electrophoresis.

2.7. Flow cytometry

5×10^5 human lymphocytes were suspended with ice-cold PBS and fixed in 70% ethanol at −20 °C for at least 1 h. After fixation, cells were washed twice, incubated in 0.5 ml of 0.5% Triton X-100/PBS at 37 °C for 30 min with 1 mg/ml of RNase A, and stained with 0.5 ml of 50 μg/ml propidium iodide for 10 min. Fluorescence emitted from the propidium iodide–DNA complex was quantified after excitation of the fluorescent dye by FACScan flow cytometry (Becton Dickenson, San Jose, CA, USA).

2.8. Caspase activity

Cells were collected and washed with PBS and suspended in 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothione, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A and 10 μg/ml leupeptin after treatment. Cell lysates were clarified by centrifugation at 12,000 *g* for 20 min at 4 °C. Caspase activity in the supernatant was determined by a fluorogenic assay (CaspACE™ Assay System; Promega Corp., Madison, WI, USA). In brief, 50 g of total protein, as determined by bicinchoninic acid assay (Promega Corp.), was incubated with 50 μM substrate acetyl-Asp-Glu-Val-Asp-methylcoumaryl-7-amide (Ac-DEVD-AMC), acetyl-Ile-Glu-Thr-Asp-aminotrifluoromethyl coumarin (Ac-IETD- AMC) or acetyl-Leu-Glu-His-Asp-methylcoumaryl-7-amide (Ac-LEHD-AMC) at 30 °C for 1 h. The release of methyl coumaryl-7-amine (MCA) was measured by excitation at 360–400 nm and emission at 460–505 nm, respectively using a fluorescence spectrophotometer (Hitachi F-2500; Hitachi, Tokyo, Japan).

2.9. Statistical analysis

Data are expressed as mean ± SD. Statistical significance was performed using analysis of variance (ANOVA) for comparison among the means. Difference was considered significant at a P value of less than 0.05.

3. Results

3.1. Determination of 8-epi-PGF2 in urine samples collected from rats treated with CH or CH/and vitamin E

The degree of lipid peroxidation was estimated by the 8-*epi*-PGF2α level in urine. As shown in Fig. 1, the

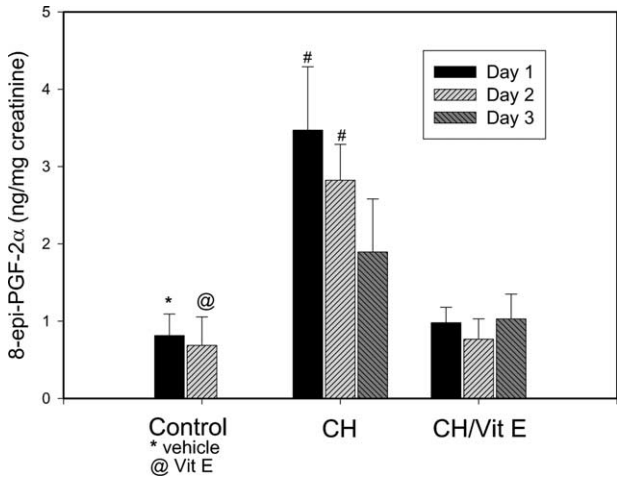

Fig. 1. Effects of CH and CH/Vit E on urinary 8-*epi*-prostaglandin F2α in rats. Five rats per group were injected ip with CH and combined CH/vitamin E at doses of 100 and 100 mg/kg plus 100 mg/kg. Urine samples were collected from the metabolic cages at 24, 48 and 72 h after treatment. Each value represents the mean±S.D. for five animals. # $P<0.001$ vs control.

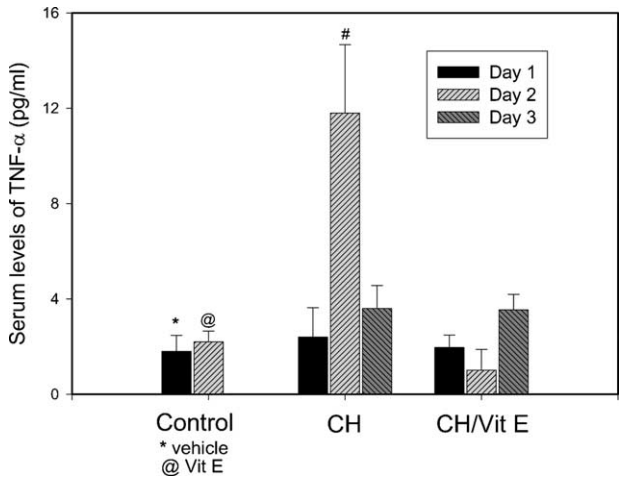

Fig. 2. Effects of CH and CH/Vit E on serum levels of TNF-α in rats. Five rats per group were injected ip with CH and combined CH/vitamin E at doses of 100 and 100 mg/kg plus 100 mg/kg. Urine samples were collected from the metabolic cages at 24, 48 and 72 h after treatment. Each value represents the mean±S.D. for five animals. # $P<0.001$ vs control.

basal level of 8-*epi*-PGF2α in control rats was about 0.81 ng/mg creatine. When rats were treated with a single dose of 100 mg/kg vitamin E for 1 day, levels of 8-*epi*-PGF2α in urine were lower than those of control rats, but the difference was not significant. When rats were treated with a single dose of 100 mg/kg CH for up to 3 days, significantly higher levels of urinary 8-*epi*-PGF2α were found. The increase of urinary 8-*epi*-PGF2α in rats treated with CH from day 1 through day 3 was about two- to five-fold. Levels of 8-*epi*-PGF2α were higher on day 1 in comparison with day 2 and day 3. When rats were treated with CH and vitamin E simultaneously, levels of urinary 8-*epi*-PGF2α were significantly lower than those of the CH-treated rats.

3.2. Determination of serum TNF- levels in rats treated with CH or CH/and vitamin E

Serum levels of TNF-α were examined to elucidate its possible role during the toxic process after acute treatment of CH. As shown in Fig. 2, basal levels of TNF-α in control rats were about 1.8 pg/ml. When rats were treated with a single dose of 100 mg/kg vitamin E for 1 day, their serum levels of TNF-α were slightly higher than those of control rats, but the difference was not significant. When rats were treated with a single dose of 100 mg/kg CH for 1–3 days, significantly higher levels of serum TNF-α could only be found in the rats treated for 2 days (roughly seven-fold higher than control) but not those treated for 1 or 3 days. When the rats were treated with CH and vitamin E simultaneously, levels of serum TNF-α were significantly lower than those of CH-treated rats.

3.3. Cytotoxicity effects of CH in Chang liver cells and human lymphocytes

As seen in Fig. 3, CH at levels greater than 2 mM showed significant toxicity to Chang liver cells. At 24 and 48 h after exposure to 2 mM CH, the viability of Chang liver cells was about 65 and 50%. An elevation of the CH concentration to 4 mM and more resulted in 45 and 70% more cell deaths at 24 and 48 h, respectively. Human lymphocytes were more resistant to the toxicity of CH (Fig. 4). At 24 and 48 h after exposure to 2 mM CH, the viability of human lymphocytes was about 90 and 70%. An elevation of the CH concentration to 4 mM

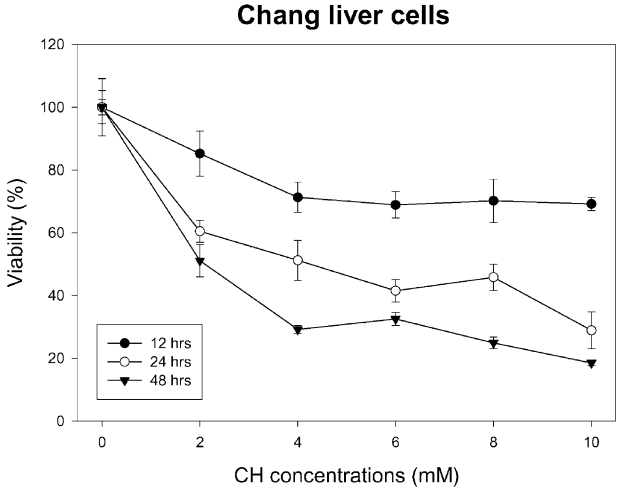

Fig. 3. Effects of varying concentrations of chloral hydrate on cell death of human Chang liver cells. Cell viability was determined at 12, 24 and 48 h after chloral hydrate treatment based on the MTT assay. Data expressed as mean±S.D.

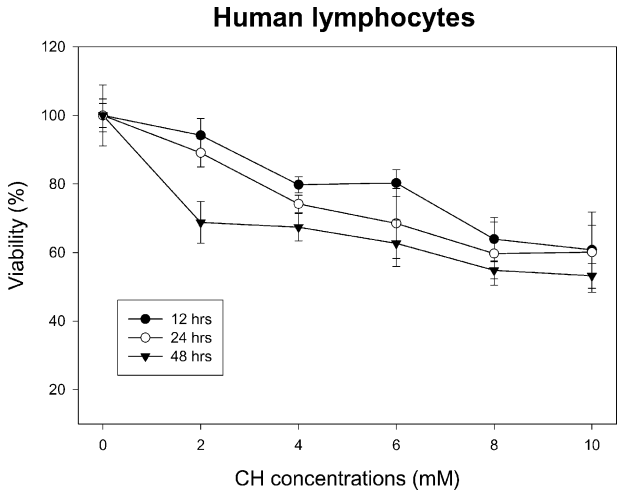

Fig. 4. Effects of varying concentrations of chloral hydrate on cell death of human lymphocytes. Cell viability was determined at 12, 24 and 48 h after chloral hydrate treatment based on the trypan blue exclusion method. Data expressed as mean ± S.D.

and more resulted in only 25% and 30–40% more of cell deaths at 24 and 48 h.

3.4. Mechanisms of cell death caused by CH in human lymphocytes

To evaluate DNA fragmentation, a characteristic of apoptosis, human lymphocytes were harvested at the indicated times after treatment with different concentrations of CH. The dose-dependent experiments showed that DNA fragmentation could be induced by CH at 48 h after exposure to doses greater than 0.5 mM in human lymphocytes (Fig. 5A). When Chang liver cells were exposed to 0.5–5 mM of CH for 48 h, agarose gel electrophoresis showed no DNA fragmentation but a slight smear of DNA due to random cleavage of base pairs during the necrosis process (data not shown). Quantitative analyses of DNA fragmentation were conducted by flow cytometry. The ratios of the sub-G1 population in human lymphocytes treated with 0.5, 1.5 and 3.0 mM of CH for 48 h were about 8, 15 and 27% (Fig. 5B,C).

3.5. Stimulation of caspase activity during CH-induced apoptosis

Caspases are activated in a sequential cascade of cleavages from their inactive forms. Once activated, caspases can subsequently cleave their substrates at specific sites (Zou et al., 1997; Abu-Oare and Abou-Donia, 2001). To monitor the enzymatic activity of caspases during CH-induced apoptosis, we used three fluorogenic peptide substrates. Ac-DEVD-AMC is a specific substrate for caspase-3, while both Ac-IETD-AMC and Ac-LEHD-AMC detect caspase-8 and caspase-9 activities. As shown in Fig. 6, 3.0 mM of CH induced a significant increase in LEHD-specific caspase-9 activity in human lymphocytes, approximately 3.5- and 7.5-fold of those in the control group at 24 h and 36 h, but the activity decreased after longer incubation periods. The same treatment also induced mild increases in caspase-3 activities. Roughly 3.0- and 3.5-fold increased activities compared with the control group could be observed in cells at 36 and 48 h. However, no significant increase in caspase-8 specific activities was found.

4. Discussion

Induction of endogenous lipid peroxidation by xenobiotics through generation of free radical species has been reported to result in alterations of cellular functions, genotoxic damage as well as tumorigenesis (Klaunig et al., 1989; Chang et al., 1992). 8-*epi*-PGF2α is a major F2-isoprostane that has been shown to increase during free-radical-mediated arachidonic acid oxidation in both experimental animal studies and in human studies (Basu, 1998, 1999; Davi et al., 1999). To the best of our knowledge, there were no studies that used 8-*epi*-PGF2α as a biomarker of lipid peroxidation induced by CH both in vitro and in vivo. Our results showed that after one single dose of CH treatment, a significant increase in urinary 8-*epi*-PGF2α was observed on day 1, and mild decreases were observed on day 2 and day 3. One day after ip injection may not be the optimal sampling time, and the peak value of oxidative damage might occur earlier. The maximum plasma concentration of CH was observed at 15 min after administration in rats. By 1 h, the concentration dropped substantially, and by 3 h CH could no longer be detected (Beland, 1999). Trichloroacetic acid (TCA) and trichloroethanol (TCOH) were the major metabolites detected in the plasma. Both CH and TCA can generate free radicals and induce lipid peroxidation in the present of liver microsomes (Beland, 1999). In addition to the liver tissue, extrahepatic tissues were also exposed to CH continuously. A metabolic study of CH indicated that TCA and TCOH could be formed in blood and liver of mouse, rat and human (Lipscomb et al., 1996). Measurements of urinary 8-*epi*-PGF2α served as an indicator of oxidative stress in the whole body, not only in the liver.

The non-invasive measurement of 8-*epi*-PGF2α is a promising approach for studying the possible roles of lipid peroxidation under normal conditions and in the pathology of human diseases (Pratico, 1999). However, only a few animal studies have investigated the effects of vitamin E on F_2-isoprostanes (Sodergren et al., 2000, 2001). The protective effect of vitamin E against lipid peroxidation in rats treated with CH was assessed in the present study, and the result showed that lipid

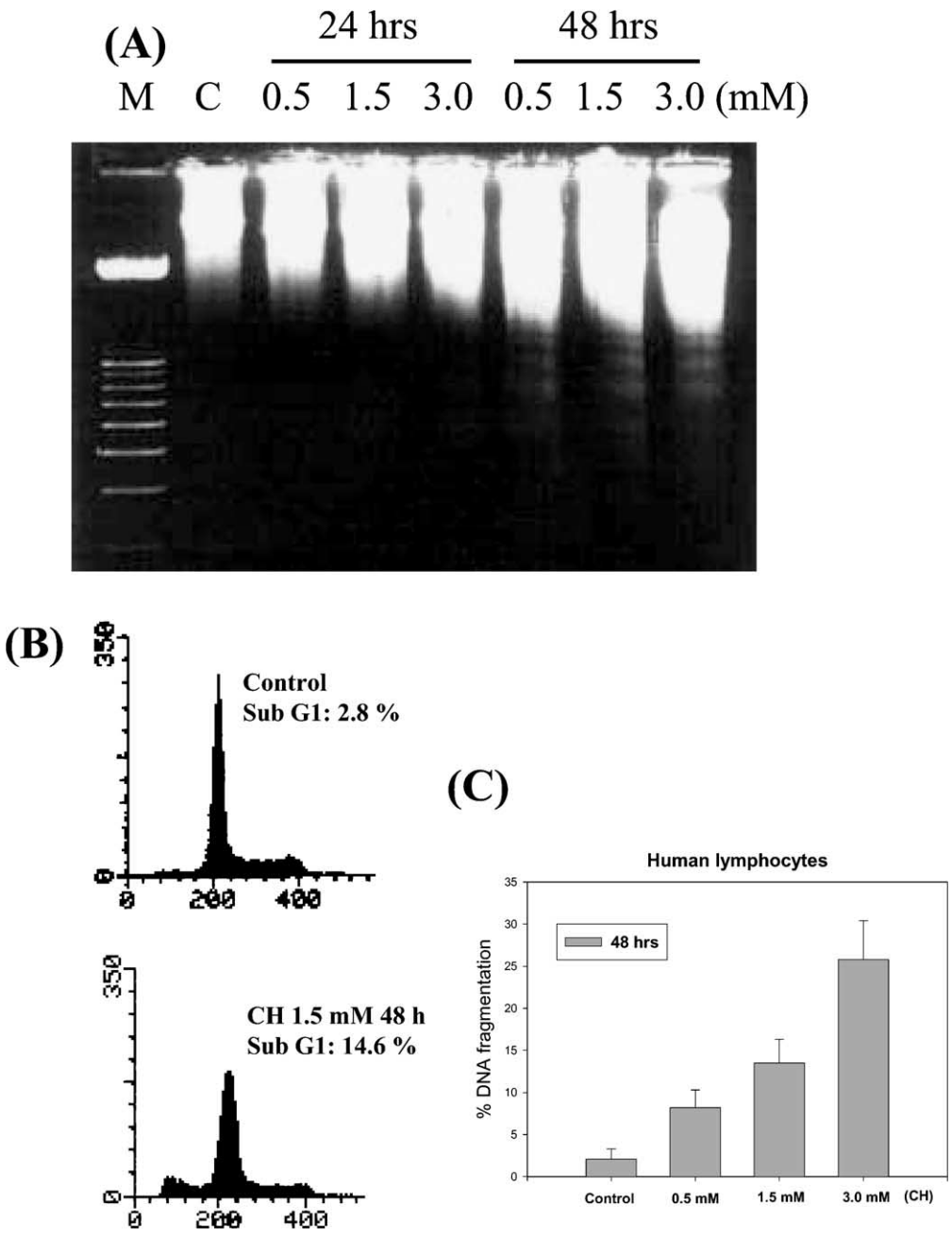

Fig. 5. Analysis of DNA fragmentation in human lymphocytes. (A) Agarose gel analysis of DNA fragmentation in human lymphocytes treated with 0.5, 1.5 and 3.0 mM CH for 24 and 48 h. Cells produced a DNA ladder characteristic of apoptosis after treatment with CH for 48 h. (B) Measurement of percentage of sub-G1 cells in human lymphocytes treated with CH by flow cytometry. The ratio of sub-G1 cells was about 14.8% in human lymphocytes treated with 1.5 mM CH for 48 h. (C) Quantification of DNA fragmentation in human lymphocytes treated with different concentrations of CH for 48 h by flow cytometry.

peroxidation was suppressed by vitamin E. Basal levels of 8-*epi*-PGF2α in vitamin E-treated rats (100 mg/kg, ip) were lower than those in control rats. Many different biomarkers of lipid peroxidation could be used to evaluate the oxidative damage. Different responses may be represented by different biomarkers because they reflect different stages of the lipid peroxidation process. Vitamin E, a chain-breaking antioxidant with the particular function of preventing lipid peroxidation in membrane systems, was proven to be efficiently against the oxidative damages induced by CH.

An over-production of free radical species has been demonstrated to stimulate the expression and synthesis of various inflammatory cytokines, such as TNF-α and IL-1, which are thought to associate with the pathophysiology of liver disease (Koga et al., 1992; Liu et al., 2001). Recently, TNF-α was proposed to be the central mediator of tumor promotion and releasing of TNF-α

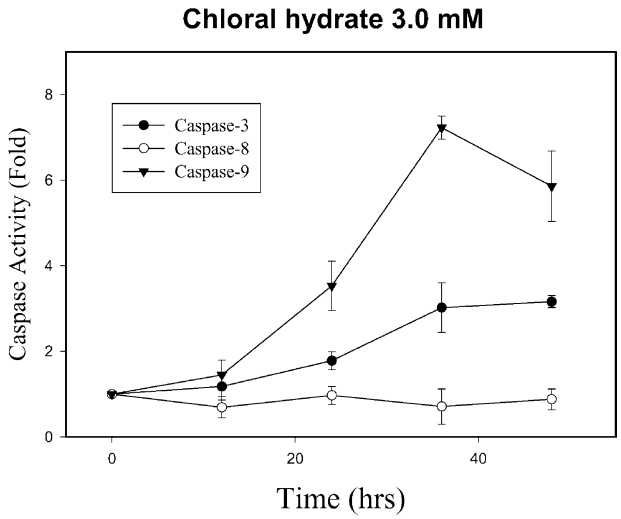

Fig. 6. Induction of caspase activities in human lymphocytes. Cells were treated with 3.0 mM CH for different time periods. Caspase activities were analyzed as described in Materials and methods. Data represent means±S.D. for three determinations.

from initiated cells or various neighboring cells was believed to be able to induce clonal growth in these initiated cells (Moore et al., 1999). TNF-α was proposed to be the essential molecule common to carcinogenesis in various organs, and IL-1 was suspected to be involved in the later stages of carcinogenesis (Suganuma et al., 1999). A recent report indicated that hepatoadenoma prevalence and multiplicity were significantly increased in animals treated with CH. CH was shown to be carcinogenic in mice following a lifetime exposure through drinking water (George et al., 2000). Our results showed that serum TNF-α level was induced on day 2 treated with CH, but lipid peroxidation could be induced in less than 1 day, implicating that CH induced oxidative stress may serve as a mediator of TNF-α release and facilitate at least in part the toxic manifestations of CH. We also found that vitamin E treatment not only lowered the generation of lipid peroxidation but also decreased the production of TNF-α secretion induced by CH. Thus, CH-induced oxidative stress may play a critical role in the induction of lipid peroxidation and production of cytokines. An aspect of related interest that is under intensive investigation is lipid peroxidation mediated stress signaling, which may evoke a variety of cellular responses, ranging from induction of antioxidant enzymes to apoptotic death (reviewed in Girotti, 1998). Further investigation of these processes will facilitate the understanding of disease states associated with peroxidative stress.

Cytotoxicity, cell death mechanisms and apoptosis-related caspases activities were investigated in human Chang liver cells and lymphocytes treated with CH. Using the trypan blue exclusion method and MTT assay to detect cytotoxicity, exposure to relatively mild insults (levels below 1 mM CH) led to slower onset and less overall cell death than exposure to more intense insults (levels greater than 2 mM CH). The concentration of CH required to cause 50% cell death in human lymphocytes was about five-fold greater than that required in human Chang liver cells. CH is very rapidly metabolized to TCA and TCOH; both can be formed in liver and blood. Furthermore, the metabolism of CH may accounts for its toxicity (Lipscomb et al., 1996). After exposure by the oral route, which most often occurs with CH administration, the first-pass effect may occur in portal blood rather than liver. Indeed, given the large volume of blood, it is possible that less than one-half of the total CH metabolism may actually occur in the liver (Lipscomb et al., 1996). However, in a 2-year animal study, CH increased the prevalence of neoplasia only in liver but not any other organ site (George et al., 2000). A higher sensitivity of liver cell to CH-induced toxicity may partly explain why liver is the target organ of carcinogenicity of CH.

Cell death mechanisms induced by CH were investigated in human Chang liver cells and lymphocytes. The results suggest a CH-mediated apoptosis and DNA laddering in human lymphocytes. But cell deaths were more characteristic of necrosis than apoptosis in Chang liver cells. The distinction between apoptosis and necrosis in cell culture systems can be confusing because of the lack of scavenging cells; thus, the phagocytic step after apoptosis may not occur. It is important to look for features of apoptosis at various time points after the insult and not to unduly delay these observations, since secondary necrosis may intervene and therefore obfuscate the true nature of the injury to the compromised cell (Bonfoco et al., 1995). The selection of incubation time for treatment of human lymphocytes with CH is based on a time-course pilot study, and the results showed that 48 h is optimal for observing DNA fragmentation. As seen in Fig. 5, a dose-dependent DNA laddering could be observed in human lymphocytes treated with different concentrations of CH for 48 h, indicating that CH was an inducer that can trigger apoptotic cell death in human lymphocytes. This time period for induction of apoptosis in cells seems to be unusual, because apoptosis generally occurs within 24 h after induction of damage in the cell. However, it has been reported that apoptosis may occur after a considerable delay and that in such a situation, cell death is often not due to the primary damage only but also may result from the accumulation of secondary changes during the cytostatic phase (Halicka et al., 1997). Thus, we suggest a postmitotic apoptosis pathway in human lymphocytes exposed to CH. How CH triggers the induction of postmitotic apoptosis in human lymphocytes is unclear and has never been reported.

Disruption of mitochondria transmembrane potential and loss of phospholipid asymmetry of the plasma membrane are early features of apoptosis and can be

detected in cells that still lack obvious morphologic signs of apoptosis (Kroemer et al., 1997). One critical consequence of the mitochondria alteration is the rapid release of caspase activators such as cytochrome c from the intermembrane space into the cytosol. Released cytochrome c may trigger caspase-9 and caspase-3 activation via the Apaf-1 pathway (Reed, 1997; Green and Reed, 1998). In the present study, we found that CH induced significant activation of caspase-9 but only a mild increase in caspase-3. A number of recent studies indicated that caspase-9, caspase-3 and caspase-activated DNA fragmentation factors may all be a linear, non-redundant pathway during acute apoptosis (Liu et al., 1997; Bernardi et al., 2001). Nevertheless, in our study, we found only a slight increased of caspase-3 activity during the CH-induced apoptotic processes in human lymphocytes. It has been reported recently that premitotic and postmitotic apoptosis differ from each other not only in cell-cycle sensitivity but also in intracellular apoptotic pathways (Shinomiya et al., 2000). Treatment with a caspase-3-specific inhibitor remarkably suppressed premitotic apoptosis but showed little effect on postmitotic apoptosis (Shinomiya et al., 2000). The precise mechanism differences between these two types of apoptosis remain not well understood and need further investigation.

In summary, the data presented here indicate that oxygen species may be involved in the mechanisms of CH intoxication because urinary 8-*epi*-PGF2α can be induced by CH and attenuated by vitamin E. The generation of oxygen species not only induces lipid peroxidation but also modulates the production of TNF-α. Lipid peroxidation and cytokines may play a role in CH-induced injury. After treatment of CH, a stronger cytotoxic effect can be observed in human Chang liver cells than in human lymphocytes. Apoptosis features were found only in human lymphocytes but not in Chang liver cells treated with CH. CH-induced cell damage in lymphocytes may offer signals for the induction of caspases activation. Further information about the relationship between caspase activation and the cleavage of other death substrates during postmitotic apoptosis in human lymphocytes needs further investigation.

Acknowledgements

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