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# Cytotoxicity and Apoptosis Produced by Cytochrome P450 2E1 in Hep G2 Cells

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## ABSTRACT

Two Hep G2 subclones overexpressing CYP2E1 were established with the use of transfection and limited dilution screening techniques. The Hep G2-CI2E1–43 and -47 (E47) cells (transduced Hep G2 subclones that overexpress CYP2E1) grew at a slower rate than parental Hep G2 cells or control subclones that do not express CYP2E1, but remained fully viable. When GSH synthesis was inhibited by treatment with buthionine sulfoximine, GSH levels rapidly declined in E47 cells but not control cells, which is most likely a reflection of CYP2E1-catalyzed formation of reactive oxygen species. Under these conditions of GSH depletion, cytotoxicity and apoptosis were found only with the E47 cells. Low levels of lipid peroxidation were found in the E47 cells, which became more pronounced after GSH depletion. The antioxidants vitamin E, vitamin C, or trolox prevented the lipid peroxidation as well as the cytotoxicity and apoptosis, as did transfection with plasmid containing antisense CYP2E1 or overexpression of Bcl-2. Levels of ATP were lower in E47 cells because of damage to mitochondrial complex I. When GSH was depleted, oxygen uptake was markedly decreased with all substrates in the E47 extracts. Vitamin E completely prevented the decrease in oxygen uptake. Under conditions of CYP2E1 overexpression, two modes of CYP2E1-dependent toxicity can be observed in Hep G2 cells: a slower growth rate when cellular GSH levels are maintained and a loss of cellular viability when cellular GSH levels are depleted. Elevated lipid peroxidation plays an important role in the CYP2E1-dependent toxicity and apoptosis. This direct toxicity of overexpressed CYP2E1 may reflect the ability of this enzyme to generate reactive oxygen species even in the absence of added metabolic substrate.

Direct exposure of various cell types to such oxidants as hydrogen peroxide or lipid hydroperoxides can directly induce apoptosis; in many experimental models, pretreatment of the cells with antioxidants has been shown to protect against this form of cell death (Nobel *et al.*, 1995; Talley *et al.*, 1995). The prototypic regulator of mammalian apoptosis is the proto-oncogene *bcl-2*. Overexpression of Bcl-2 leads to protection for many cell types against apoptosis induced by exposure to a wide variety of adverse conditions and stimuli, including lipid peroxidation, which suggests that Bcl-2 controls a distal step in a signal transduction pathway leading to apoptosis (Hockenberry *et al.*, 1993; Reed, 1994; Armstrong *et al.*, 1996). Mitochondria are important in generating a membrane potential and producing ATP and can release factors that seem to activate caspases, which initiate the apoptotic process (Martin and Green, 1995; Whyte and Evan, 1995). Damage to mitochondria and membrane permeability transitions occur in ROS-induced apoptosis (Schulze-Osthoff *et al.*, 1992; Kripper *et al.*, 1996).

Cytochrome P450 2E1 (CYP2E1), the ethanol-inducible form, is of interest because of its ability to metabolize and activate many toxicologically important substrates to more toxic products (Yang *et al.*, 1990; Guengerich *et al.*, 1991; Koop, 1992). A variety of mechanisms have been suggested to play important roles in pathways of ethanol toxicity to the liver. Induction of CYP2E1 and CYP2E1-dependent oxidative stress is one area of active research (Nordmann *et al.*, 1992). CYP2E1 from rat and rabbit liver exhibits enhanced NADPH

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**ABBREVIATIONS:** GSH, reduced glutathione; 4-HNE, 4-hydroxy-2-nonenal; 4-MP, 4-methylpyrazole; A14, Hep G2-CIA-14, a Hep G2 subclone transduced with vector containing antisense bcl-2 cDNA; B28, Hep G2-CIBcl-2–28, a transduced Hep G2 subclone that overexpresses Bcl-2; BSO, buthionine sulfoximine; C34, Hep G2-CI-34, a Hep G2 subclone transduced with pCI-neo vector lacking any CYP2E1 cDNA insert; C37, Hep G2-CI-37, a Hep G2 subclone transduced with pCI-neo vector lacking any CYP2E1 cDNA insert; DMSO, dimethylsulfoxide; E43, Hep G2-CI2E1–43, a transduced Hep G2 subclone that overexpresses CYP2E1; E47, Hep G2-CI2E1–47, a transduced Hep G2 subclone that overexpresses CYP2E1; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LDH, lactate dehydrogenase; MDA, malondialdehyde; MEM, minimum essential medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PNP, *p*-nitrophenol; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; VitE, vitamin E.

oxidase activity and elevated production of ROS, in that it seems to be poorly coupled with NADPH-cytochrome P450 reductase (Gorsky *et al.*, 1984; Ekstrom and Ingelman-Sundberg, 1989; Rashba-Step *et al.*, 1993). CYP2E1 was shown to be more active in catalyzing lipid peroxidation than several other forms of cytochrome P450 enzymes (Ekstrom and Ingelman-Sundberg, 1989). Microsomes from rats treated with ethanol to induce CYP2E1 displayed elevated rates of production of a variety of ROS, including superoxide, $H_2O_2$, hydroxyl and preferryl-type oxidants (Thurman, 1973; Ekstrom *et al.*, 1986; Ekstrom and Ingelman-Sundberg, 1989; Rashba-Step *et al.*, 1993). CYP2E1 is also reactive in catalyzing formation of the 1-hydroxyethyl radical from ethanol (Reinke *et al.*, 1990; Albano *et al.*, 1991).

To directly demonstrate that CYP2E1 can promote the hepatotoxicity of various agents, a Hep G2 cell line that constitutively expresses the human CYP2E1 was established (Dai *et al.*, 1993). Microsomes from the Hep G2-MV2E1–9 cells that express CYP2E1 were much more reactive in production of superoxide radical and $H_2O_2$ and in catalyzing lipid peroxidation than control microsomes (Dai *et al.*, 1993). The addition of acetaminophen, ethanol, or arachidonic acid (as a representative polyunsaturated fatty acid) resulted in cytotoxicity to those Hep G2 cells that expressed CYP2E1 but not to the control cells (Dai and Cederbaum, 1995; Wu and Cederbaum, 1996; Chen *et al.*, 1997). Toxicity of these agents was enhanced when cellular GSH was lowered by treatment with BSO. Ethanol and arachidonic acid toxicity was associated with elevated lipid peroxidation and could be prevented by a variety of antioxidants (Wu and Cederbaum, 1996; Chen *et al.*, 1997).

Formation of ROS by microsomes from the CYP2E1-containing cells was not influenced by the presence of CYP2E1 substrates or ligands such as PNP, aniline, ethanol, or 4-methylpyrazole (Dai *et al.*, 1993). This is probably a reflection of the "loose coupling" associated with CYP2E1 (Gorsky *et al.*, 1984; Ekstrom *et al.*, 1986; Ekstrom and Ingelman-Sundberg, 1989). In the Hep G2-MV2E1–9 cells, although hepatotoxicity was observed upon addition of exogenous toxin (acetaminophen, ethanol, or arachidonic acid), no apparent toxicity was caused by the expression of CYP2E1 itself in the absence of exogenous toxins. Because ROS could be generated by CYP2E1 even in the absence of substrate, the lack of toxicity was assumed to reflect the relatively low level of expression of CYP2E1 and ability of the cellular antioxidant defense to protect against ROS-induced cytotoxicity. The goal of this study was to evaluate whether establishing a cell line with enhanced expression of CYP2E1 could serve as a model to demonstrate direct toxicity of the overexpressed CYP2E1, in the absence of added toxin. Indeed, it was observed that overexpression of CYP2E1 resulted in a decrease in growth rate of the Hep G2 cells and, when the cells were treated with BSO, which inhibited *de novo* GSH synthesis, intracellular GSH was quickly depleted and cytotoxicity and apoptosis occurred.

## Materials and Methods

**Cells and chemicals.** Hep G2 cells and its transduced subclones, A14, B28, C34, C37, E43, and E47 cells, were cultured in MEM, supplemented with 10% fetal calf serum, 100 units of penicillin per milliliter, 100 mg/ml of streptomycin, and 2 mM glutamine in a humidified atmosphere in 5% $CO_2$ at 37°. Most reagents were purchased from Sigma Chemical (St. Louis, MO). Specific reagents are described below. The protein content of cell lysates or isolated microsomes was determined with the DC-20 Protein Assay kit (Bio-Rad Laboratories, Hercules, CA).

**Establishment of Hep G2 subclones that overexpress CYP2E1.** Human CYP2E1 cDNA, excised from a plasmid p91023(B)-2E1 (kindly provided by Dr. F. J. Gonzalez, National Cancer Institute, Bethesda, MD), was inserted into the *Eco*RI restriction site of pCI-neo expression vector (Promega, Madison, WI) in the sense and antisense orientation (confirmed by restriction mapping) to form the plasmids pCI-2E1 and pCI-as-2E1. Transfections of Hep G2 cells were carried out with the use of LipofectAMINE reagent (Life Technologies, Grand Island, NY) as described by Hawley-Nelson *et al.* (1993). Eighteen hours after transfection, fresh MEM containing 0.8 mg of geneticin per milliliter was added and the cells were incubated for an additional 2 days. The cells were harvested by trypsinization for geneticin-selection and Western blot analysis. About $1.5 \times 10^6$ transfected Hep G2 cells were seeded into 100-mm Petri dishes with MEM containing 0.8 mg of G418 per milliliter. Seven days later, survivors were harvested by trypsinization and seeded into 96-well tissue culture plates at densities of 0.5, 1, and 2 cell/well (limited dilution). Monoclones were formed in about 3 weeks. Colonies were grown to large scale, and subjected to Western blot analysis and PNP oxidation activity assay. Positive clones were subjected to another two rounds of limited dilution screening to create stable cell lines.

**Establishment of Hep G2 subclones overexpressing Bcl-2.** A full-length human bcl-2 cDNA, excised from pSFFV-bcl-2 expression vector (kindly provided by Drs. George Acs and Beatriz Pogo, Mount Sinai School of Medicine, New York, NY), was inserted into the *Eco*RI restriction site of pCI-neo expression vector in the sense and antisense orientation to form the expression vectors pCI-bcl-2 and pCI-as-bcl-2. These vectors were used for transfections of Hep G2 cells using the LipofectAMINE reagent as described above. After transfection, geneticin selection and limited dilution screening, stable new clones were analyzed by immunoblotting using anti-Bcl-2 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

**Western blot analysis.** Cell lysates were produced by vortexing and boiling $1 \times 10^6$ cells in 0.2 ml SDS-PAGE running buffer. A 20-$\mu$l aliquot of pretreated cell sample was resolved on 10% SDS polyacrylamide gels and transblotted onto nitrocellulose sheets (Bio-Rad) for Western blot analysis (Towbin *et al.*, 1979). Rabbit anti-human CYP2E1 polyclonal antibody (provided by Dr. J. M. Lasker, Mt. Sinai School of Medicine, New York, NY) was used as the primary antibody followed by treatment with alkaline phosphatase conjugated to goat anti-rabbit IgG (Bio-Rad) as the second antibody. Staining intensity was developed with the nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate mixture (Promega).

**PNP oxidation assay.** Cells were washed once with PBS (100 mM phosphate; 154 mM NaCl, pH 7.4) and harvested by scraping and subsequent sonication for 45 sec. Microsomes were prepared by differential centrifugation and resuspended in PBS containing 20% glycerol. Oxidation of PNP was determined using 100 $\mu$g of microsomal protein in a 100-$\mu$l reaction-system containing PBS, 0.4 mM PNP, and 1 mM NADPH. All reactions were carried out in duplicate, initiated with NADPH, incubated at 37°, and stopped after 60 min by addition of 30 $\mu$l of 20% trichloroacetic acid. Absorbance of the final product of the reaction was measured at 586 nm, and activity was determined using an extinction coefficient of 9.4 $mM^{-1}cm^{-1}$.

**Cytotoxicity assay.** DNA fragmentation as a biochemical marker of apoptosis (Harmon *et al.*, 1979; Caron-Leslie and Cidlowski, 1991) was determined by agarose gel electrophoresis as described previously (Chen *et al.*, 1997). Pictures of the gels were taken by UV transillumination. Cytotoxicity was measured primarily with the use of the MTT assay (Mosmann, 1983), using the Cell Titer 96 Nonradioactive Cell Proliferation Assay kit (Promega) as described previously (Chen *et al.*, 1997). The absorbance of the reaction solution at 570 nm was recorded. The absorbance at 630 nm was used as

reference. The net $A_{570nm}$–$A_{630nm}$ was taken as the index of cell viability. The net absorbance from the wells of cells cultured with control medium was taken as the 100% viability value. The percent viability of the treated cells was calculated by the formula: $(A_{570nm}–A_{630nm})_{sample}/(A_{570nm}–A_{630nm})_{control} \times 100$. LDH leakage was measured as another index of cytotoxicity using the LDH Assay kit LD-L20 (Sigma) as described previously (Chen *et al.*, 1997). LDH activity in sonicated cell extracts and in aliquots of tissue culture medium was determined, and the cytotoxicity index was expressed as the ratio of $LDH_{out}/LDH_{in}$. The lipid peroxidation end products MDA and 4-HNE were assayed using the LPO-586 kit (Calbiochem-Novabiochem, La Jolla, CA) as described previously (Chen *et al.*, 1997).

**Transient transfection with pCI-2E1 plasmids.** Transfection of B28, A14, and C34 cells with pCI-2E1 was carried out with the LipofectAMINE reagent. Cells ($1.5 \times 10^6$) were seeded into a 100-mm culture dish and grown to 50–70% confluence. Cells were rinsed with serum-free MEM before transfection. pCI-2E1 plasmid DNA (15 $\mu$g) and 100 $\mu$l of LipofectAMINE reagent were used to transfect each culture dish of B28, A14, or C34 cells. To prevent expression of CYP2E1 protein, 20 $\mu$g of pCI-as-2E1 plasmid DNA and 100 $\mu$l of LipofectAMINE reagent were used to transfect one culture dish of E47 cells. The cells were collected by trypsinization and used for Western blot analysis and for studies of cell growth and cytotoxicity.

**GSH assay.** Cells ($5 \times 10^6$) were subcultured into a 10-mm culture dish overnight before BSO was added. After varying times of incubation, the cells were harvested by scraping. Cells scraped before BSO treatment were considered the 0-time sample. Cells were washed with PBS and resuspended in PBS and sonicated for 10 sec. After protein assay, cell lysate equivalent to 2 mg of protein was used to measure the content of intracellular GSH by the Bioxytech GSH-400 Assay Kit (OXIS International, Portland, OR). Briefly, an initial sample volume of 200 $\mu$l was incubated with 50 $\mu$l of the chromogenic reagent R1, thoroughly mixed, followed by addition of 50 $\mu$l of 30% NaOH (solution R2) and incubation for 30 min at 37°. Under alkaline conditions, the assay is specific for GSH. The final absorbance at 400 nm was measured. Reduced GSH was used to prepare a standard curve. The intracellular GSH value was standardized against the protein concentration of the mixture.

**ATP assay.** The intracellular ATP level was assayed using kit 366-A (Sigma). The measurement is based on the reactions catalyzed by the enzyme's phosphoglyceric acid phosphokinase, which catalyzes conversion of 3-phosphoglycerate to 1,3-diphosphoglycerate by consuming ATP, and glyceraldehyde-3-phosphate dehydrogenase, which catalyzes reduction of 1,3-diphosphoglycerate to glyceraldehyde-3-phosphate. The overall reactions consume one ATP molecule and one NADH. The ATP concentration was estimated by the consumption of NADH monitored as the decrease in absorbance at 340 nm using an extinction coefficient of 6.22 $mM^{-1}cm^{-1}$.

**Mitochondrial $O_2$ consumption.** A Clarke oxygen electrode was used to measure the oxygen consumption with various substrates as described by Kripper *et al.* (1996) with minor modification. Transduced Hep G2 cells were harvested by trypsinization, washed, and resuspended in respiration buffer (0.25 M sucrose, 0.1% bovine serum albumin, 10 mM $MgCl_2$, 10 mM HEPES, 5 mM $KH_2PO_4$, pH 7.2) at a final concentration of $3 \times 10^7$ cells/ml. One milliliter of the suspension was added into a chamber, maintained at 37°, containing 2.0 ml of air-saturated respiration buffer plus 1 mM ADP. The cells were permeabilized with digitonin added to a final concentration of 0.005%, and the sequential substrates and inhibitors were added in the following order and final concentrations: 5 mM malate + 5 mM pyruvate; 100 nM rotenone; 5 mM succinate; 50 nM antimycin; 1 mM ascorbate + 0.4 mM tetramethyl-*p*-phenylenediamine; and 5 mM $NaN_3$. Oxygen concentration was calibrated with air-saturated $H_2O$ assuming 214 $\mu$M $O_2$ at 37°.

**Statistics.** Results refer to mean ± standard deviation and are averages of three to five values per experiment; each experiment was repeated at least three times.

## Results

**Establishment of E47 and E43 cell lines overexpressing human CYP2E1.** E47 and E43 clones were selected from Hep G2 cells transfected with a pCI-2E1 plasmid. After G-418 selection, three-time limited dilution screening, and nearly one year of continuous tissue culture, the two clones seem to be stable with respect to expression of CYP2E1 and microsomal PNP oxidation activity. Western blot analysis of cell extracts from E47 (Fig. 1A, *lane 4*) or E43 (Fig. 1A, *lane 3*) cells showed a clear band at a molecular mass of about 54

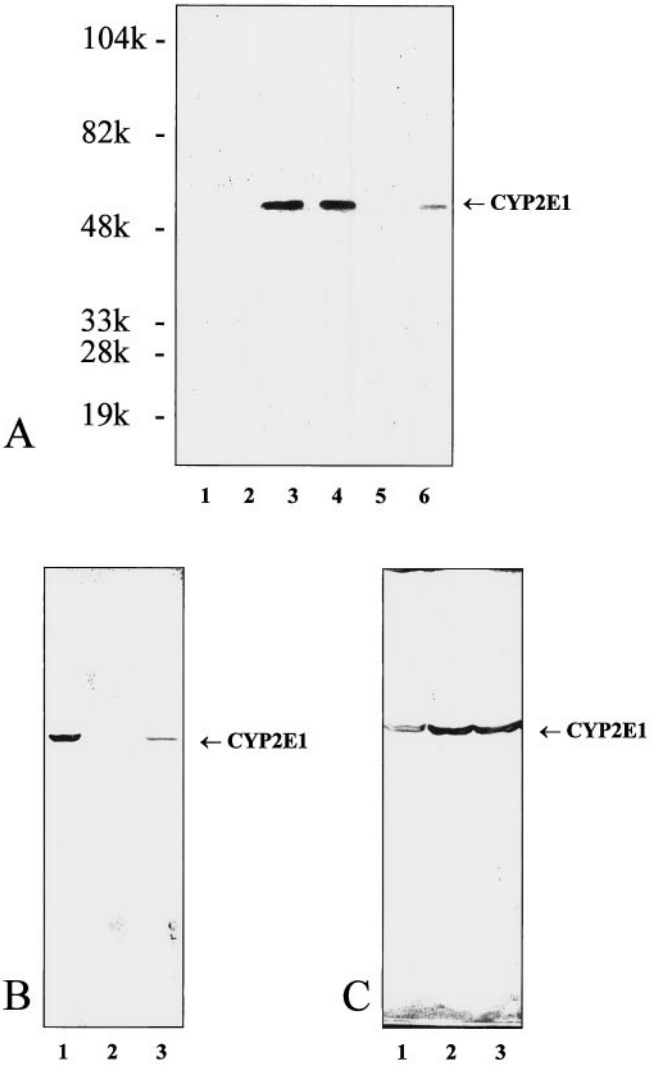



**Fig. 1.** Western blot analysis of CYP2E1 in Hep G2 cell subclones. Cell lysate, 50 $\mu$g, was loaded into each lane for SDS-PAGE, followed by Western blot analysis with polyclonal rabbit anti-human CYP2E1 antibody as described in Materials and Methods. A, CYP2E1 expression levels in parental Hep G2 cells (*lane 5*), Hep G2 subclones C34 (*lane 1*), C37 (*lane 2*), E43 (*lane 3*), and E47 (*lane 4*), as well as Hep G2-MV2E1–9 cells (*lane 6*). B, CYP2E1 expression levels in E47 cells 4 days after transfection with pCI-neo (*lane 1*) and pCI-as-2E1 plasmids (*lane 3*). The nondetectable level of CYP2E1 in cultures of C34 cells (*lane 2*) is shown for comparative purposes. C, CYP2E1 expression levels in E47 cells 2 days after culture in MEM (control, *lane 1*), medium containing 2 mM 4-MP (*lane 2*), or 25 mM DMSO (*lane 3*).

kDa, which is identical to the band generated by human liver microsomes (data not shown) and to cell lysate from a previously established Hep G2-MVh2E1–9 subclone (Fig. 1A, *lane 6*). The content of CYP2E1 in the E47 or E43 cells was ~10- to 15-fold greater than that of the Hep G2-MVh2E1–9 cells as determined by densitometric analysis. The constitutive expression of CYP2E1 in E47 and E43 cells is promoted by the human cytomegalovirus immediate-early enhancer/promoter. For the purpose of comparative study, we also established two control cell clones, C34 and C37, through pCI-neo plasmid transfection. As in the parental Hep G2 cells, the C34 and C37 cells do not express detectable CYP2E1 (Fig. 1A, *lanes 1, 2,* and *5*).

Enzymatic activities of E47, E43, C34, and C37 cells were determined by the ability of their microsomes to carry out a typical CYP2E1-dependent reaction, *p*-nitrophenol oxidation. As shown in Table 1, the average PNP oxidation activity values of the microsomes prepared from E47, E43, C34, and C37 cells were ~ 340, 180, 5, and 3 pmol/min/mg of microsomal protein, respectively. Although there is considerable variability in the CYP2E1 content of human liver microsomes, the PNP oxidation activities of E47 and E43 microsomes are lower but within a reasonable range of that found with human liver microsomes (0.4–1 nmol/min/mg of microsomal protein). However, the PNP activity by microsomes from the E47 and E43 cells was 4- to 8-fold greater than that by microsomes from the previously established Hep G2-MV2E1–9 cells (30–60 pmol/min/mg of microsomal protein) (Dai *et al.*, 1993).

**Growth inhibition effect of CYP2E1 on Hep G2 cells.** The expression of CYP2E1 caused an apparent decrease in the growth curve for the Hep G2 cells. As shown in Fig. 2, the C34 and C37 cells grow at a rate similar to that of the parental Hep G2 cells, whereas the increase in cell numbers with time are lower with the E47 and E43 cells. The doubling time for E47 or E43 cells is ~30 or ~28 hr, respectively, which is longer than control C34 or C37 cells (21 hr) or parental Hep G2 cells (20 hr) (Table 1). However, cell morphology of E47 or E43 cells appears to be normal and similar to that of C34 cells, C37 cells, or parental Hep G2 cells (Fig. 3A, *top*). In addition, no significant LDH leakage was observed with E47 cells (Fig. 3B). Thus the overexpression of CYP2E1 seems to decrease cell growth, but the cells remain viable.

To validate that the decrease in cell growth is a reflection of the expression of CYP2E1 rather than clonal variation, the effect of transfecting Hep G2 cells with plasmid containing CYP2E1 cDNA and control plasmid was determined. The growth rate of the Hep G2 cells transfected with pCI-2E1 plasmid was slower than the cells transfected with pCI-neo. For example, after plating the same number of cells, the number of cells after 7 days was $4.5 \times 10^6$ for the pCI-neo transfectant, and $1.4 \times 10^6$ for the pCI-2E1 transfectant;

TABLE 1

Comparison of Hep G2 subclones

The oxidation of PNP by microsomes from parental Hep G2 cells and transduced Hep G2 subclones was determined as described in Materials and Methods. The doubling time was estimated after 20 days of cell number counting using the formula: DT (hr) = $20 \times 24/\log_2$ [cell number]$_{20}$/[cell number]$_0$. DT refers to doubling time, and [cell number]$_{20}$ or [cell number]$_0$ refers to cell number at day 20 or day 0, respectively.

| Subclones | Oxidation of PNP | Doubling time |
|---|---|---|
| | *nmol/min/mg of microsome* | *hr* |
| Hep G2 | < 0.01 | 20 ± 1 |
| C34 | < 0.01 | 21 ± 1 |
| C37 | < 0.01 | 21 ± 1 |
| E43 | 0.19 | 28 ± 2 |
| E47 | 0.34 | 30 ± 1 |

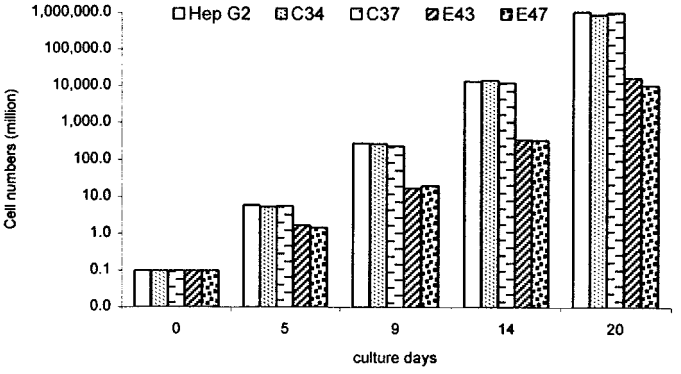
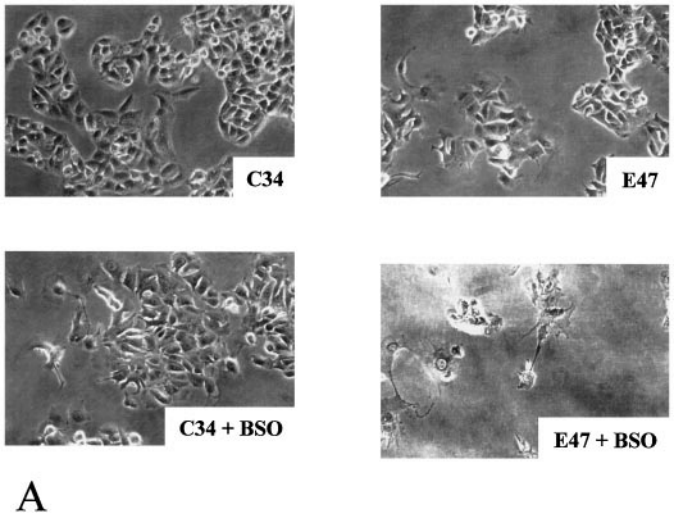



**Fig. 2.** Differential growth rates of Hep G2 cell subclones. Cells, $1 \times 10^5$, were cultured in MEM. After the indicated days of culture, cells were counted with a hemocytometer and $1 \times 10^5$ cells were subcultured into fresh MEM for next cell number counting.



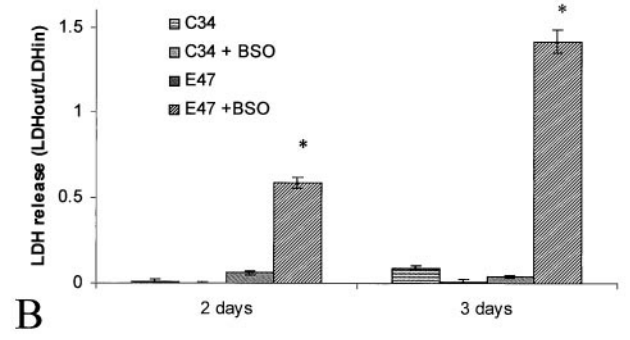




**Fig. 3.** Morphology and LDH leakage of C34 and E47 cells in the absence or presence of BSO. A, C34 and E47 cells were cultured in MEM in the absence or presence of 0.1 mM BSO for 4 days and visualized under the light microscope (magnification, 200 ×). B, C34 and E47 cells were cultured in MEM with or without 0.1 mM BSO for 2 or 3 days. Supernatants were collected, and the cells were harvested by scraping for measurement of LDH as described in Materials and Methods. The cytotoxicity and the membrane integrity were determined by the ratio of $LDH_{out}/LDH_{in}$. *, $p < 0.001$ compared with C34, C34 + BSO, and E47 cells.

after 12 days, the number of cells was $2.4 \times 10^7$ for pCI-neo transfectant, and $3.3 \times 10^6$ for the pCI-2E1 transfectant.

**GSH level in E47 and C34 cells.** GSH is among the most important intracellular antioxidants. ROS generated from CYP2E1 or other sources can be removed either by direct reaction with GSH or by the glutathione peroxidase reaction. The intracellular GSH level is effectively maintained by recycling oxidized glutathione back to GSH and by *de novo* synthesis. BSO is an effective inhibitor of γ-glutamylcysteine synthetase, the rate-limiting enzyme for glutathione synthesis. The intracellular GSH level of E47 and C34 cells with or without BSO treatment was evaluated. In the absence of BSO treatment, the E47 cells cultured in normal MEM had a level of GSH similar to or slightly higher than the C34 cells (Fig. 4). As expected, BSO-treatment resulted in decreasing levels of GSH. However, BSO-treatment caused an accelerated rate of decline of intracellular GSH in E47 cells compared with C34 cells (Fig. 4). In fact, GSH levels were only slightly decreased after 24-hr incubation of C34 cells with BSO, which suggests that these cells, in contrast to the E47 cells, are not under oxidative stress.

**Cytotoxicity and apoptosis in Hep G2 cells expressing CYP2E1.** As discussed above, the E47 and E43 cells seem fully viable in the absence of BSO treatment. However, after treatment with BSO for 4 days, substantial morphological changes of E47 cells were observed (Fig. 3A). Many E47 cells were detached and floated to the top of the culture medium, membrane blebbing and cytoplasmic shrinkage were observed, cells were dispersed, and a monolayer was not formed. No such changes in morphology were evident when BSO was added to culture medium of C34 cells (Fig. 3A). Moreover, LDH leakage was observed 2 days after BSO-treatment of the E47 cells, but not after adding BSO to the C34 cells (Fig. 3B) or Hep G2 cells (data not shown). The cytotoxicity in the E47 cells was quantified with an MTT assay. As shown in Fig. 5A, about 40–50% of the Hep G2 cells expressing CYP2E1 (E47 and E43) died after 2 days of BSO treatment, whereas no loss in viability was observed with the C34 cells, C37 cells, or Hep G2 cells. A time course for the decrease in MTT reduction is shown in Fig. 5B. Loss of cell viability upon BSO treatment of the E47 cells was evident after 2 days of culture and became much more pronounced with increasing time of culture. A lower MTT reading ($A_{570nm}$–$A_{630nm}$) was also observed for the E47 cells in the absence of BSO, compared with the C34 cells with or without BSO treatment (Fig. 5B); this is probably a reflection of the decrease in cell growth of the E47 cells rather than a significant loss of viability, because cell morphology was intact and LDH leakage was minimal (Fig. 3). The effect of BSO treatment on viability of Hep G2 cells transiently transfected with vector containing CYP2E1 cDNA or control vector pCI-neo was determined with the MTT assay. Four days after trans-

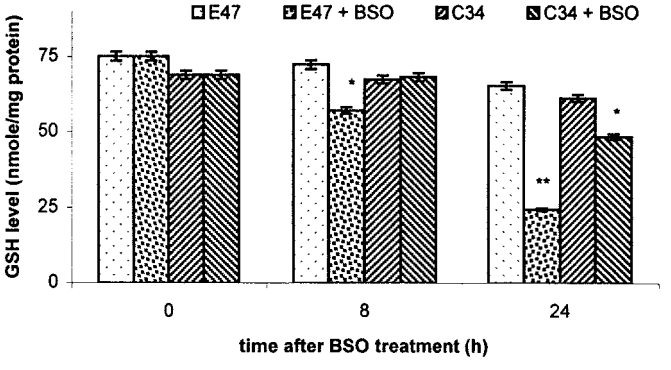



**Fig. 4.** GSH level of C34 and E47 cells after BSO treatment. Cells were cultured in the presence of 0.1 mM BSO, and harvested by scraping 8 hr or 24 hr after BSO addition. Cells cultured in the absence of 0.1 mM BSO were collected as 0 time. The intracellular GSH level was measured as described in Materials and Methods. *, $p < 0.01$; *, $p < 0.001$ compared with E47 or C34 cells without BSO treatment.

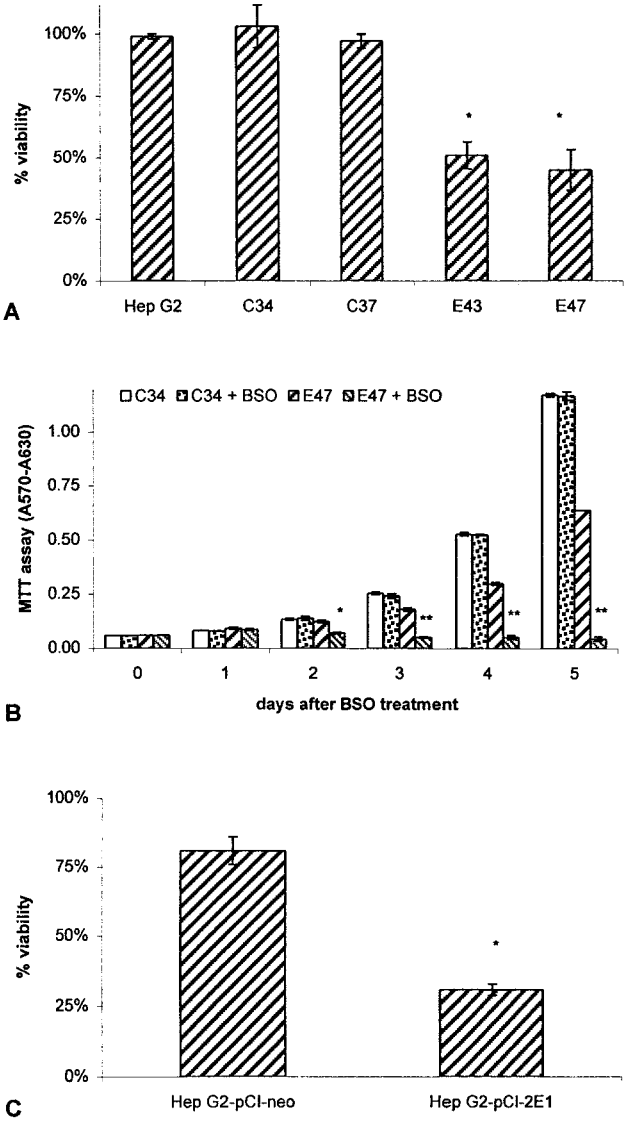



**Fig. 5.** Cytotoxicity in Hep G2 cells overexpressing CYP2E1 after depletion of GSH. A, Hep G2 cells or Hep G2 subclones were cultured in MEM in the absence or presence of 0.1 mM BSO for 2 days. The viability was measured using the MTT assay as described in Materials and Methods. The percentage viability was calculated by the formula: $(A_{570} - A_{630})_{+BSO}/(A_{570} - A_{630})_{-BSO} \times 100$; $(A_{570} - A_{630})_{+BSO}$ are the readings taken from cells cultured in the presence of BSO; $(A_{570} - A_{630})_{-BSO}$ are the readings taken from cells cultured in the absence of BSO. *, $p < 0.001$ compared with Hep G2 cells. B, Time course curve of cell viability. C34 and E47 cells were cultured in MEM with or without 0.1 mM BSO for 1–5 days and absorbance associated with the MTT assay determined. *, $p < 0.01$, **, $p < 0.001$ compared with E47 cells without BSO treatment. C, Hep G2 cells were transiently transfected with vectors pCI-neo or pCI-2E1. Four days after transfection, the cells were subjected to BSO treatment for 2 days. Cell viability was measured by the MTT assay. *, $p < 0.001$.

fection, the cells were treated with BSO, and viability was assayed after an additional 2 days in culture. There was a 3-fold decrease (percentage-wise) in cell viability when Hep G2 cells were transfected with pCI-2E1 compared with pCI-neo (Fig. 5C). These results suggest that when GSH levels are lowered in the CYP2E1-expressing cells, there is a dramatic loss of cell viability, whereas no such loss in viability occurs in the control cells that do not express CYP2E1.

The extent of DNA fragmentation, a biochemical hallmark of apoptotic cell death, was determined to further validate CYP2E1-dependent toxicity. DNA fragmentation in Hep G2 cells expressing CYP2E1 treated for 2 days with 0.1 mM BSO was analyzed by an agarose gel electrophoresis assay. As shown in Fig. 6, a "DNA ladder" composed of DNA fragments at an interval of about 200 base pairs was produced by the DNA purified from BSO-treated E47 cells (Fig. 6, *lane 8* and (especially) *lane 9*); C34 cells with or without BSO treatment (Fig. 6, *lanes 1* to *3*), and E47 cells not treated with BSO (Fig. 6, *lane 7*), did not produce the same pattern of DNA fragmentation. Therefore, the cytotoxicity induced in E47 cells seemed to be apoptotic and was only induced after GSH was depleted.

**Role of CYP2E1 in growth inhibition and cytotoxicity of E47 cells.** The only apparent difference between E47 and C34 cells, as well as between pCI-2E1 and pCI-neo transfectants, is the expression of CYP2E1. It appears that the growth inhibition effect and cytotoxicity or apoptosis (after BSO-treatment) in Hep G2 cells containing CYP2E1 is caused by the presence of CYP2E1 in these cells. To further validate this idea, the plasmid pCI-as-2E1, which contains cDNA encoding antisense CYP2E1, was transfected into E47 cells to inhibit CYP2E1 production. Western blot analyses of the CYP2E1 content after transfection with the pCI-as-2E1 plasmid indicated that the expression of CYP2E1 was decreased about 70% with pCI-as-2E1 (Fig. 1B, *lane 3*) compared with control transfection with pCI-neo plasmid (Fig. 1B, *lane 1*). pCI-as-2E1 transfected E47 cells grew faster than pCI-neo transfected cells as measured by cell counting 7 days after transfection (Fig. 7A). Viability in the cells transfected with pCI-as-2E1 plasmid and treated with BSO for 2 days was higher (about 65% viable) than that in cells transfected with the control vector pCI-neo (less than 25% viable) (Fig. 7B). Thus, the observed growth inhibition effect in the absence of BSO treatment and toxicity after BSO-treatment

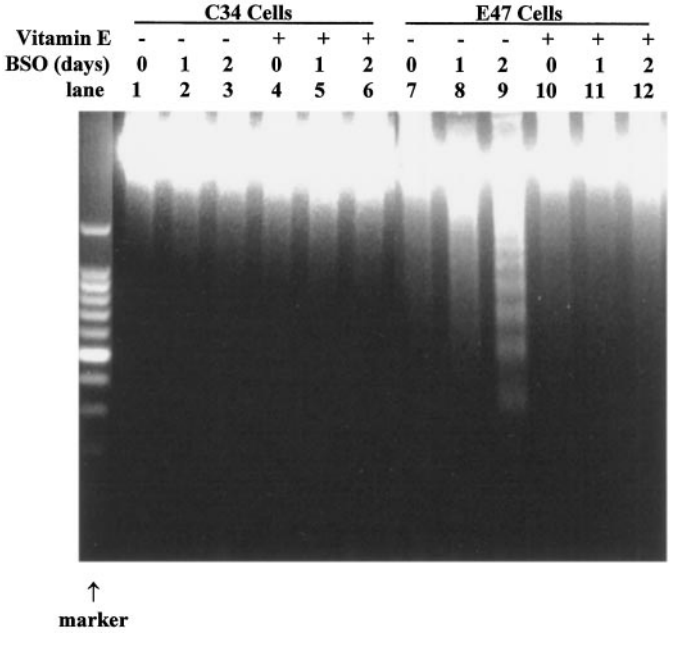



**Fig. 6.** Apoptosis produced in E47 cells after BSO treatment and the effect of VitE. C34 (*lanes 1–6*) and E47 (*lanes 7–12*) cells were treated with 0.1 mM BSO for 1 (*lanes 2, 5, 8,* and *11*) or 2 (*lanes 3, 6, 9,* and *12*) days with or without 20 $\mu$M VitE. Cells cultured in MEM in the absence of BSO were collected as controls (*lanes 1, 4, 7,* and *10*). Cells were harvested by scraping, followed by DNA separation, and DNA ladders were visualized by agarose gel electrophoresis as described under Materials and Methods.

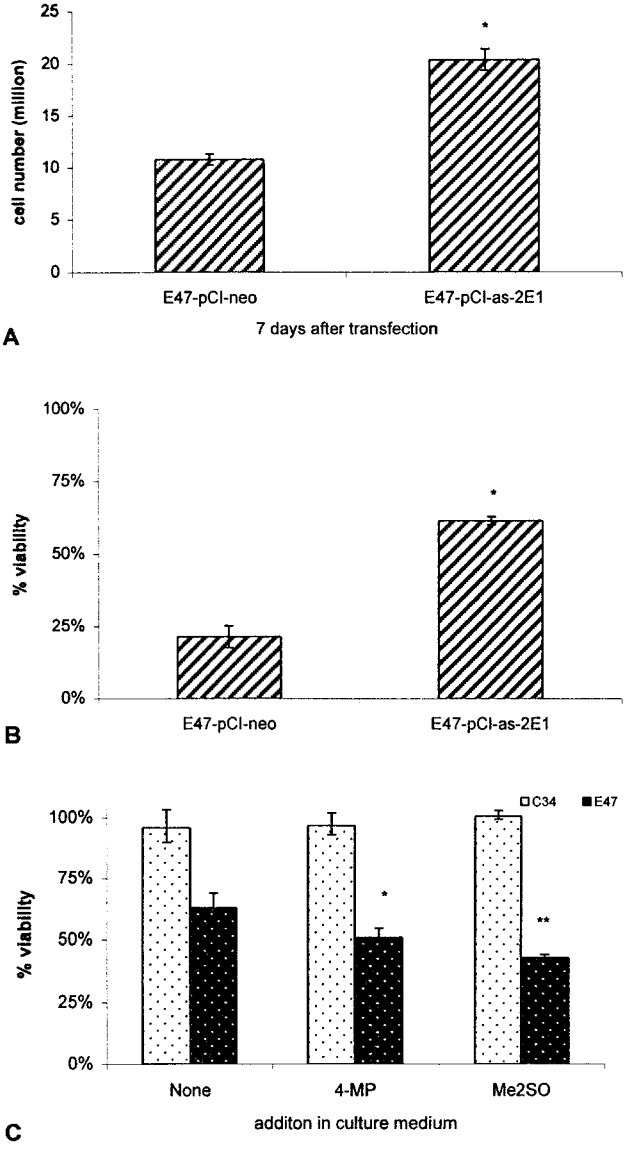



**Fig. 7.** Role of CYP2E1 in growth inhibition and cytotoxicity induced in E47 cells. A, The same number of E47 cells were transiently transfected with pCI-neo or pCI-as-2E1 plasmid. Seven days after transfection, the cell numbers were counted. *, $p < 0.001$. B, Four days after transfection, pCI-neo or pCI-as-2E1 transfectants were treated with BSO for 2 days, followed by assaying for viability using the MTT reaction as described in Materials and Methods. *, $p < 0.001$. C, C34 and E47 cells were cultured in MEM with or without 4-MP or DMSO ($Me_2SO$) for 2 days and then subjected to 0.1 mM BSO treatment for an additional 2 days. Cell viability was determined by the MTT assay. The percentage viability was calculated as described in the legend to Fig. 5. *, $p < 0.05$; **, $p < 0.001$ compared with no addition (*none*).

in Hep G2 cells are dependent upon CYP2E1 expression under these experimental conditions.

4-MP and DMSO are ligands for CYP2E1, and have been shown to stabilize CYP2E1 in rat hepatocyte cultures (Song *et al.*, 1986; Koop and Tierney, 1990) and in the Hep G2 cells (Yang and Cederbaum, 1997). After culture in 4-MP- or DMSO-containing medium for 2 days, E47 cells contain an increased level of CYP2E1 compared with E47 cells cultured in normal MEM (Fig. 1C). Cells cultured in MEM + 4-MP (47% loss in viability) or DMSO (57% loss in viability) showed an enhanced loss of viability after treatment with BSO compared with E47 cells (33% loss in viability), which suggests a correlation between the expression level of CYP2E1 and cytotoxicity (Fig. 7C).

**Evidence of lipid peroxidation in Hep G2 cells expressing CYP2E1.** One consequence of ROS formation may be lipid peroxidation. Lipid peroxidation of E47 and C34 cells was assessed by measuring production of lipid peroxidation end products, MDA and 4-HNE. As shown in Table 2, there is no detectable lipid peroxidation in C34 cells; a low level of lipid peroxidation could be observed in the E47 cells during the regular cell culture. BSO-treatment did not induce lipid peroxidation in C34 cells. However, lipid peroxidation was elevated in the E47 cells after BSO treatment for 2 days. The significant difference in lipid peroxidation between the two cell subclones suggests that overexpression of CYP2E1 caused lipid peroxidation, especially when GSH was depleted, possibly through the CYP2E1-induced generation of ROS. Subsequent studies were carried out to evaluate whether the enhanced lipid peroxidation was responsible for the cytotoxicity found when GSH was depleted from the E47 cells.

**Effect of antioxidants on CYP2E1 cytotoxicity.** To further characterize the nature of the CYP2E1 cytotoxicity, several antioxidants were added to the culture medium and their effect on the cytotoxicity produced upon GSH depletion was determined. As shown in Fig. 8A, VitE, trolox, and vitamin C were protective against CYP2E1 cytotoxicity to the E47 cells. VitE also blocked the DNA fragmentation induced in E47 cells upon GSH depletion (Fig. 6, *lanes 11* and *12*) and prevented the enhanced lipid peroxidation (Table 2). Furthermore, VitE also increased the growth rate of E47 cells as reflected by the increase in MTT absorbance (Fig. 8B). These results suggest that the CYP2E1 induced growth inhibition and cytotoxicity are related to lipid peroxidation and development of a state of oxidative stress.

**Bcl-2 protects Hep G2 cells against CYP2E1 toxicity.** Bcl-2 has been shown to be protective against apoptosis in several reaction systems (Hockenberry *et al.*, 1993; Reed, 1994; Armstrong *et al.*, 1996; Chen *et al.*, 1997). Hep G2 cells contain a low level of Bcl-2, as shown by Western blot analysis (Fig. 9A, lane 6). To determine the effect of Bcl-2 on the CYP2E1 toxicity, two Hep G2 subclones, B27 and B28, were established after transfection of Hep G2 cells with the expression vector pCI-bcl-2, which contains bcl-2 cDNA; two other Hep G2 subclones, A14 and A15, were obtained from the transfection with vector pCI-as-bcl-2, which contains an antisense bcl-2 cDNA. As shown in Fig. 9A, Hep G2 (*lane 6*) and C34 (*lane 5*) cell lysates produced a low level of endogenous Bcl-2 protein; B27 and B28 cells produced a significant higher level of Bcl-2 (Fig. 9A, *lanes 3* and *4*) whereas A14 and A15 cells did not display a detectable Bcl-2 expression (*lanes 1* and *2*).

To study the effect of Bcl-2 on CYP2E1 toxicity, B28, A14, and C34 cells were transfected with the same amount (1 $\mu$g of plasmid DNA/1 $\times$ $10^5$ cells) of pCI-2E1 plasmid. Four days after transfection, B28-CYP2E1, A14-CYP2E1, and C34-CYP2E1 transfectants were subjected to BSO treatment. As shown in Fig. 10A, B28-CYP2E1 cells were relatively resistant to BSO-treatment (87% viable). C34-CYP2E1 cells showed a similar loss of cell viability, as found previously with E47 and E43 cells (52% viable). A14-CYP2E1 cells were the most sensitive to BSO-treatment (41% viable). On an

TABLE 2

Lipid Peroxidation in Hep G2 Subclones

C34 and E47 cells were cultured in MEM with or without 0.1 mM BSO and/or 20 $\mu$M VitE for 2 days. The lipid peroxidation levels in C34 and E47 cells were monitored by the production of lipid peroxidation end products, MDA and 4-HNE, as described in Materials and Methods. *, Compared to C34 cells (±BSO), $p < 0.001$.

| Cells | Addition | LPO |
|---|---|---|
| | | *nmol of 4-HNE + MDA/mg of protein* |
| C34 | | 0 |
| C34 | BSO | 0 |
| E47 | | 0.03 ± 0.01* |
| E47 | BSO | 0.16 ± 0.03* |
| E47 | VitE | 0 |
| E47 | VitE + BSO | 0.01 ± 0.01 |

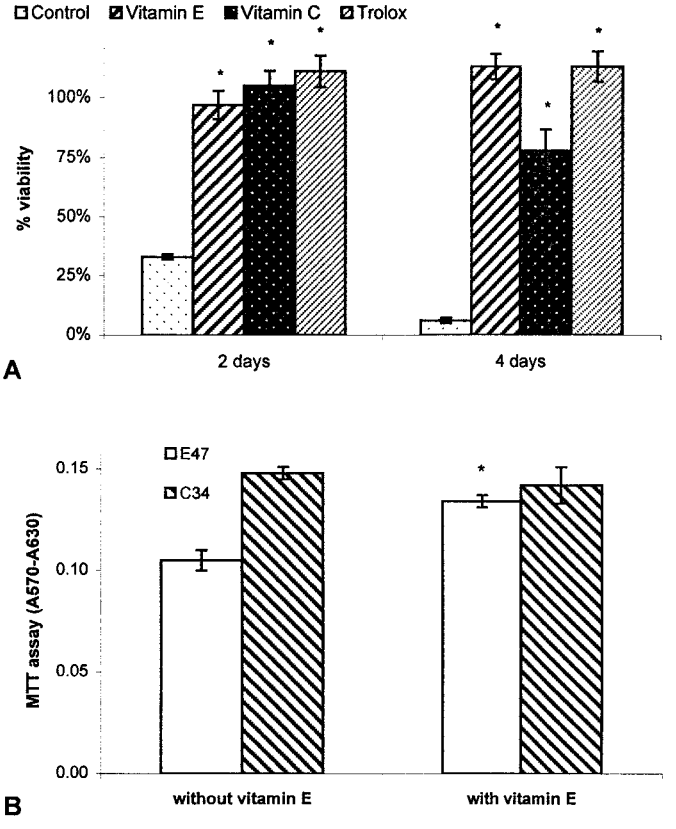



**Fig. 8.** Effect of antioxidants on CYP2E1 cytotoxicity. A, E47 cells were treated with BSO for 2 or 4 days in the absence or presence of the antioxidants, 5 $\mu$M VitE, 0.2 mM vitamin C, or 20 $\mu$M trolox, followed by assaying for viability using the MTT reaction as described in Materials and Methods. The percentage viability was calculated as described in the legend to Fig. 5. *, $p < 0.001$. B, E47 and C34 cells were cultured in MEM with or without 20 $\mu$M vitamin E for 3 days, and seeded into 24-well-plate wells, 10,000 cells/well. Two days later, the MTT assay was determined as described in Materials and Methods. *, $p < 0.001$ compared with cells in MEM without VitE.

agarose gel electrophoresis experiment, the B28-CYP2E1 transfectant did not produce a "DNA ladder" as observed in the C34-CYP2E1 and A14-CYP2E1 transfectants (Fig. 10B). As shown in Fig. 9B, the expression levels of CYP2E1 in the three transfectants were similar. Thus, the apparent difference among the three transfectants, which explains the varying loss of viability, was the Bcl-2 level. The protection by Bcl-2 against the CYP2E1 cytotoxicity is consistent with the observation that the CYP2E1 cytotoxicity seems to be apoptotic in nature.

**Intracellular ATP concentration of Hep G2 cells.** The observed growth inhibition effect of CYP2E1 on the Hep G2 cells does not seem to reflect a loss of cell viability attributable to ROS-induced damage, because normally cultured E47 cells appeared to be morphologically similar to either Hep G2 or C34 cells, LDH leakage was not observed, and a low level of lipid peroxidation was observed in E47 cells in the absence of 0.1 mM BSO. We explored other possible mechanisms to explain the slow growth. ATP serves as an essential energy source for most intracellular synthetic reactions and is necessary for cellular repair of damaged macromolecules and maintenance of adequate levels of GSH. Decreased levels of ATP could contribute to the slow growth rate of the Hep G2 cells overexpressing CYP2E1. Indeed, the ATP level in E47 cells ($0.48 \pm 0.01$ nmol per $1 \times 10^6$ cells) was about 30% lower than that in C34 cells ($0.70 \pm 0.02$ nmol per $1 \times 10^6$ cells).

**Mitochondrial damage in Hep G2 cells that overexpress CYP2E1.** The lower content of ATP in the E47 cells may reflect antioxidative reactions that consume ATP and/or a decreased rate of production of ATP by mitochondria. Mitochondrial electron flow is transported through four multisubunit complexes, designated complexes I to IV, which reside in the inner mitochondrial membrane. Complexes I and II accept electrons from NADH and succinate, respectively, then pass the electrons to complex III via ubiquinone, and

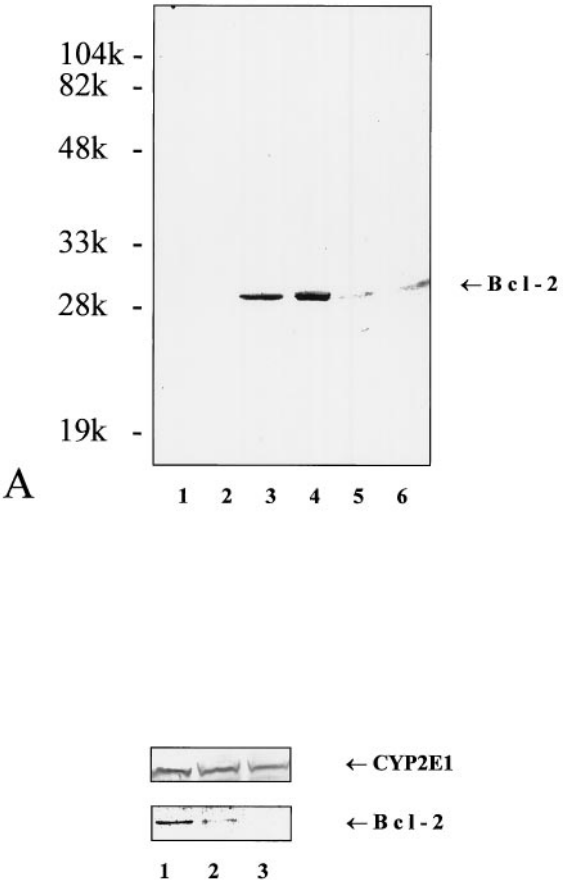



**Fig. 9.** Western blot analysis of Bcl-2 and CYP2E1 expression in Hep G2 subclones. Cell lysate, 50 $\mu$g, was loaded into each lane for SDS-PAGE, followed by Western blot analysis as described in Materials and Methods. A, Bcl-2 expression levels in Hep G2 subclones, A14 (*lane 1*), A15 (*lane 2*), B27 (*lane 3*), B28 (*lane 4*), and C34 (*lane 5*), as well as parental Hep G2 cells (*lane 6*) were assessed by Western blot analysis with mouse anti-human Bcl-2 monoclonal antibody. A14 and A15 cells are Hep G2 subclones transfected with a vector containing anti-sense bcl-2 cDNA, whereas B27 and B28 cells are Hep G2 subclones transfected with a vector containing a full length human bcl-2 cDNA in the sense orientation. B, B28 (*lane 1*), C34 (*lane 2*), and A14 (*lane 3*) cells were transiently transfected with the same amount of pCI-2E1 vector. Four days after transfection, CYP2E1 and Bcl-2 expression levels were assessed by Western blot analysis with polyclonal rabbit anti-human CYP2E1 IgG (*top*) or mouse anti-Bcl-2 monoclonal antibody (*bottom*).

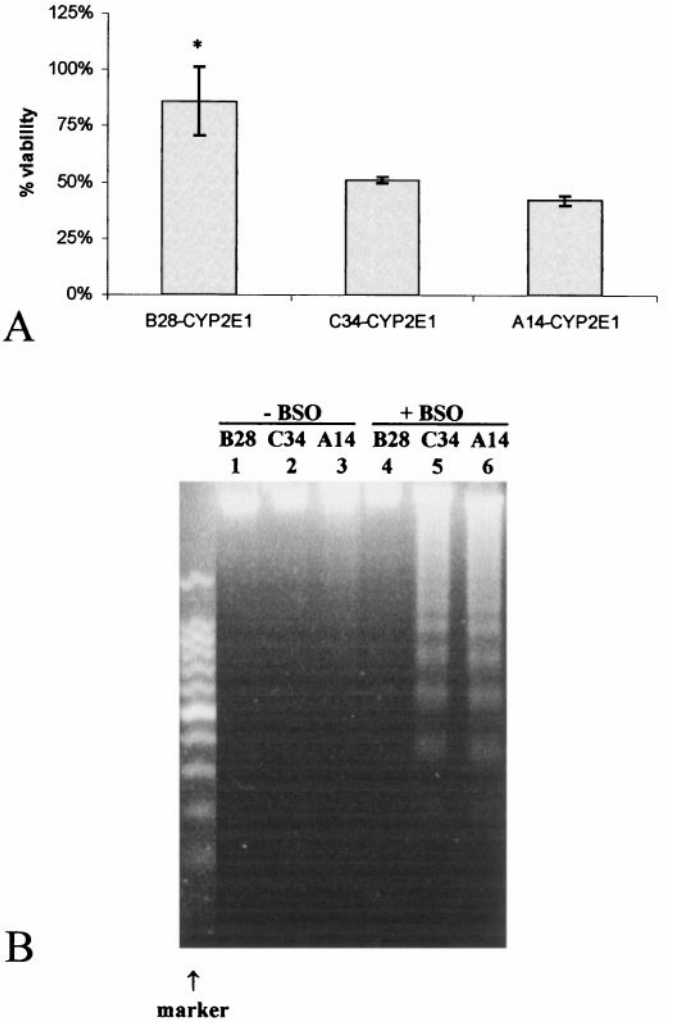



**Fig. 10.** Effect of Bcl-2 on CYP2E1-induced cytotoxicity and apoptosis. A14, B28, and C34 cells were transiently transfected with the same amount of pCI-2E1 plasmid. Four days after transfection, the transfectants were treated with BSO for 2 days, followed by determining viability using the MTT assay (A) or determining "DNA ladders" as visualized by agarose gel electrophoresis (B). *, $p < 0.05$ compared with the C34-CYP2E1.

then to complex IV, which is mediated by cytochrome *c*. The respiratory function of the mitochondria from C34 or E47 cells was assayed by measuring oxygen consumption after the sequential addition of substrates, which are specific for each electron transport pathway, to a respiration buffer containing cells treated with 0.005% digitonin, a condition which permeabilizes the cellular membrane and mitochondrial outer membrane. As shown in Table 3, the oxygen consumption rate with pyruvate-malate, which produces NADH and donates electrons to complex I was significantly lower with mitochondria of E47 cells compared with mitochondria of the C34 cells. However, oxygen consumption with succinate or ascorbate-tetramethyl-*p*-phenylenediamine, which donate electrons through complex II or complex IV, respectively, was the same with mitochondria from E47 cells and C34 cells. After BSO-treatment, the oxygen consumption rates with substrates donating electrons through all three complexes were lower in E47 mitochondria than in C34 mitochondria (BSO did not change the oxygen consumption rate of C34 mitochondria).

VitE, which prevented lipid peroxidation and apoptosis in the BSO-treated E47 cells, completely protected against the damage to complex I in E47 cells and to all three complexes in BSO-treated E47 cells (Table 3). These results suggest that ROS generated by CYP2E1 caused damage to mitochondria in E47 cells, and that the presence of GSH limited this damage mainly to complex I.

## Discussion

The primary goal of the present study was to investigate the toxic effect of CYP2E1 protein in the absence of added toxin in a human liver cell line, which has been transduced to express CYP2E1. Induction of CYP2E1, formation of ROS, elevation of lipid peroxidation, and cytotoxic damage are features observed in alcohol-induced hepatotoxicity, but direct linkage between these events has been difficult to evaluate. In the intragastric infusion model of ethanol feeding, liver injury occurred when the rats consumed diets containing PUFA but not saturated fatty acid; injury was associated with striking elevation of CYP2E1 (Nanji *et al.*, 1989; Tsukamoto *et al.*, 1990; Castillo *et al.*, 1992; French, 1993; Nanji *et al.*, 1994; Morimoto *et al.*, 1994). In a transduced Hep G2 cell model that expresses CYP2E1 (Hep G2-MVh2E1–9), significant cytotoxicity was observed when the CYP2E1 expressing cells were treated with acetaminophen (Dai and Cederbaum, 1995) or ethanol (Wu and Cederbaum, 1996) or were preloaded with a representative PUFA, arachidonic acid (Chen *et al.*, 1997). In these models, elevated lipid peroxidation was evident and seemed to correlate with CYP2E1 levels. However, there is no evidence of direct toxicity observed in these cells in the absence of added agents such as ethanol, acetaminophen, $CCl_4$, or PUFA. No toxicity was observed, even when *de novo* GSH synthesis was inhibited. Possible explanations are that CYP2E1 itself is not directly toxic in the absence of added toxin that requires metabolism by CYP2E1, or the CYP2E1 expression level in the Hep G2-MV2E1–9 cells is still relatively low. The significance of the current study is that the new established Hep G2 subclones E47 and E43 express CYP2E1 at levels 4- to 8-fold higher than the Hep G2-MVh2E1–9 cells as show by Western blot analysis and microsomal PNP-oxidation activity. Under these conditions of enhanced expression, direct effects of CYP2E1 on the rate of cell growth and cellular viability can readily be observed in the absence of any added agent. However, the possible presence of endogenous substrates that may be activated by CYP2E1 to reactive, toxic intermediates or that may promote uncoupling of the mixed-function oxidase reaction cannot be ruled out.

The E47 and E43 cells grew at a slower rate than the control C37 or C34 cells, or parental Hep G2 cells. Despite this slow growth rate, the E47 or E43 cells remain viable as shown by morphology, lack of LDH leakage, and capacity for vital dye reduction. Maintenance of cellular viability in the presence of elevated CYP2E1 expression seems to reflect maintenance of cellular levels of GSH. We initially predicted a possible decline of GSH in the E47 cells, because GSH is a primary antioxidant to remove the ROS generated by CYP2E1. However, the GSH level in E47 cells was not decreased compared with C34 cells or parental Hep G2 cells. This suggests a possible up-regulation of GSH production and/or increased oxidized glutathione-GSH turnover. Because increased synthesis of GSH or turnover requires ATP, it is interesting to speculate that the slower rate of growth of the CYP2E1-expressing cells may reflect the increased demand for ATP for maintaining cellular GSH levels in the face of elevated production of ROS. Indeed, ATP levels were found to be ~30% lower in the CYP2E1-expressing cells. Studies to evaluate GSH synthesis and turnover in these cells are planned.

Depletion of GSH by treatment with BSO resulted in a striking loss of viability of the E47 cells, without any effect on control or parental Hep G2 cells. After BSO addition, GSH levels declined more rapidly in the E47 cells than in the

TABLE 3

Oxygen Consumption Rate of Permeablized C34 and E47 Cells

C34 and E47 cells were cultured in MEM with or without BSO and VitE for 2 days. Oxygen consumption was determined as described under Materials and Methods. Numbers in parentheses and statistics refer to the change in the rate of oxygen consumption by the E47 cells compared with the corresponding (−/+ BSO) C34 cells. *, $p < 0.01$; **, $p < 0.05$.

| Cells | Addition | Oxygen consumption rates after substrate addition | | |
|---|---|---|---|---|
| | | Pyruvate-Malate | Succinate | Ascorbate-TMPD |
| | | *nmol/million cells/min* | | |
| C34 | | 0.15 | 0.16 | 0.36 |
| C34 | BSO | 0.19 | 0.18 | 0.35 |
| E47 | | 0.10 (−33%*) | 0.15 (−6%) | 0.39 (+7%) |
| E47 | BSO | 0.03 (−84%*) | 0.03 (−83%*) | 0.12 (−66%*) |
| E47 | VitE | 0.15 (−0%) | 0.16 (−0%) | 0.41 (+14%) |
| E47 | VitE + BSO | 0.14 (−26%**) | 0.16 (−11%) | 0.39 (+11%) |

control cells, which clearly indicates that the CYP2E1-expressing cells are under elevated oxidative stress. Thus, the E47 model depicts two modes of CYP2E1 toxicity: a slower growth rate when cellular GSH levels are maintained and a loss of cellular viability when cellular GSH levels are not maintained. In the E47 cells, very low lipid peroxidation was found when GSH levels were maintained. After GSH depletion, an increased level of lipid peroxidation was found in the E47 cells, consistent with an elevated state of oxidative stress. No lipid peroxidation was observed in the C34 cells even after BSO treatment, consistent with the maintenance of GSH levels. Antioxidants and inhibitors of lipid peroxidation, such as trolox, VitE, and ascorbate, prevented the elevated lipid peroxidation as well as cytotoxicity and apoptosis. These results suggest that elevated lipid peroxidation plays an important role in the CYP2E1-dependent toxicity.

The role of CYP2E1 in the growth inhibition and cytotoxicity is established by comparison between two CYP2E1 expression subclones (E47 and E43) and control cells (C34, C37, and parental Hep G2) and by comparison between Hep G2 cells transfected with vector containing CYP2E1 cDNA or control vector that does not contain CYP2E1 cDNA. To provide further evidence for a direct role of CYP2E1, experiments were performed with a vector containing antisense CYP2E1 cDNA. In the pCI-as-2E1 transfected E47 cells, the CYP2E1 expression was 70% less than that of the original E47 cells. Viability of these cells in BSO-containing medium was about 3-fold higher than that of the E47 cells transfected with the control pCI-neo vector, and cell growth rate was about 2-fold faster. 4-MP and DMSO also increased the CYP2E1 content in the E47 cells, and the E47 cells treated with 4-MP or DMSO were more sensitive to the BSO treatment. The role of ligands in CYP2E1 toxicity might be difficult to interpret; besides increasing the content of CYP2E1, these compounds may cause other effects (e.g., change the rate of electron flow to CYP2E1 or become metabolized to reactive products). DMSO is a good scavenger of ·OH; this, however, should provide protection against toxicity and not exacerbation of the injury. We have shown previously that 4-MP did not alter *in vitro* microsomal production of superoxide and $H_2O_2$ (Dai *et al.*, 1993). Taken as a whole, these data are consistent with the suggestion that a higher level of CYP2E1 content is related to a greater extent of cytotoxicity.

DNA fragmentation and typical morphological changes associated with apoptosis, such as membrane blebbing, cytoplasmic shrinkage, as well as massive destruction into apoptotic bodies, were observed after treatment of E47 cells with BSO. VitE prevented CYP2E1-dependent DNA fragmentation in Hep G2 cells, suggesting that lipid peroxidation played a role in the developing apoptosis and in the cytotoxicity. Bcl-2 inhibits many types of apoptotic cell death, although the mechanism is not completely clear. Bcl-2 is localized to intracellular sites of ROS generation, including mitochondria, endoplasmic reticulum, and nuclear membranes (Hockenberry *et al.*, 1993; Reed, 1994). The B28 subclone, which overexpresses Bcl-2, was more resistant to the CYP2E1 toxicity; in these cells, apoptosis did not develop after 2 days of BSO treatment. Interestingly, in the A14 subclone, which does not express detectable Bcl-2, CYP2E1 transfection caused somewhat more toxicity compared with the C34 subclone, which expresses a low level of endogenous Bcl-2.

The ATP levels in E47 cells are about 30% lower than those of C34 cells. The lower level of ATP may be a result of the need for increased GSH production and GSH turnover related to the oxidative stress generated by CYP2E1. On the other hand, mitochondrial ATP production may also be altered. The mitochondrial complex I, which mediates electron transfer from NADH, seemed to be affected as a consequence of the CYP2E1 expression as the oxygen consumption rate of E47 mitochondria with pyruvate-malate as substrate was about 35% lower than that of C34 mitochondria. VitE completely restored the oxygen consumption rate with pyruvate/malate, and it also increased the growth rate of E47 cells. Therefore, it seems that the low level of lipid peroxidation that occurs in E47 cells in the absence of BSO treatment is sufficient to cause specific damage to complex I; this damage probably contributes to the lower content of ATP, which subsequently decreases the growth rate of the cells. Maintaining intracellular GSH levels prevents widespread damage to the mitochondria; BSO treatment caused a strong elevation in lipid peroxidation in E47 cells and decreased oxygen consumption was observed with all substrates. The decreased electron flow through all complexes was prevented by VitE. Why complex I is especially sensitive to CYP2E1-dependent lipid peroxidation or whether there is a regulative effect on expression of complex I protein(s) is unknown, but it could relate to the high concentration of iron-sulfur clusters in this complex. Chronic ethanol consumption was shown previously to damage complex I (Cederbaum *et al.*, 1974), and there were decreased ESR signals associated with iron-sulfur clusters of NADH dehydrogenase, but not succinic dehydrogenase in submitochondrial particles of chronic ethanol-fed rats (Thayer *et al.*, 1980).

In summary, experiments have been carried out that demonstrate a growth inhibition effect and a cytotoxic effect of CYP2E1 in a transduced liver hepatoma cell line. These effects occur in the absence of externally added toxin or agent and therefore seem to be caused by high levels of expression of CYP2E1 itself. The slow growth may be a result of mitochondrial damage, the need to maintain cellular GSH level, and lower level of intracellular ATP content. The cytotoxicity is apoptotic in nature and is initiated by the depletion of GSH by CYP2E1-related oxidative stress and elevated lipid peroxidation. The direct toxicity of overexpressed CYP2E1 may be a reflection of the ability of this isoform to produce ROS even in the absence of substrate. These experiments demonstrate the potential of overexpressed CYP2E1 to cause toxicity in a Hep G2 model in the absence of adequate protection against oxidative stress.

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