Adenovirus-Mediated Expression of CYP2E1 Produces Liver Toxicity in Mice

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Induction of cytochrome P450 2E1 by ethanol is believed to be one of the central pathways by which ethanol generates a state of oxidative stress and causes hepatotoxicity. In order to evaluate the biochemical and toxicological actions of CYP2E1 and its sensitization of hepatotoxic-induced injury, an adenovirus which can mediate overexpression of CYP2E1 was constructed. Injecting this virus into mice through the tail vein elevated CYP2E1 protein and activity twofold in the liver of the mice compared with the mice injected with Ad-LacZ or saline. Transaminase levels were dramatically increased in mice injected with the CYP2E1 adenovirus. Histological evaluation of liver specimens of mice infected with Ad-2E1 showed liver cell injury. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay demonstrated that more cells were stained positively in the liver of the mice infected with Ad-2E1 than in the liver of the mice infected with Ad-LacZ. 3-Nitrotyrosine protein adducts and protein carbonyl adducts were increased in the liver of the mice infected with Ad-2E1 compared with Ad-LacZ. This potentiated toxicity most likely reflects interactions between CYP2E1- and adenovirus-mediated toxicity pathways. These results show that adenovirus-mediated overexpression of CYP2E1 could induce liver toxicity in mice and suggests a mechanism involving oxidative/nitrosative stress.

Key Words: adenovirus; CYP2E1; liver toxicity; oxidative stress.

Cytochrome P450 2E1 (CYP2E1), the ethanol-inducible form of cytochrome P450, is involved in metabolizing and activating many toxicologically important substrates including ethanol, carbon tetrachloride, acetaminophen, and N-nitrosodimethylamine to more toxic products (Guengerich et al., 1991; Koop, 1992; Yang et al., 1990). CYP2E1 is induced under a variety of conditions such as fasting, diabetes, obesity, high-fat diets, and some models of nonalcoholic steatohepatitis (Johansson et al., 1988; Raucy et al., 1991; Weltman et al., 1996, 1998) and by drugs and solvents besides ethanol (Lieber, 1997, 1999; Ryan et al., 1985) and by certain hormones (Hong et al., 1990; Peng and Coon, 1998). Induction of cytochrome P450 2E1 by ethanol appears to be one of the central pathways by which ethanol causes hepatotoxicity (Bondy, 1992; Cederbaum, 1991; Dianzani, 1985). Increases in formation of reactive oxygen species (ROS) by microsomes isolated from ethanol-treated rats are prevented by anti-CYP2E1 IgG (Ekström and Ingelman-Sundberg, 1989; Kukiela and Cederbaum, 1994). Hepatic CYP2E1 enzyme activity is significantly higher in alcoholic patients with liver disease than alcoholic patients without signs of liver disease (Dupont et al., 1998). In the intragastric model of ethanol feeding, ethanol-induced liver pathology has been shown to correlate with CYP2E1 levels and elevated lipid peroxidation and to be blocked by inhibitors of CYP2E1 (Castillo et al., 1992; Morimoto et al., 1994; Nanji et al., 1994). More recently, a cytochrome P450 2E1 transgenic mouse has been produced that expresses human CYP2E1 in the liver. When fed a nutritionally complete alcohol diet, transgenic mice develop more liver damage than nontransgenic mice (Morgan et al., 2002). There are, however, some reports which suggest that CYP2E1 may not play a significant role in early alcohol liver injury (Kono et al., 1999; Koop et al., 1997); this has been previously discussed (Cederbaum et al., 2001).

Studies in HepG2 cells overexpressing human CYP2E1 demonstrated that ethanol, polyunsaturated fatty acids (PUFA), and iron were cytotoxic to cells that overexpress CYP2E1 and not to the control cells lacking detectable CYP2E1 (Cederbaum, 2003; Chen et al., 1998; Sakurai and Cederbaum, 1998; Wu and Cederbaum, 1996). The cytotoxicity and apoptosis found with ethanol and PUFA in cells overexpressing CYP2E1 were prevented by several antioxidants, especially vitamin E and the vitamin E analog trolox. Induction of a state of oxidative stress appears to play a central role in the CYP2E1-dependent cytotoxicity. Adenovirus-mediated overexpression of catalase in cytosolic and mitochondrial compartments as well as Cu/Zn- or Mn-superoxide dismutase (SOD) protect against CYP2E1-dependent toxicity in CYP2E1-expressing HepG2 cells after treatment with arachidonic acid plus iron (Bai and Cederbaum, 2001; Perez and Cederbaum, 2003).

To further characterize the biochemical and toxicological actions of CYP2E1, we established an adenovirus which can...
overexpress human CYP2E1 (Ad-2E1). Using this adenovirus to infect HepG2 cells, CYP2E1 expression was increased, and as proof of principle, these cells were shown to be more sensitive to acetaminophen-induced toxicity compared with control cells infected with Ad-LacZ (Bai and Cederbaum, 2004). In the current study, the effects of CYP2E1 overexpression on liver viability in mice were evaluated after injecting Ad-LacZ or Ad-2E1 into mice through the tail vein.

MATERIALS AND METHODS

Reagents. Polyclonal antibody raised in rabbit against human CYP2E1 was obtained from Dr. Jerome M. Lasker (Institute for Biomedical Research, Hackensack, NJ) as a kind gift. Anti-β2 glycoprotein I polyclonal antibody was purchased from Upstate Inc. (Charlottesville, VA). Anti-carbonyl adducts’ monoclonal antibody, horseradish peroxidase (HRP) conjugated to goat anti-rabbit IgG, Minimal essential medium, fetal bovine serum, and all most chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Recombinant adeno virus production. Ad5 adenoviral vector with compensating deletions in the early region 1 (E1), as developed by Graham and coworkers (Bett et al., 1994), was purchased from Microbix Biosystems Inc. (Ontario, Canada) Adenovirus containing CYP2E1 and β-galactosidase was developed as described previously using Ad5 adenoviral vector (Bai and Cederbaum, 2004). Briefly, the plasmid shuttle vectors pAd5-CMV-CYP2E1 and pAd5-CMV-LacZ were constructed by inserting human CYP2E1 cDNA or β-galactosidase cDNA into the Ad5 shuttle vector. These adenoviral shuttle plasmids together with the Ad5 genomic DNA were transfected into human embryonic kidney 293 cells for producing adenovirus. Viruses possessing CYP2E1 (Ad-2E1) and β-galactosidase (Ad-LacZ) were plaque purified two times and amplified in 293 cells. Purified high-titer stocks of recombinant adenovirus were generated by two sequential rounds of CsCl density purification. The titer of each viral stock was determined by plaque assay on 293 cells; titers were consistently 1 × 1011 plaque-forming units (pfu)/ml.

Animal and adenovirus infection. All studies were performed according to the American Association for the Accreditation of Laboratory Animal Care guidelines. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Mount Sinai School of Medicine. Male C57BL/6 mice (20–25 g) were fed a standard mouse chow diet and were divided into three groups (group 1, control; group 2, Ad-LacZ; and group 3, Ad-2E1) with four mice in each group. Purified recombinant adenovirus (3 × 108 pfu) was diluted in 0.2 ml normal saline and injected into the tail vein. Mice in group 1 were injected with 0.2 ml saline. Mice were sacrificed under sodium pentobarbital anesthesia 14 days after the injection of adenovirus. Blood was collected from the abdominal aorta; serum was taken to assay for ALT and AST levels in serum using diagnostic kits made by Sigma Chemical Co.

ALT and AST assay. ALT and AST levels were measured in serum using diagnostic kits made by Sigma Chemical Co.

Western blot. To detect CYP2E1 protein level and protein carbonyl adducts, 30 μg of homogenate was resolved on a 10% SDS-polyacrylamide gel and electroblotted onto nitrocellulose membranes (Bio-Rad Hercules, CA). The membrane was incubated with the primary antibodies, followed by incubation with HRP-conjugated secondary antibody. Detection by the chemiluminescence reaction was carried out for 1 min using the ECL kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). All specific bands detected via Western blot analysis were quantified with the Automated Digitizing System (UN-SCAN-IT gel programs, version 5.1, Silk Scientific Corp., Orem, UT).

CYP2E1 activity. The CYP2E1 activity was measured by the rate of oxidation of p-nitrophenol to p-nitrocatechol in the presence of NADPH and O2. Oxidation of p-nitrophenol was determined using 200 μg of homogenate in a 100-μl reaction system containing 100mM potassium phosphate buffer (pH 7.4), 0.2mM PNP, and 1mM NADPH. All reactions were carried out in duplicate, initiated with NADPH, incubated at 37°C, and stopped after 60 min by addition of 30 μl of 20% trichloroacetic acid. The supernatant was treated with 10 μl of 10M sodium hydroxide and absorbance determined at 546 nm; activity was determined using a formula of PNP activity (pmol/min/mg of protein) = OD546/9.53/0.2/60/7.1 × 103.

TUNEL assay. DNA fragmentation was detected by using the in situ TUNEL assay with an ApopTag in situ apoptosis detection kit (Serological Corporation, Atlanta, GA). Slides containing liver tissue were pretreated with proteinase K and H2O2 and incubated with the reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and digoxigenin-conjugated dUTP for 1 h. The labeled DNA was visualized with HRP-conjugated antidigoxigenin antibody with diaminobenzidine as the chromogen. For a negative control, the TdT enzyme was omitted from the reaction mixture. Samples were counterstained with 0.5% methyl green. The quantitative analysis of positive nuclei was performed by counting the average number of apoptotic nuclei per visual field (original magnification ×400 [25 visual fields]).

Caspase activities. Caspase-8, -3, and -9 activities were determined in liver tissue homogenates by measuring proteolytic cleavage of the luminogenic substrates (0.1 mmol/l) Carbobenzoxy-Ile-Glu-Thr-Asp-7-amino-4-trifluoromethyl coumarin, Acetyl-Ile-Glu-Val-Asp-7-amino-4-methyl coumarin, and Ac-Leu-Glu-His-Asp-7-amino-4-trifluoromethyl coumarin (Calbiochem, La Jolla, CA), which are cleaved by caspase-8, caspase-3, and caspase-9, respectively. Fluorescence was determined with a spectrophuorometer (PerkinElmer, Wellesley, MA) based on the amount of released 7-amino-4-trifluoromethyl coumarin (caspase-8, -9 activities, λex = 400, λem = 505) or 7-amino-4-methyl coumarin (caspase-3 activity, λex = 380, λem = 460). The results were expressed as relative units compared with control as 1.

Statistics. Data are presented as mean ± SD. The experimental and control values were compared using unpaired Student’s t-test. Differences at p values of < 0.05 were considered significant. Results with the Ad-2E1 treatment were compared not only to saline-injected mice but also to the Ad-LacZ treatment since the adenovirus alone may cause some toxicity.

RESULTS

Adenovirus-Mediated Overexpression of β-Galactosidase or CYP2E1 in Mouse Liver

In order to evaluate the expression of adenovirus in vivo, purified recombinant adenoviruses Ad-2E1 or Ad-LacZ (3 × 108 pfu) were diluted in 0.2 ml normal saline and injected into the tail vein of male C57BL/6 mice. Mice injected with 0.2 ml saline were used as control. Fourteen days after virus injection, mice were sacrificed under sodium pentobarbital
anesthesia. To evaluate the adenovirus infectious efficiency, x-gal staining using frozen sections was carried out; most of the cells in the liver were stained with blue color (Fig. 1A). Interestingly, some cells showed intense staining, suggesting expression of large amount of CYP2E1 in those cells. Western blot analysis of liver homogenates 2 weeks after injection of the Ad-2E1 or Ad-LacZ showed that expression of CYP2E1 was about twofold higher in liver of the mice injected with Ad-2E1 compared with mice injected with Ad-LacZ or saline (Fig. 1C). CYP2E1 activity, analyzed by the oxidation of PNP in liver homogenates from mice injected with Ad-2E1, was 0.49 nmol/min/mg of protein, twofold higher than the activity in homogenates from mice injected with Ad-LacZ (0.25 nmol/min/mg of protein) (Fig. 1D). Time-course experiments of CYP2E1 activity at 2, 4, and 6 weeks after infection indicated rates of PNP oxidation of 0.50, 0.38, and 0.33 nmol/min/mg of protein, respectively (data not shown). Hence, experiments below were carried out 2 weeks after administration of Ad-2E1, the time point of the highest CYP2E1 activity.

Adenovirus-Mediated Overexpression of CYP2E1 Induces Liver Toxicity in Mice

To study whether liver toxicity related to adenovirus-mediated CYP2E1 overexpression occurs, mice were sacrificed at 14 days after adenovirus injection. Liver toxicity was assayed by evaluating the level of ALT and AST in serum, liver histopathology, and TUNEL assay. Results are the average from four mice. The levels of AST and ALT in mice injected with Ad-2E1 were up to 1300 U/l and 1500 U/l, respectively, values that were six- to sevenfold higher than that in mice injected with Ad-LacZ (250 U/l and 300 U/l, respectively) (Figs. 2A and 2B). Compared with the mice injected with saline, mice injected with Ad-LacZ also showed some increase in the levels of AST and ALT, but these increases were much lower than those produced by Ad-2E1. The liver of mice injected with Ad-LacZ did not show any significant pathological change in hematoxylin-eosin-stained liver tissue. However, the liver of mice injected with Ad-2E1 showed cell death (Fig. 3A). TUNEL assay showed a large number of positive cells in the liver of the mice injected with Ad-2E1 (around 25 positive cells in each magnification = ×400 visual field). This increase was much more pronounced than that found in mice injected with Ad-LacZ (around two positive cells per visual field) (Fig. 3B, brown spots, and Fig. 3C for quantitative analysis). No positive cells were detected in the liver of the mice injected with saline (data not shown).

Activities of caspase-3, -8, and -9 were evaluated (Fig. 4). In the liver of the mice injected with Ad-2E1 or Ad-LacZ, only caspase-3 activity was significantly increased compared with that in the liver of mice injected with saline. However, there is no significant difference in activities of caspase-3, -8, or -9 between the liver of mice injected with Ad-2E1 and that of mice injected with Ad-LacZ (Fig. 4).
Adenovirus-Mediated Overexpression of CYP2E1 Increases 3NT Protein Adducts and Protein Carbonyls in Mouse Liver

To evaluate possible mechanisms by which CYP2E1 expression causes liver toxicity, 3NT protein adducts and protein carbonyls were assayed by immunohistochemistry and Western blot, respectively. Immunohistochemistry showed that 3NT protein adducts were increased in mice infected with Ad-2E1 compared with mice infected with Ad-LacZ (Fig. 5A). Carbonyl protein adducts were increased 1.5-fold in mice infected by Ad-2E1 compared with those in mice infected by Ad-LacZ (Figs. 5B and 5C).

DISCUSSION

CYP2E1 activates many toxicologically important compounds and is induced under a variety of physiological and pathophysiological conditions. Several mechanisms have been suggested to play important roles in pathways of ethanol toxicity to the liver. A major interest is the role of oxidative stress and ethanol generation of reactive radical species in mechanisms by which ethanol is toxic. Induction of CYP2E1 by ethanol appears to be a central pathway in ethanol-induced oxidative stress. Further studies on the biological and toxicological properties of CYP2E1 are important to provide mechanistic information on the actions of CYP2E1 in the liver. CYP2E1 transgenic or knockout mice have provided us with valuable models to study the function of CYP2E1, although the life-long change of CYP2E1 in these models may cause the change of other forms of CYPs, e.g., CYP4A10 and CYP4A14 (Leclercq et al., 2000), or reductase activity (Kono et al., 1999) or other genes such as antioxidants enzyme Glutathione S-transferase, SOD, or Glutathione Peroxidase. The possibility that adenoviral delivery of CYP2E1 might be another model in addition to the above in evaluating CYP2E1 actions was the goal of the current study.

The adenovirus-mediated gene transfer method has been commonly used in vivo studies of liver toxicity (Uesugi et al., 2001; Wheeler et al., 2001a,b). We have developed an adenovirus...
which expresses human CYP2E1 by inserting the CYP2E1 cDNA into an adenovirus vector Ad5. The CYP2E1 expressed by this adenovirus is catalytically active, and it potentiated acetaminophen-induced toxicity in HepG2 cells (Bai and Cederbaum, 2004).

The infectious efficiency of the adenovirus is high as shown by x-gal assay. CYP2E1 protein and activity were increased twofold in the liver of the mice infected with Ad-2E1 compared with the mice infected with Ad-LacZ. Other CYPs such as CYP1A, CYP2A5, CYP2A6, CYP3A4, and P450 reductase were evaluated by activity measuring or Western blot analysis. No difference was detected between the mice injected with Ad-2E1 and Ad-LacZ in any of these enzymes (data not shown). Since adenovirus-mediated gene expression is transient (Connelly, 1999; Lai et al., 2002), the CYP2E1 activities at 2, 4, and 6 weeks after injection were evaluated and were highest at 2 weeks after adenoviral infection, the time point used for pathology studies. CYP2E1 immunohistochemistry confirmed that CYP2E1 was overexpressed in hepatocytes, and some hepatocytes expressed large amounts of CYP2E1 (Fig. 1B). Perhaps of significance is that unlike the expression of basal CYP2E1, which is confined to cells surrounding the pericentral vein, CYP2E1 was expressed throughout the liver after infection with Ad-2E1. This characteristic of CYP2E1 distribution can occur with respect to the CYP2E1 distribution in alcohol liver disease. In livers of nonalcoholics, CYP2E1 staining was seen in the perivenular zone only, whereas in samples of alcoholics, the staining was perivenular to midzonal and also, to a lesser degree, periportal (Cohen et al., 1997). Takahashi et al. (1993) showed that CYP2E1 mRNA levels were highest in the perivenular zone, lower in the midzone, and lowest in the periportal zone of livers of abstaining alcoholics. These levels were elevated in all three zones of recently drinking alcoholics; in fact, the fold increase (fourfold) was highest in the periportal zone than midzone (twofold) or perivenular zone (60%), although levels still remained highest in the perivenular zone (Takahashi et al., 1993). In addition, CYP2E1 has a rapid turnover in absence of an inducer, e.g., ethanol, and levels vary considerably as a function of ethanol consumption by rodent model (Lieber, 1997, 1999). However, constant elevated levels of CYP2E1 should be expressed after infection with Ad-2E1. Because adenovirus-mediated CYP2E1 expression is a transient event, little change was shown to occur in other forms of cytochrome P450 or components of the mixed-function oxidase, e.g., reductase as found as a response to permanent long-lasting changes in CYP2E1 transgenic or knockout mouse models. Therefore, this model has certain advantages for studying the toxicological actions of CYP2E1 itself and for future studies on ethanol liver toxicity related to CYP2E1.

Adenovirus-mediated overexpression of CYP2E1 causes liver toxicity compared to Ad-LacZ expression. Serum ALT and AST levels were significantly higher in mice injected with Ad-2E1 than in mice injected with Ad-LacZ or saline. Liver histology showed cell death in mice injected with Ad-2E1; while there was no significant change in the liver of the mice injected with Ad-LacZ (Fig. 3A). Increasing hepatocyte apoptosis has been reported in human alcoholic hepatitis (Natori et al., 2001), in CYP2E1 transgenic mice fed alcohol (Morgan et al., 2002), and in HepG2 cell lines overexpressing CYP2E1 and exposed to ethanol (Wu and Cederbaum, 1999).
Using the TUNEL assay, we detected many positive cells in the liver of the mice injected with Ad-2E1, and this increase was much more pronounced than that in mice injected with Ad-LacZ (Figs. 3B and 3C). Of interest is the finding that the liver pathology and TUNEL staining appeared to be panlobular, consistent with the widespread expression of CYP2E1 throughout the liver. These findings indicated that the overexpression of CYP2E1 via adenoviral delivery is able to directly induce liver toxicity. We believe that even though the increase in CYP2E1 was “only” twofold in total liver, constant expression of high levels of CYP2E1, the widespread expression of CYP2E1 throughout the liver, and the very high expression in some hepatocytes contribute to the resulting toxicity despite the modest quantitative change. Since adenovirus vectors can cause liver injury by themselves, and indeed the Ad-LacZ infection did elevate transaminase levels and caspase-3 activity, it is possible that in this model, the CYP2E1 overexpression synergizes with and potentiates the adenoviral-induced liver injury. Such synergistic hepatotoxic interactions between CYP2E1 and hepatitis B or hepatitis C core proteins have been reported (Kim et al., 2001; Otani et al., 2005; Wen et al., 2004).

The mechanism by which CYP2E1 induces or potentiates liver toxicity is believed to be through production of ROS such as superoxide, hydrogen peroxide, and hydroxyl radical. Enhanced lipid peroxidation seems to be a key feature in the ethanol-induced liver injury observed in the intragastric infusion model (Kamimura et al., 1992; Tsukamoto et al., 1995). However, lipid peroxidation was not significantly changed in mice injected with Ad-2E1 compared with the mice injected with Ad-LacZ or saline (data not shown). This may be due to the regular chow diet utilized in these experiments as the Purina pellets do not contain anywhere as much unsaturated fats as the liquid diet used in the intragastric infusion model. Future studies will evaluate the effect of Ad-2E1 in mice consuming the high-fat Lieber-DeCarli diet, with or without ethanol. No significant difference in inducible nitric oxide synthase levels was detected between mice injected with Ad-2E1 and those injected with Ad-LacZ by Western blot analysis (data not shown). Since increases in 3NT protein adducts were detected in the liver of the mice injected with Ad-2E1 compared with the mice injected with Ad-LacZ, this suggests that the increased production of peroxynitrite (precursor of the 3NT protein adducts) may reflect elevated lipid peroxidation as a consequence of CYP2E1 expression and not an increase in nitric oxide production. Increased carbonyl formation also serves as an indicator of oxidative stress (Davies, 1987). An increase of protein carbonyls in the livers from rats or mice fed ethanol has been reported (Bailey et al., 2001; Kessova et al., 2003; Rouach et al., 1997). Protein carbonyls were increased 1.5-fold in mice injected with Ad-2E1 compared with the mice injected with Ad-LacZ. Future experiments will evaluate whether antioxidants and inhibitors of CYP2E1 such as chlorimethiazole can prevent the CYP2E1 toxicity.

In summary, we have produced an adenovirus containing human CYP2E1 cDNA which can express catalytically active CYP2E1 protein after injecting in vivo into mice or after addition to liver cells (Bai and Cederbaum, 2004). This adenovirus-mediated overexpression of CYP2E1 caused liver toxicity in mice compared to infection with Ad-LacZ. Further studies to investigate the role of overexpression of CYP2E1 in alcohol liver toxicity using this adenovirus are underway.

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REFERENCES