conductance of Bax-VDAC channel was calculated as 4-fold and 10-fold greater than that of VDAC or Bax homotypic channels (35).

The overall objective of the present study was to examine the mechanisms by which acute ethanol intoxication induces mitochondrial cytochrome *c* release and apoptosis. To address this objective, we formulated the following questions: *1*) Does Bax translocate from the cytosol to mitochondria on acute ethanol treatment? *2*) Is Bax translocation oxidative stress dependent? *3*) Does Bax form a complex by either homotypic oligomerization or heterotypic interactions with VDAC? *4*) Are the Bax-VDAC interactions essential for ethanol-induced apoptosis? and *5*) Is Bax-VDAC complex formation cyclosporin-A dependent? To assess these questions, rat primary hepatocytes were used in this study. Hepatocytes were treated with ethanol (50 mM), a sufficient concentration to induce apoptosis as established in previous studies (12, 22).

MATERIALS AND METHODS

Materials and reagents. Anti-human VDAC monoclonal antibody (31HL) was purchased from Calbiochem-Novabiochem (La Jolla, CA). Rabbit anti-VDAC antibody for microinjection was kindly provided by Dr. S. Shimizu (Osaka University, Osaka, Japan). Mouse anti-Bax and rabbit anti-Bax polyclonal antibodies were from Pharmingen (Eugene, OR). CsA, *N*-acetyl-cysteine (NAC), and actinomycin D (ActD) were purchased from Sigma (St. Louis, MO). Recombinant mouse TNF-α was purchased from R&D Systems (Minneapolis, MN). *N*′,*N*′-dimethylthiourea (DMTU) was purchased from Janssen Chimica (cat. no. B-2440; Geer, Belgium). 2′,7′-Dichlorofluorescin diacetate (DCFH-DA), 5-(and-6-chloromethyl-2′,7′-dichlorofluorescein diacetate (CM-H_2DCFDA) Hoechst 33342, and MitoTracker Red CMXRos were purchased from Molecular Probes (Eugene, OR). Green fluorescent protein (GFP) was purchased from BD Biosciences Clontech (Palo Alto, CA).

Experimental protocol. Male Wistar rats with an average body weight of 250–300 g were used for the cell preparation. All animals received humane care in compliance with the National Research Council's criteria for humane care as outlined in "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Science and published by the National Institutes of Health. Rat hepatocytes were isolated and cultured as previously described (6). The viability of isolated cells was >95% as determined by the trypan blue dye exclusion test. Cells were seeded on culture dishes at a concentration of 5×10^6 cells/cm^2 and incubated in DMEM (Sigma) containing 10% fetal calf serum (Invitrogen, Carlsbad, CA) for 24 h at 37°C in 5% CO_2. Every precaution was taken to ensure that the additives, medium, and plastic materials used were free of endotoxin as determined by the Limulus Amebocyte Lysate Test Kit (Whittaker Bioproducts, Walkersville, MD), which has a sensitivity of 0.1 ng/ml. Rat hepatocytes were cultured for 24 h after isolation before ethanol (50 mM) exposure. This concentration of ethanol (50 mM), which is known to be toxicologically relevant, is sufficient to induce apoptosis in cultured rat hepatocytes (22). To compare the apoptotic machineries between ethanol and TNF-related apoptotic models, TNF-α (30 ng/ml) plus ActD (0.2 μM) were exposed to hepatocytes. ActD was added to cultured hepatocytes 1 h before being added to TNF-α. In some experiments, the PTP inhibitor CsA (10 μM); the antioxidant NAC (5 mM) or DMTU (10 mM); a small, permeable, and relatively nontoxic scavenger of hydrogen peroxide; and the hydroxyl radical were added to the culture medium before treatment with ethanol or TNF-α plus ActD.

Determination of reactive oxygen species. To investigate subcellular localization of oxidative stress in hepatocytes, DCFH-DA was used according to the methods of Cathcart et al. (5) with minor modification (23). Briefly, cultured rat hepatocytes on 35-mm glass-bottom Microwell culture dishes (MatTek, Ashland, MA) were incubated with DMEM (pH 7.4) containing 1 μM DCFH-DA for 30 min at 37°C in the dark. The cells were washed three times with phenol red-free DMEM to remove the extracellular fluorescence and were observed on an inverted fluorescence microscope (Diaphot TMD-2S; Nikon, Tokyo, Japan). Mitochondria were labeled by incubation of hepatocytes with 200 nM of MitoTracker Red CMXRos. A PlanApochromat ×63 oil immersion objective and laser scanning confocal microscope system (Zeiss 410; Zeiss, Thornwood, NY) were used for visualization. Confocal images of 2′7′-dichlorofluorescein (DCF; an oxidized form of DCFH) fluorescence was collected by using a 488-nm excitation light from an argon/krypton laser, a 560-nm dichroic mirror, and a 500- to 550-nm band-pass barrier filter. Images of MitoTracker Red fluorescence were collected by using 568-nm excitation light from the argon/krypton laser, a 560-nm dichroic mirror, and a 590-nm long-pass filter. The intracellular formation of reactive oxygen species (ROS) was measured by using CM-H_2DCFDA. Cells (2×10^5 cells) were harvested in 24-well culture plates (Corning, Acton, MA) and loaded with 1 μM CM-H_2DCFDA for 30 min at 37°C. After free probes were washed with Hanks' balanced salt solution (Invitrogen, Carlsbad, CA), fluorescence was analyzed before and after ethanol treatment (20 min) under fluorescent plate reader (FLUOstar OPTIMA; BMG Labtechnologies, Durham, NC). ROS production was expressed as ROS generation equivalent to H_2O_2 (μmol/l) exposure for 10 min determined from an H_2O_2 standard, which was obtained from a fluorescence intensity from 2×10^5 cells exposed to 10–1000 μM of H_2O_2 for 10 min.

Immunocytochemistry of Bax. Hepatocytes were cultured on glass chamber slides (LAB-TEK; Nalge Nunc, Hanover Park, IL) and incubated with ethanol. MitoTracker Red was used for mitochondrial labeling as described in *Determination of reactive oxygen species.* After being washed with PBS three times, cells were fixed for 5 min using 4% paraformaldehyde in PBS and then permeabilized with 0.1% Triton X for 5 min. After being blocked with 10% fetal calf serum, cells were incubated with 1:100 dilution of rabbit anti-Bax polyclonal antibody (13686E) for 2 h at 37°C. After being washed three times, cells were incubated for 30 min with 1:250 dilution of an Oregon Green-conjugated goat anti-rabbit secondary antibody (Molecular Probes) for 45 min at 37°C. Fluorescence images were visualized by using confocal microscopy.

Preparation of protein extracts. Hepatocytes cultured on 90-mm culture dishes (Asahi Techno Glass, Tokyo, Japan) were collected by centrifugation and washed with ice-cold PBS. Cells were resuspended in 5 vol of extraction buffer (in mM: 250 sucrose, 20 HEPES pH 7.5, 1.5 $MgCl_2$, 10 KCl, 1 sodium-EDTA, 1 sodium-EGTA, 1 dithiothreitol, 0.1 PMSF, with 10 μg/ml leupeptin and 10 μg/ml aprotinin), incubated for 30 min on ice, and lysed by homogenization with 10 strokes of a Teflon homogenizer. Homogenates were centrifuged at 750 *g* for 10 min to remove cell debris. The supernatants were transferred to a fresh tube and centrifuged at 10,000 *g* for 15 min to pellet the mitochondria. The pellet (mitochondria) was resuspended in RIPA buffer (50 mM Tris·HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS). The supernatants were then centrifuged at 100,000 *g*, and the resulting supernatants were designated as the cytosolic fraction (S-100). Cytosolic and mitochondrial fractions were used for immunoblot analysis. Protein concentration was determined by the bicinchoninic acid assay using BSA as the standard.

Immunoblotting. Immunoblotting for cytochrome *c* was performed by using the cytosolic S-100 fraction. Immunoblotting for Bax was performed by using mitochondrial or whole cell lysates from hepatocytes. Samples were resuspended in 20 μl of SDS-sample buffer and boiled at 90°C for 2 min, separated by 12% SDS-PAGE, and transferred to PVDF membranes (Immobilin-P; Millipore, Bedford, MA). After being blocked with 1% wt/vol skim milk and 3% wt/vol BSA in 20 mM Tris, 0.5 M NaCl, and 0.05% Tween 20, pH 7.0, for 30 min, membranes were incubated for 60 min with the primary antibodies:

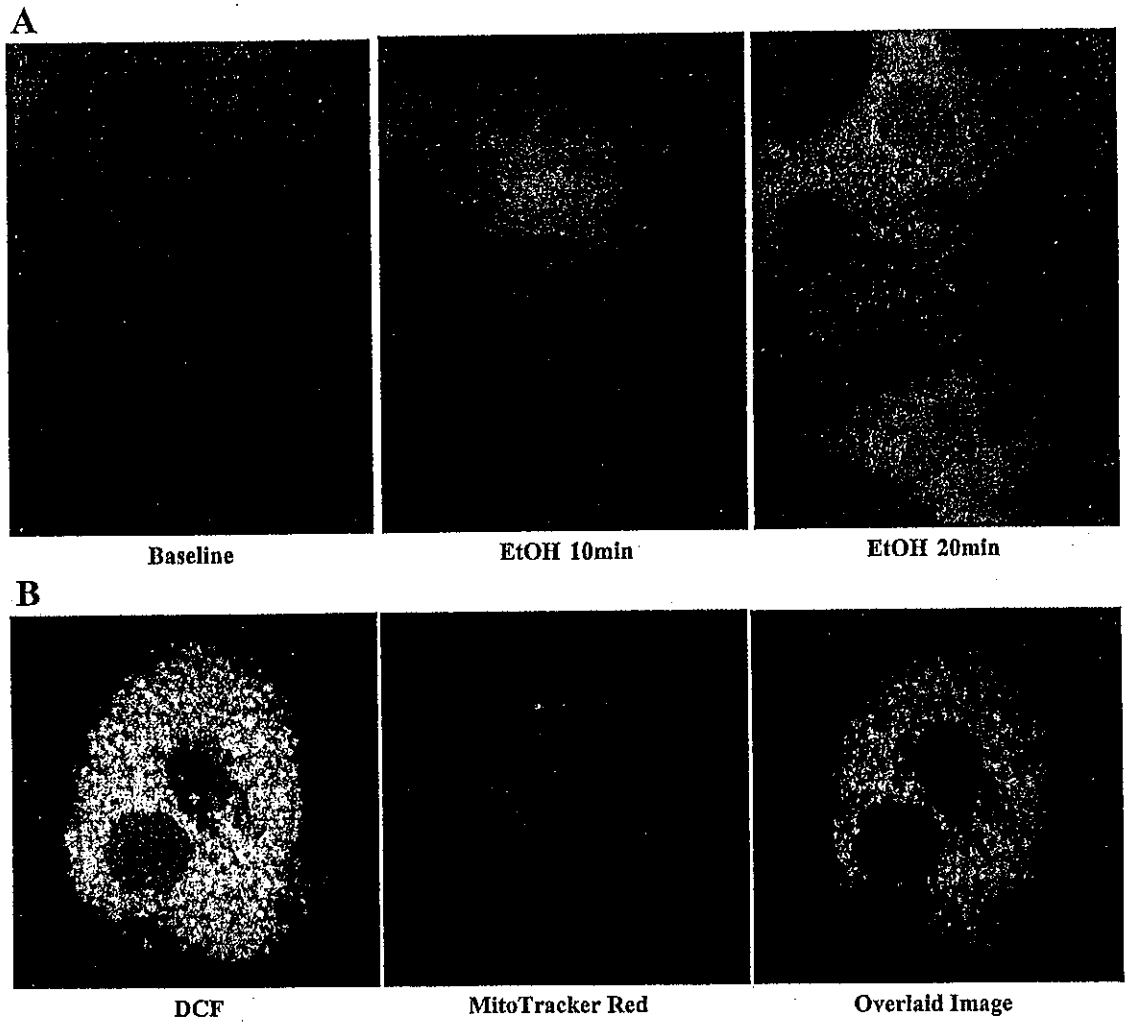

Fig. 1. Ethanol (EtOH)-induced 2′7′-dichlorofluorescein (DCF) oxidation was observed predominantly in mitochondria. Rat primary hepatocytes were incubated in the presence of 2′,7′-dichlorofluorescin diacetate (DCFH-DA; 1 μM) for 30 min. After being washed, cells were incubated with or without EtOH (50 mM). *A*: representative imaging of DCF fluorescence by fluorescent microscopy. DCF fluorescence increased in rat hepatocytes exposed to EtOH within 10 min and further increased in 20 min. *B*: hepatocytes were double-stained with MitoTracker Red CMXRos and DCFH-DA and observed by confocal microscopy. DCF-associated green fluorescence, MitoTracker Red-associated red fluorescence, and overlaid image are shown. Note that DCF fluorescence is colocalized with the MitoTracker Red fluorescence.

mouse anti-Bax (1:500 dilution) or mouse anti-cytochrome *c* (1:1,000 dilution). After being washed three times, membranes were further incubated for 60 min with peroxidase-conjugated goat anti-mouse IgG secondary antibodies (1:3,000 dilution) (Amersham, Arlington Heights, IL). Bound antibodies were detected by using enhanced chemiluminescent substrate (Amersham) and exposed to Kodak X-OMAT film. Results were confirmed by triplicate analysis.

In vivo protein cross-linking and immunoprecipitation. In vivo cross-linking for identifying Bax oligomerization or Bax-VDAC interactions was performed as described previously (1, 29). Briefly, we used the cross-linkers bis-(sulfosuccinimidyl)suberate (BS^3) and disuccinimidyl suberate (DSS) (Pierce Chemical, Rockford, IL) for Bax oligomerization or 3,3′-dithio-bis(succinimidylproprionate) (DSP) and dimethyl 3,3′-dithio-bis(proprionate)·2HCl (DTBP) (Pierce Chemical) for Bax-VDAC interaction, respectively. Cells were treated with 2 mM of cross-linkers in PBS for 30 min at room temperature. After the reaction was quenched with 50 mM Tris·HCl for 10 min at 4°C, cells were washed in PBS. Cells were then lysed with lysis buffer (in mM: 10 Tris·HCl, pH 7.4, 142.5 KCl, 5 $MgCl_2$, 1 EDTA, 1 PMSF, with 0.5% Nonidet P-40, and 20 μM leupeptin) for 30 min on ice and centrifuged to remove insoluble debris.

Immunoprecipitation was carried out as follows. Samples were precleared by mixing with 50 μl of 50% (vol/vol) protein G-Sepharose beads for 60 min at 4°C, and the beads were removed by centrifugation. The resultant supernatants were incubated with appropriate antibodies (2 μg/ml) at 4°C for 2 h. Immunoprecipitates were collected by incubating with protein G-Sepharose for 60 min, followed by centrifugation for 2 min at 4°C. The pellets were washed with lysis buffer three times. After the final wash, the beads were suspended in SDS-sample buffer, and the samples were analyzed by SDS-PAGE and Western blotting as described in *Immunoblotting*.

Microinjection. Microinjection was performed by using a micromanipulator (Narishige, Tokyo, Japan) as described previously (36). The rabbit anti-VDAC blocking antibodies were used. This antibody was reported to inhibit Bax-mediated cytochrome *c* release and membrane potential loss, without inhibiting mitochondrial respiration of cells (36). Normal rabbit IgG (NRI; Santa Cruz Biotechnology, Santa Cruz, CA) was used as a control. Either anti-VDAC antibodies (15 μg/μl) or NRI

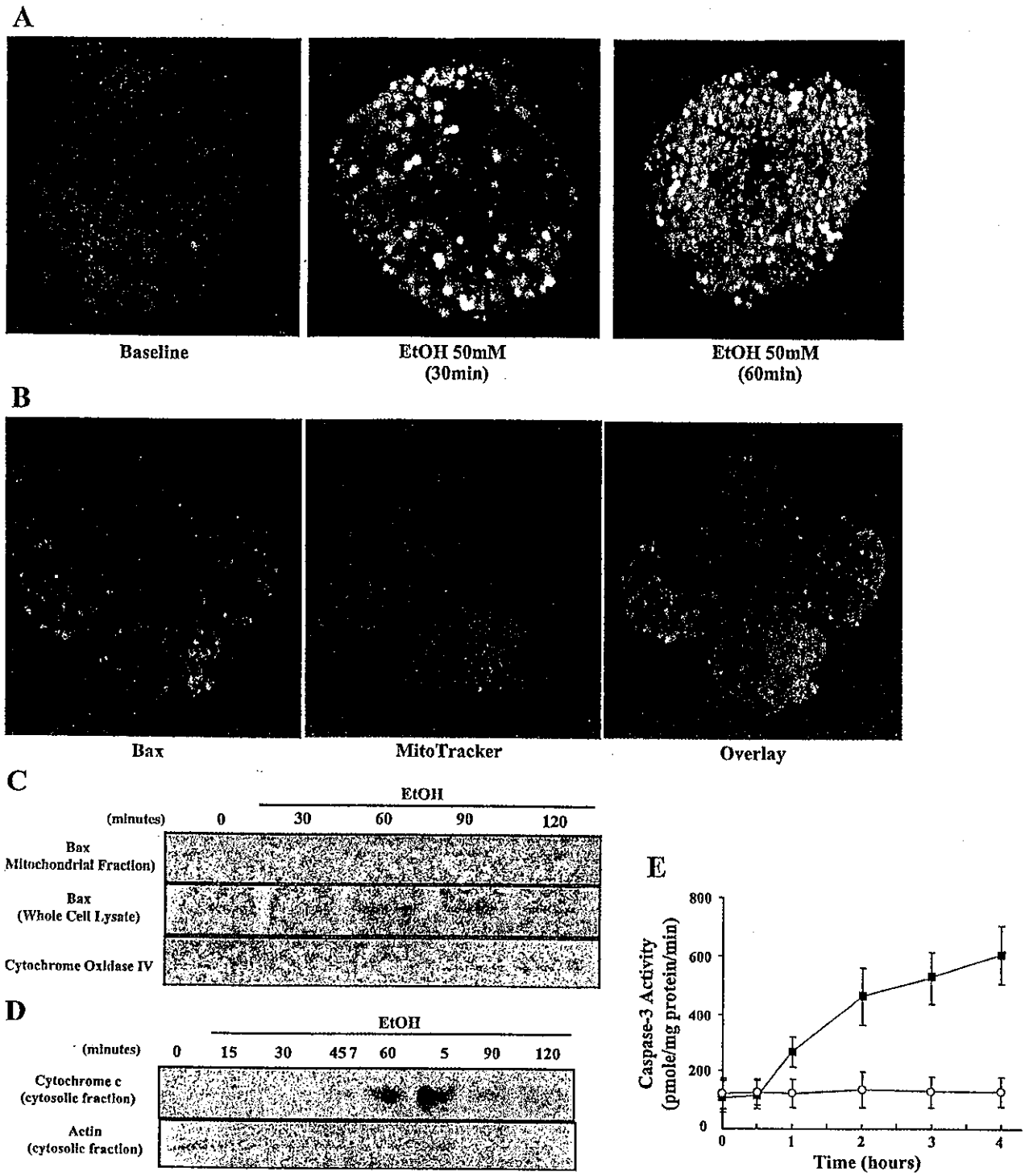

Fig. 2. Bax is predominantly observed on mitochondria in EtOH-treated hepatocytes. Hepatocytes were incubated with or without EtOH (50 mM). Immunofluorescence staining for Bax and immunoblotting for Bax and cytochrome *c* were carried out. Experiments were repeated 3 times, and the representative images were depicted. *A*: Bax immunofluorescence was visualized under a confocal microscope. Subcellular localization of Bax was altered overtime by EtOH treatment. *B*: hepatocytes were subjected to a double staining of anti-Bax and MitoTracker Red. Bax-associated fluorescence and the MitoTracker Red fluorescence are colocalized in the overlay image. *C*: mitochondrial fraction (*top*) and the whole cell lysates (*bottom*) were subjected to immunoblot analysis. Mitochondrial Bax increased within 30 min after EtOH exposure, whereas the expression level of Bax in whole cell lysates was unchanged. *D*: release of cytochrome *c* from mitochondria to the cytosol was observed by immunoblot analysis. At selected time intervals, cytosolic extracts were collected from EtOH-treated hepatocytes and exposed to an immunoblot analysis. Note that cytochrome *c* is detected in cytosolic fraction at 60 min after the exposure to EtOH. *E*: caspase-3 activity was evaluated by measuring a fluorogenic substrate Ac-Asp-Glu-Val-Asp-α-(4-methyl-coumaryl-7-amide) (DEVD-MCA) cleavage activity. Caspase-3 activity was increased at 60 min of EtOH treatment and was further elevated at the following time points.

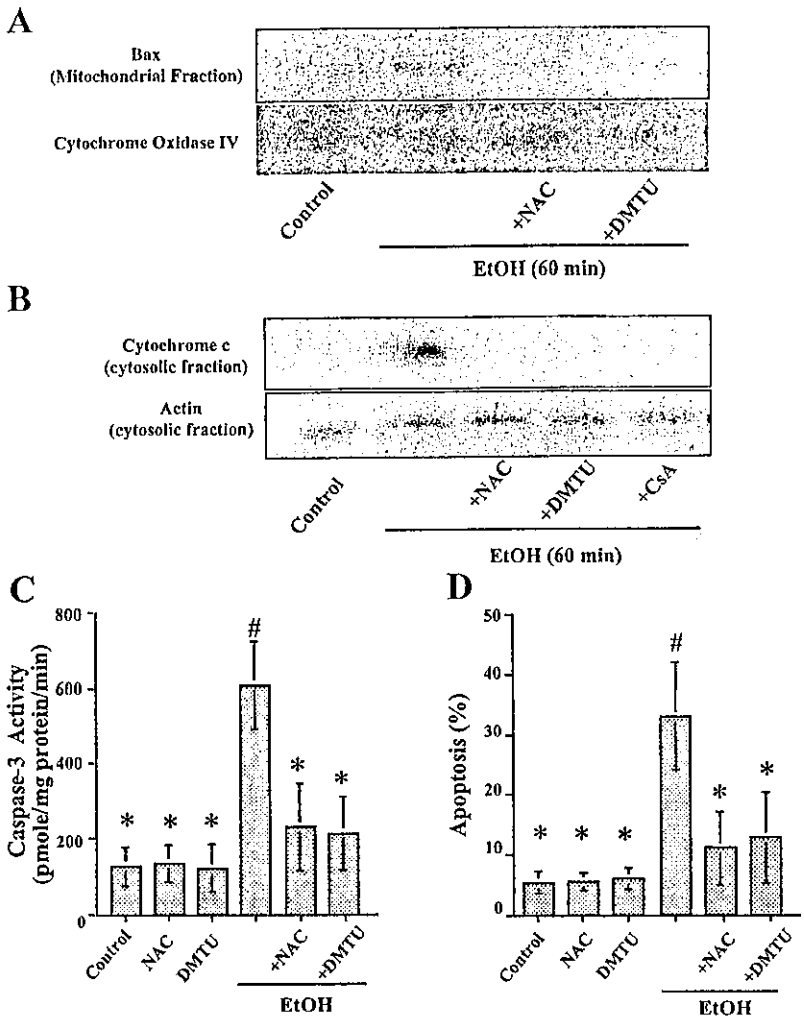

Fig. 3. Bax transmigrates to mitochondria in an oxidative stress-dependent manner. Hepatocytes were incubated with EtOH in the presence or absence of indicated inhibitors. At indicated time points, cells were harvested for mitochondria and whole cell lysate preparation. Effects of *N*-acetyl-cysteine (NAC; 5 mM), *N*′,*N*′-dimethylthiourea (DMTU; 10 mM), or cyclosporin A (CsA; 10 μM) on EtOH-induced Bax translocation to mitochondria (at 60 min) (*A*), mitochondrial cytochrome *c* release (at 60 min) (*B*), caspase-3 activation (at 2 h) (*C*), and apoptosis (at 8 h) (*D*) were evaluated. *A* and *B*: results were representative of 3 independent experiments. *C* and *D*: data were expressed as means ± SD from 5 independent experiments. #$P < 0.05$ vs. control, *$P < 0.05$ vs. EtOH by ANOVA.

was mixed with GFP (3 μg/μl) as a marker of microinjected cells and then microinjected into the cytosol of cultured hepatocytes. One hour after the injection, cells were treated with ethanol for the following 6 h.

Quantitation of apoptosis. A cell membrane-permeable nuclear binding dye Hoechst 33342 was used for evaluation of apoptosis (12). Cells were incubated with 10 μM of Hoechst 33342 for 15 min before the addition of ethanol. The blue fluorescence was visualized by using a fluorescence microscope (excitation: 330–380 nm, emission: 460 nm). Apoptosis was evaluated by morphological criteria, i.e., condensed chromatin and fragmented nuclei, and the number of cells with apoptotic nuclei was determined within a field of view at a magnification of ×400. A total of 10 randomly prechosen fields were counted per well, and the number of apoptotic cells was averaged to obtain an apoptotic index.

Caspase activity assay. Cytosolic extracts for the enzyme assay were prepared as previously described (16) with minor modifications. In brief, cells were homogenized in hypotonic buffer (in mM: 25 HEPES, 5 $MgCl_2$, 1 EGTA, 0.5 PMSF, with 2 μg/ml pepstatin and 2 μg/ml leupeptin, pH 7.5), and centrifuged for 10 min at 1,000 *g*. Caspase activity was measured by adding 50 μl of cytosol to 450 μl of assay buffer containing 25 mM HEPES (pH 7.5), 10 mM DTT, 0.1% CHAPS, 0.5 mM PMSF, 100 U/ml aprotinin, and 20 μM of fluorogenic tetrapeptide substrates Ac-Asp-Glu-Val-Asp-α-(4-methyl-coumaryl-7-amide) (DEVD-MCA; Peptide Institute, Osaka, Japan) for caspase-3 or Ac-Ile-Glu-Thr-Asp-α-(4-methyl-coumaryl-7-amide) (IETD-MCA; Peptide Institute) for caspase-8. Fluorescence (excitation: 380 nm, emission: 450 nm) was quantitated by using a fluorometer (Hamamatsu Photonics, Hamamatsu, Japan) as described previously (16).

Statistical analysis. All data represent at least three independent experiments and are expressed as the means ± SD, unless otherwise indicated. Differences between groups were compared by using ANOVA for repeated measures and a post hoc Bonferroni test to correct for multiple comparisons.

RESULTS

Ethanol causes oxidative stress predominantly within mitochondria. Because our previous study demonstrated that ethanol-induced oxidative stress targeted mitochondria (21, 22), we first evaluated subcellular localization of oxidative stress by using the oxidant-sensitive fluorescence probe DCFH-DA. DCF, an oxidized form of DCFH, fluorescence was not visible in control hepatocytes; however, the fluorescence increased significantly within 10 min after ethanol (50 mM) treatment (Fig. 1*A*). The fluorescence further increased at 20 min with an increasingly dotted pattern. To determine the exact localization of the DCF, we performed dual labeling with the mitochondria-specific dye MitoTracker Red. DCF fluorescence colocalized with MitoTracker Red fluorescence (Fig. 1*B*), suggesting that ethanol-induced oxidative stress predominantly occurs within

mitochondria. These results are consistent with our previous study showing rapid mitochondrial dysfunction after oxidative stress in ethanol-treated hepatocytes (12, 21) and emphasize the importance of oxidative stress in ethanol-induced mitochondrial injury.

Ethanol induces Bax translocation to mitochondria via an oxidative stress-dependent mechanism. Because mitochondrial dysfunction can be associated with translocation of cytosolic Bax to mitochondria (40), we then determined whether Bax transmigrates to mitochondria during ethanol-induced hepatocyte apoptosis. Bax immunofluorescence was initially diffuse, consistent with cytosolic localization. However, the Bax-associated fluorescence became dotted over time after ethanol treatment (Fig. 2*A*). To clarify the subcellular localization of Bax, mitochondria were counterstained with MitoTracker Red (Fig. 2*B*). Both Bax (green) and mitochondria (red)-associated fluorescence displayed the same pattern of fluorescence, and the overlay image showed a complete colocalization of Bax with mitochondria. Thus Bax transmigrates to mitochondria during ethanol treatment.

To confirm the transmigration of Bax to mitochondria during ethanol treatment, we performed subcellular fractionation and immunoblot analysis. Bax was initially observed in the mitochondrial fraction; however, the amount of mitochondrial Bax was significantly increased at 30 min after ethanol exposure (Fig. 2*C*), whereas total Bax expression levels within whole cell lysates were unchanged at all time points tested. Release of cytochrome *c* from mitochondria to cytosol was observed at 60 min after the addition of ethanol (Fig. 2*D*). In addition, the time point when release of cytochrome *c* starts also coincides with caspase-3 activation (Fig. 2*E*). This was consistent with our previous report (12). These results suggest that Bax translocates from cytosol to mitochondria before cytochrome *c* release and caspase-3 activation.

Previous studies (12) demonstrated that mitochondrial cytochrome *c* release is blocked by either antioxidants or the PTP inhibitor CsA. Therefore, we then tested the effects of these agents on ethanol-induced Bax transmigration. Both DMTU, a cell membrane-permeable antioxidant, and NAC, a glutathione precursor, prevented the increase in Bax association with mitochondria (Fig. 3*A*). These agents have been shown to prevent ethanol-induced elevation of DCF fluorescence (12). To confirm that the suppression of Bax translocation results in a decrease in hepatocellular apoptosis, we then evaluated the effect of antioxidants on cytochrome *c* release, caspase-3 activity, and apoptosis. Indeed, either NAC or DMTU inhibited cytochrome *c* release, caspase-3 activity, and apoptosis (Fig. 3, *B–D*), suggesting that Bax transmigration and subsequent apoptotic alteration in ethanol-treated hepatocytes is oxidative stress dependent.

Bax is not oligomerized but interacts with the mitochondrial channel protein VDAC. Bax promotes mitochondrial cytochrome *c* release by either Bax homotypic oligomerization or interaction with the PTP components such as VDAC (13, 25, 29, 40). We then examined whether Bax is oligomerized in ethanol-treated hepatocyte (1). After pretreatment with the noncleavable cross-linkers BS^3 and BSP, cells were lysed and Bax oligomerization was evaluated by Bax immunoprecipitation and immunoblot analysis (Fig. 4*A*). As a positive control for Bax oligomerization, we used protein extracts from TNF-α+ActD-treated hepatocytes. The 21-, 42-, and 63-kDa forms of

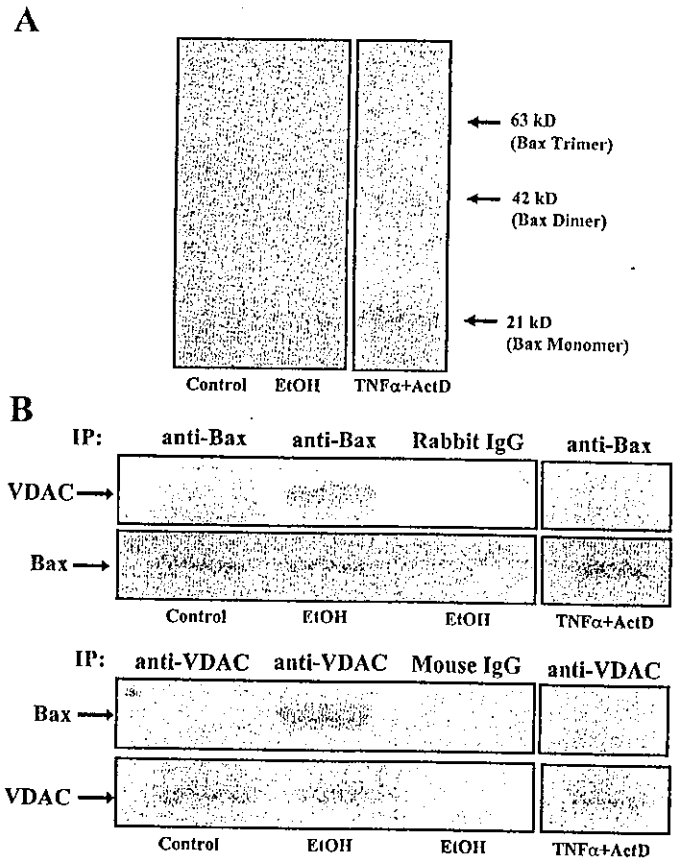

Fig. 4. Bax is not oligomerized but interacts with voltage-dependent anion channel (VDAC) in EtOH-treated hepatocytes. *A*: hepatocytes were treated with noncleavable cross-linkers bis-(sulfosuccinimidyl)suberate (BS^3) and disuccinimidyl suberate (DSS) for 30 min. Protein extracts were immunoprecipitated with rabbit anti-Bax antisera and immunoblotted with mouse anti-Bax antibody. Bax oligomerization was not observed in both control and EtOH-treated hepatocytes. Effect of TNF-α (30 ng/ml) plus actinomycin D (ActD; 0.2 μg/ml) was also evaluated. *B*: hepatocytes were treated with cleavable cross-linkers 3,3′-dithio-bis(succinimidylproprionate) (DSP) and 3,3′-dithio-bis(proprionate) (DTBP) for 30 min. Protein extracts were immunoprecipitated (IP) with either rabbit anti-Bax antisera, rabbit anti-VDAC antisera, normal rabbit IgG (NRI; as a control of anti-Bax antisera), or normal mouse IgG (as a control of anti-VDAC antisera). Immunoprecipitates were immunoblotted by indicated antibodies. Representative images were shown from 3 independent experiments. Note Bax-VDAC interactions were observed in only EtOH-treated hepatocytes.

Bax, corresponding to monomeric, dimeric, and trimeric forms of Bax, respectively, were observed in the TNF-α+ActD-treated cells. In contrast, only Bax monomers were detected in the ethanol-treated hepatocytes. In ethanol-treated hepatocytes, the density of the monomeric Bax band appears to be decreased compared with untreated controls (Fig. 4*A*). Therefore, we could not eliminate the possibility that Bax may form a larger molecular mass complex that could not be separated by the SDS-PAGE performed in the experiment.

Bax is known to form a complex with the PTP component proteins, such as VDAC, and the importance of Bax and PTP interactions in mitochondrial cytochrome *c* release has been suggested (25, 29). Therefore, we then determined whether Bax-VDAC interactions were observed in ethanol-treated hepatocytes. Hepatocytes were treated with the cleavable cross-linkers DSP and DTBP, and the cell lysates were subjected to immunoprecipitation with either anti-Bax or anti-

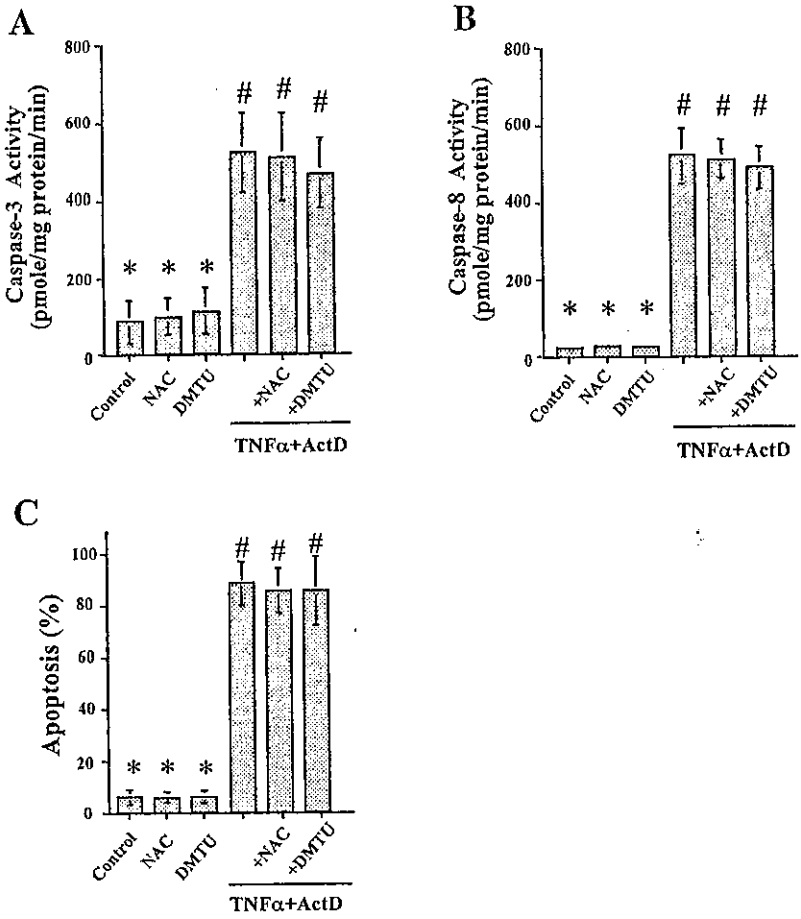

Fig. 5. TNF-α plus ActD induces caspase-8- and caspase-3-dependent apoptosis, which are not sensitive to antioxidants. Hepatocytes were incubated with or without TNF-α (30 ng/ml) plus ActD (0.2 μg/ml) in the presence or absence of indicated antioxidants. Effects of NAC (5 mM) or DMTU (10 mM) on caspase-3 (at 4 h) (*A*), caspase-8 (at 4 h) (*B*), and apoptosis (at 12 h) (*C*) were evaluated. Data were expressed as means ± SD from 5 independent experiments. #$P < 0.05$ vs. control, *$P < 0.05$ vs. TNF-α plus ActD by ANOVA.

VDAC antisera. In ethanol-treated hepatocytes, VDAC coprecipitated with Bax was observed, whereas VDAC was not observed in the precipitates from the control hepatocytes or immunoprecipitates by rabbit IgG (Fig. 4*B*). Furthermore, when the cell lysates were exposed to immunoprecipitation using an anti-VDAC antibody, Bax was only coprecipitated in ethanol-treated cells. These results suggest that Bax binds to the PTP component protein VDAC on ethanol treatment. Interestingly, the Bax-VDAC interactions were not observed in TNF-α+ActD-treated hepatocytes, suggesting that the effect of Bax on mitochondria is different between ethanol- and TNF-α+ActD-induced apoptosis.

To compare the other apoptotic machineries between these two models (ethanol vs. TNF-α+ActD), we compared other experimental manipulations in addition to an observation of BAX-VDAC interactions. TNF-α+ActD induced activation of caspase-3 and caspase-8 (Fig. 5, *A* and *B*), whereas our previous observation has shown that caspase-8 is not activated in ethanol-treated hepatocytes (12). Interestingly, antioxidants did not prevent TNF-induced caspase activation or apoptosis (Fig. 5, *A*–*C*).

Microinjection of anti-VDAC antibody inhibits ethanol-induced apoptosis. To determine whether the Bax-VDAC complex is essential for ethanol-induced hepatocyte apoptosis, we then microinjected anti-VDAC-blocking antibody into the cells before ethanol exposure. This antibody was raised against amino acids 151–165 of human VDAC1 where they are probably exposed to the cytoplasm, are specific for human and rat VDAC, and are able to prevent Bax-VDAC interactions (36). Anti-VDAC antibody or NRI was microinjected into the cytosol of hepatocytes, and then ethanol was added 1 h after the microinjection. Six hours after treatment with ethanol, 32.6 ± 6.9% of NRI-injected hepatocytes underwent apoptosis (Fig. 6). Microinjection of anti-VDAC antibody effectively attenuated ethanol-induced hepatocyte apoptosis, 13.6 ± 3.8% ($P < 0.05$, Fig. 6). These results suggest that VDAC-Bax associations are essential for ethanol-induced hepatocyte apoptosis.

The PTP inhibitor does not inhibit oxidative stress, Bax translocation to mitochondria, and Bax-VDAC interactions; however, these inhibitors effectively prevent cytochrome c release, caspase activation, and apoptosis. Time course observations of oxidative stress, cytochrome *c* release, and Bax translocation suggest that oxidative stress and Bax translocation to mitochondria are upstream of the mitochondrial permeability transition (MPT) and mitochondrial cytochrome *c* release within ethanol-mediated apoptotic signaling cascade. Therefore, to confirm this hypothesis, we tested whether CsA-sensitive permeability transition affects these events. CsA did not prevent the ROS generation (Fig. 7*A*), the mitochondrial translocation of Bax (Fig. 7*B*), and the formation of Bax-VDAC complex (Fig. 7*C*), whereas CsA effectively attenuated caspase-3 activation (Fig. 7*D*) and apoptosis (Fig. 7*E*). Thus oxidative stress is the first event that leads the MPT, Bax translocation to mitochondria, and Bax-VDAC interactions.

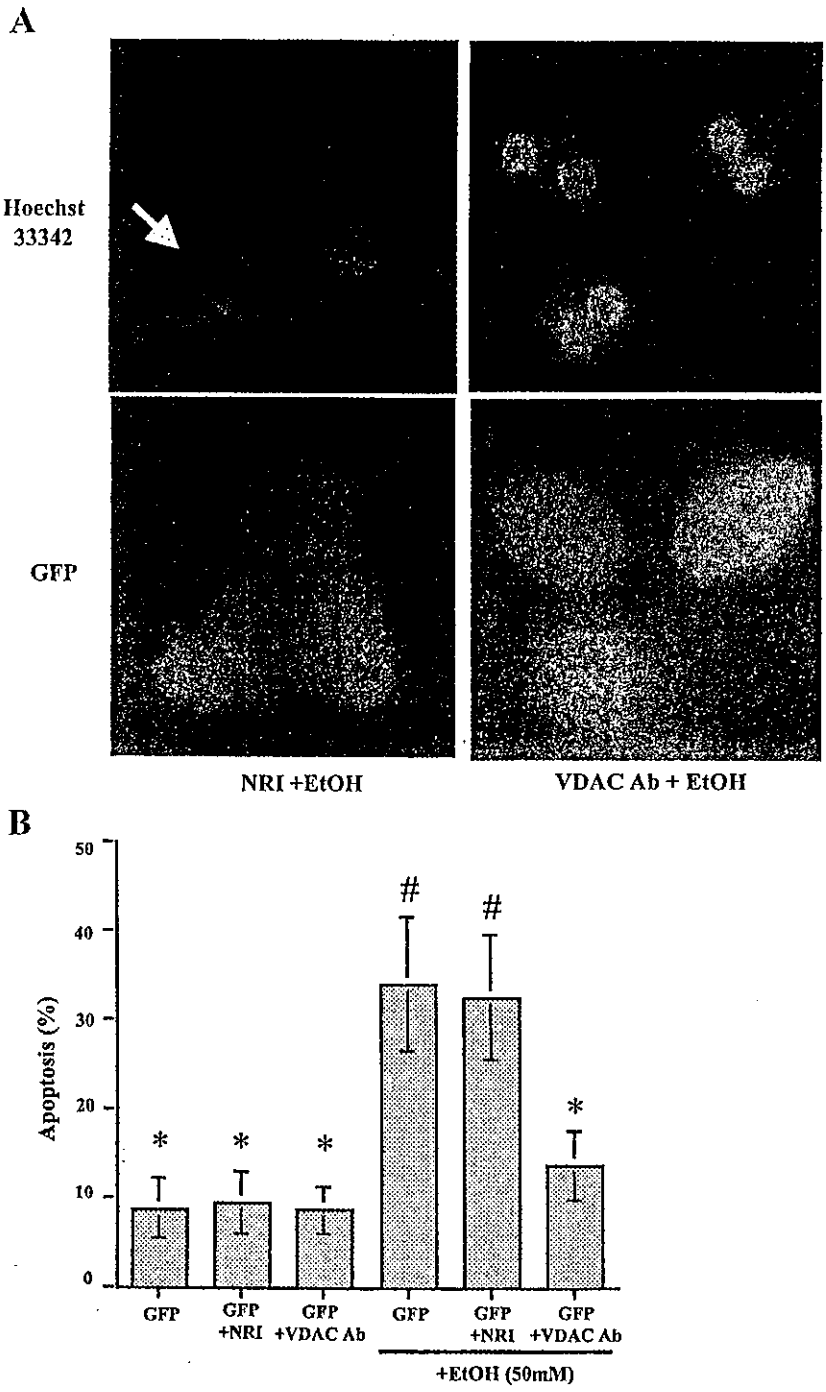

Fig. 6. Microinjection of anti-VDAC antibodies to hepatocyte inhibits EtOH-induced apoptosis. Hepatocytes were microinjected with anti-VDAC antibody or NRI at 12 μg/μl concentration. GFP (3 μg/μl) was coinjected to identify the injected cells. After incubation with EtOH (50 mM) for 8 h, cells were stained with Hoechst 33342 and observed by a fluorescent microscope. *A*: apoptosis was evaluated by morphological criteria after the Hoechst 33342 nuclear staining (blue). GFP was monitored to identify the injected cells. Representative fluorographs of control NRI-injected hepatocytes (*left*) and anti-VDAC antibody-injected hepatocytes (*right*). The arrow indicates the apoptotic cell. *B*: apoptotic nuclei were quantitated under the microscopic fields. >100 injected cells were counted for each experiment. Data were expressed as means ± SD from 5 independent experiments. #$P < 0.05$ vs. GFP + NRI-injected group, *$P < 0.05$ vs. EtOH-treated GFP + NRI-injected group by ANOVA.

DISCUSSION

The major findings of the present study relate to the cellular mechanisms of acute ethanol-induced hepatocyte apoptosis. The results indicated that *1*) acute ethanol treatment induces oxidative stress in hepatocytes within mitochondria, *2*) ethanol induces Bax translocation from the cytosol to mitochondria, *3*) Bax translocates to mitochondria before mitochondrial cytochrome *c* release, *4*) mitochondrial Bax interacts with the PTP component protein VDAC, and *5*) inhibition of Bax-VDAC interactions by anti-VDAC antibody prevents ethanol-induced apoptosis. These data implicate a role for Bax-VDAC interactions in acute ethanol-induced hepatocyte apoptosis. These observations provide further insights into mechanisms responsible for alcoholic liver injury.

Ethanol induces a hypermetabolic state in the liver that is characterized by enhanced mitochondrial respiration. The decrease in the $NAD^+/NADH$ ratio induced by acute ethanol administration may favor mitochondrial superoxide generation by increasing the electron flow along the respiratory electron transport chain (11, 15, 21). The enhanced superoxide generation increases mitochondrial lipid peroxide generation (26). Our data support these concepts by directly demonstrating

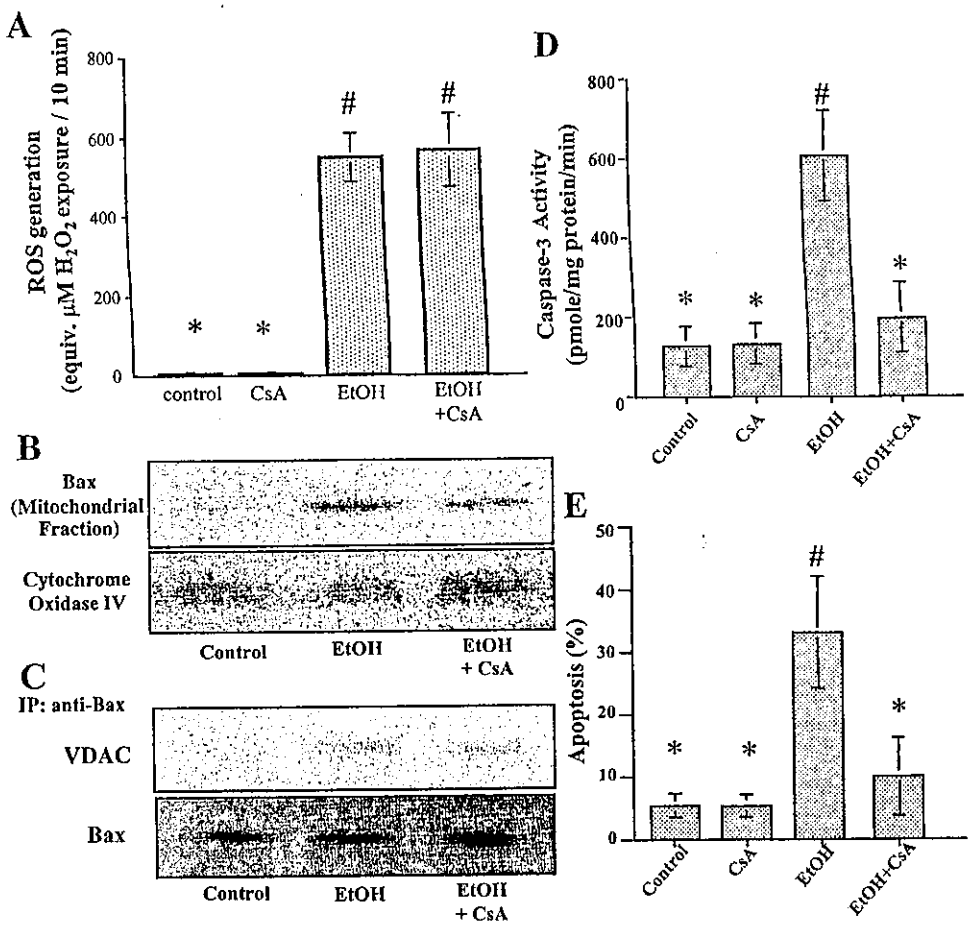

Fig. 7. Oxidative stress, Bax translocation to mitochondria, and Bax-VDAC interaction are upstream events of apoptotic mitochondrial permeability transition. Hepatocytes were incubated with or without 50 mM of EtOH in the presence or absence of CsA (10 μM). Effects of CsA on reactive oxygen species (ROS) generation determined by DCF fluorescence (*A*), Bax translocation to mitochondria (*B*), Bax-VDAC interaction (*C*), caspase-3 activity (*D*), and apoptosis (*E*) were evaluated. CsA failed to prevent oxidative-stress-associated DCF fluorescence, Bax translocation, and Bax-VDAC interaction. *A*, *D*, and *E*: data were expressed as means ± SD from 5 independent experiments. #$P < 0.05$ vs. control, *$P < 0.05$ vs. EtOH by ANOVA. *B* and *C*: results were representative of 3 independent experiments.

mitochondrial oxidative stress as visualized by an oxidant-sensitive fluorescent probe DCFH-DA during acute ethanol intoxication. Because mitochondria are the major source of ethanol-associated oxidant production, they are therefore also likely to be the first target in oxidative stress-associated injury.

In the present study, Bax was observed to translocate from the cytoplasm to mitochondria before mitochondrial cytochrome *c* release during exposure to ethanol. This observation suggests that Bax may play an important role in mitochondrial cytochrome *c* release. Bax-mediated mitochondrial cytochrome *c* release has been implicated in both death receptor-mediated and nondeath receptor pathway of apoptosis (27, 37). However, our data suggest that Bax association with mitochondria in ethanol-treated hepatocytes is distinct from the TNF-mediated death receptor signaling pathway. In the ethanol-treated hepatocytes, Bax forms a complex with the PTP component protein VDAC. In contrast, this heterotypic interaction of Bax and VDAC was not observed in TNF-α-treated cells. In death receptor-mediated pathway, Bax may form a homotypic oligomer channel on the death receptor-mediated tBid signaling (7, 20). The Bax homooligomerization may result in a formation of various oligomers of Bax complexes including dimers and trimers (1, 7). Consistent with the previous reports, we observed monomeric, dimeric, and trimeric forms of Bax in the TNF-α-treated hepatocytes. In ethanol-treated hepatocytes, Bax homotypic oligomerization was not observed. Thus ethanol may predominantly induce Bax-VDAC heterotypic interactions, whereas TNF-α may induce Bax homooligomerization.

The differences in Bax molecular complex formation may account for differences in apoptotic signals between two models (ethanol vs. TNF-α). The most significant difference is caspase-8 dependency. In the case of TNF-α, it is well accepted that death receptors such as TNF-receptor 1 can activate caspase-8. Activated caspase-8 cleaves and activates Bid. Bid and Bax (or Bak) cooperate to induce mitochondrial cytochrome *c* release on mitochondrial outer membrane. In contrast, our previous study (12) has shown that ethanol-mediated apoptosis is not mediated by caspase-8 and Bid. In the present study, we report some additional findings regarding the difference of apoptosis signaling between these two models: *1*) antioxidants effectively inhibited Bax translocation and subsequent apoptotic signals in ethanol model, whereas antioxidants failed to inhibit TNF-induced apoptosis; and 2) ethanol induces Bax-VDAC interaction, whereas TNF does not induce detectable interaction of these two molecules. Interestingly, it has been reported that inhibitors of MPT reduced oxidative stress, whereas antioxidants reduced mitochondrial permeability in a certain caspase-8-mediated apoptosis such as bile acid (41). It would be possible that oxidative stress is more important to signal (or initiate) apoptosis in a caspase-8-independent apoptosis model.

Our previous study (12) demonstrated that acute ethanol induced an increase in the mitochondrial membrane permeabil-

ity leading to massive cytochrome *c* release. The increase in the mitochondrial permeability was evaluated by mitochondrial calcein release assay (an indicator of the permeability of both inner and outer membranes) and was likely mediated by the PTP opening because it was prevented by the PTP inhibitor CsA. In the present study, we further evaluated whether the mitochondrial Bax transmigration and the Bax-VDAC interactions are sensitive to PTP inhibitor. CsA failed to attenuate ethanol-induced mitochondrial translocation of Bax or its interaction with VDAC, suggesting that Bax-VDAC interactions observed in the ethanol-treated hepatocytes is an upstream signal of PTP opening. Because VDAC is a major component of the PTP, it would be possible that Bax-VDAC interactions may alter the PTP status, which allows cytochrome *c* to leave mitochondria. Indeed, CsA effectively prevented ethanol-induced mitochondrial cytochrome *c* release, caspase-3 activation, and apoptosis (12).

In conclusion, the present study provides an additional mechanism for acute ethanol-induced hepatocyte apoptosis. Ethanol-associated oxidative stress induces Bax transmigration to the mitochondria. Bax interacts with the PTP component protein VDAC and likely causes PTP opening, cytochrome *c* release, caspase activation, and apoptosis. Prevention of the Bax-VDAC interactions by specific anti-VDAC antibody prevented the hepatocyte apoptosis. Therefore, Bax-VDAC interaction would be a potential target for prevention of alcohol-related liver injury.

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REFERENCES

1. **Antonsson B, Montessuit S, Sanchez B, and Martinou JC.** Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. *J Biol Chem* 276: 11615–11623, 2001.
2. **Benedetti A, Brunelli E, Risicato R, Cilluffo T, Jezequel AM, and Orlandi F.** Subcellular changes and apoptosis induced by ethanol in rat liver. *J Hepatol* 6: 137–143, 1988.
3. **Bernardi P.** The permeability transition pore. Control points of a cyclosporin A-sensitive mitochondrial channel involved in cell death. *Biochim Biophys Acta* 1275: 5–9, 1996.
4. **Beutner G, Ruck A, Riede B, and Brdiczka D.** Complexes between porin, hexokinase, mitochondrial creatine kinase and adenylate translocator display properties of the permeability transition pore. Implication for regulation of permeability transition by the kinases. *Biochim Biophys Acta* 1368: 7–18, 1998.
5. **Cathcart R, Schwiers E, and Ames BN.** Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay. *Anal Biochem* 134: 111–116, 1983.
6. **Drochmans P, Wanson JC, and Mosselmans R.** Isolation and subfractionation on ficoll gradients of adult rat hepatocytes. Size, morphology, and biochemical characteristics of cell fractions. *J Cell Biol* 66: 1–22, 1975.
7. **Eskes R, Desagher S, Antonsson B, and Martinou JC.** Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. *Mol Cell Biol* 20: 929–935, 2000.
8. **Finucane DM, Bossy-Wetzel E, Waterhouse NJ, Cotter TG, and Green DR.** Bax induced caspase activation and apoptosis via cytochrome *c* release from mitochondria is inhibitable by Bcl-xL. *J Biol Chem* 274: 2225–2233, 1999.
9. **Goldin RD, Hunt NC, Clark J, and Wickramasinghe SN.** Apoptotic bodies in a murine model of alcoholic liver disease: reversibility of ethanol-induced changes. *J Pathol* 171: 73–76, 1993.
10. **Green DR and Reed JC.** Mitochondria and apoptosis. *Science* 281: 1309–1312, 1998.
11. **Handler JA and Thurman RG.** Redox interactions between catalase and alcohol dehydrogenase pathways of ethanol metabolism in the perfused rat liver. *J Biol Chem* 265: 1510–1515, 1990.
12. **Higuchi H, Adachi M, Miura S, Gores GJ, and Ishii H.** The mitochondrial permeability transition contributes to acute ethanol-induced apoptosis in rat hepatocytes. *Hepatology* 34: 320–328, 2001.
13. **Hsu YT, Wolter KG, and Youle RJ.** Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. *Proc Natl Acad Sci USA* 94: 3668–3672, 1997.
14. **Huser J, Rechenmacher CE, and Blatter LA.** Imaging the permeability pore transition in single mitochondria. *Biophys J* 74: 2129–2137, 1998.
15. **Ishii H, Kurose I, and Kato S.** Pathogenesis of alcoholic liver disease with particular emphasis on oxidative stress. *J Gastroenterol Hepatol* 12: S272–S282, 1997.
16. **Jones B, Roberts PJ, Faubion WA, Kominami E, and Gores GJ.** Cystatin A expression reduces bile salt-induced apoptosis in a rat hepatoma cell line. *Am J Physiol Gastrointest Liver Physiol* 275: G723–G730, 1998.
17. **Jurgensmeier JM, Xie Z, Deveraux Q, Ellerby L, Bredesen D, and Reed JC.** Bax directly induces release of cytochrome *c* from isolated mitochondria. *Proc Natl Acad Sci USA* 95: 4997–5002, 1998.
18. **Kaplowitz N and Tsukamoto H.** Oxidative stress and liver disease. *Prog Liver Dis* 14: 131–159, 1996.
19. **Kawahara H, Matsuda Y, and Takase S.** Is apoptosis involved in alcoholic hepatitis? *Alcohol Alcohol* 29, *Suppl* 1: 113–118, 1994.
20. **Korsmeyer SJ, Wei MC, Saito M, Weiler S, Oh KJ, and Schlesinger PH.** Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome *c*. *Cell Death Differ* 7: 1166–1173, 2000.
21. **Kurose I, Higuchi H, Kato S, Miura S, Watanabe N, Kamegaya Y, Tomita K, Takaishi M, Horie Y, Fukuda M, Mizukami K, and Ishii H.** Oxidative stress on mitochondria and cell membrane of cultured rat hepatocytes and perfused liver exposed to ethanol. *Gastroenterology* 112: 1331–1343, 1997.
22. **Kurose I, Higuchi H, Miura S, Saito H, Watanabe N, Hokari R, Hirokawa M, Takaishi M, Zeki S, Nakamura T, Ebinuma H, Kato S, and Ishii H.** Oxidative stress-mediated apoptosis of hepatocytes exposed to acute ethanol intoxication. *Hepatology* 25: 368–378, 1997.
23. **Kurose I, Miura S, Fukumura D, Yonei Y, Saito H, Tada S, Suematsu M, and Tsuchiya M.** Nitric oxide mediates Kupffer cell-induced reduction of mitochondrial energization in hepatoma cells: a comparison with oxidative burst. *Cancer Res* 53: 2676–2682, 1993.
24. **Lemasters JJ, Nieminen AL, Qian T, Trost LC, Elmore SP, Nishimura Y, Crowe RA, Cascio WE, Bradham CA, Brenner DA, and Herman B.** The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. *Biochim Biophys Acta* 1366: 177–196, 1998.
25. **Marzo I, Brenner C, Zamzami N, Jurgensmeier JM, Susin SA, Vieira HL, Prevost MC, Xie Z, Matsuyama S, Reed JC, and Kroemer G.** Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. *Science* 281: 2027–2031, 1998.
26. **Masini A, Ceccarelli D, Gallesi D, Giovannini F, and Trenti T.** Lipid hydroperoxide induced mitochondrial dysfunction following acute ethanol intoxication in rats. The critical role for mitochondrial reduced glutathione. *Biochem Pharmacol* 47: 217–224, 1994.
27. **Murphy KM, Streips UN, and Lock RB.** Bax membrane insertion during Fas(CD95)-induced apoptosis precedes cytochrome *c* release and is inhibited by Bcl-2. *Oncogene* 18: 5991–5999, 1999.
28. **Nanji AA.** Apoptosis and alcoholic liver disease. *Semin Liver Dis* 18: 187–190, 1998.
29. **Narita M, Shimizu S, Ito T, Chittenden T, Lutz RJ, Matsuda H, and Tsujimoto Y.** Bax interacts with the permeability transition pore to induce permeability transition and cytochrome *c* release in isolated mitochondria. *Proc Natl Acad Sci USA* 95: 14681–14686, 1998.
30. **Natori S, Rust C, Stadheim LM, Srinivasan A, Burgart LJ, and Gores GJ.** Hepatocyte apoptosis is a pathologic feature of human alcoholic hepatitis. *J Hepatol* 34: 248–253, 2001.
31. **Petit PX, Goubern M, Diolez P, Susin SA, Zamzami N, and Kroemer G.** Disruption of the outer mitochondrial membrane as a result of large

amplitude swelling: the impact of irreversible permeability transition. *FEBS Lett* 426: 111–116, 1998.
32. **Petronilli V, Miotto G, Canton M, Brini M, Colonna R, Bernardi P, and Di Lisa F.** Transient and long-lasting openings of the mitochondrial permeability transition pore can be monitored directly in intact cells by changes in mitochondrial calcein fluorescence. *Biophys J* 76: 725–734, 1999.
33. **Saito M, Korsmeyer SJ, and Schlesinger PH.** BAX-dependent transport of cytochrome *c* reconstituted in pure liposomes. *Nat Cell Biol* 2: 553–555, 2000.
34. **Shi Y.** Mechanisms of caspase activation and inhibition during apoptosis. *Mol Cell* 9: 459–470, 2002.
35. **Shimizu S, Ide T, Yanagida T, and Tsujimoto Y.** Electrophysiological study of a novel large pore formed by Bax and the voltage-dependent anion channel that is permeable to cytochrome c. *J Biol Chem* 275: 12321–12325, 2000.
36. **Shimizu S, Matsuoka Y, Shinohara Y, Yoneda Y, and Tsujimoto Y.** Essential role of voltage-dependent anion channel in various forms of apoptosis in mammalian cells. *J Cell Biol* 152: 237–250, 2001.
37. **Shimizu S, Narita M, and Tsujimoto Y.** Bcl-2 family proteins regulate the release of apoptogenic cytochrome *c* by the mitochondrial channel VDAC. *Nature* 399: 483–487, 1999.
38. **Susin SA, Zamzami N, and Kroemer G.** Mitochondria as regulators of apoptosis: doubt no more. *Biochim Biophys Acta* 1366: 151–165, 1998.
39. **Thornberry NA and Lazebnik Y** Caspases: enemies within. *Science* 281: 1312–1316, 1998.
40. **Wolter KG, Hsu YT, Smith CL, Nechushtan A, Xi XG, and Youle RJ.** Movement of Bax from the cytosol to mitochondria during apoptosis. *J Cell Biol* 139: 1281–1292, 1997.
41. **Yerushalmi B, Dahl R, Devereaux MW, Gumpricht E, and Sokol RJ.** Bile acid-induced rat hepatocyte apoptosis is inhibited by antioxidants and blockers of the mitochondrial permeability transition. *Hepatology* 33: 616–626, 2001.
42. **Zoratti M and Szabo I.** The mitochondrial permeability transition. *Biochim Biophys Acta* 1241: 139–176, 1995.

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Hepatocellular carcinoma in heavy drinkers with negative markers for viral hepatitis

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Abstract

Alcohol has been known to be associated with an increased risk of cancer. We investigated the characteristics of hepatocellular carcinoma (HCC) in heavy drinkers with negative serum markers for viral hepatitis (non-B, non-C) to determine whether ethanol enhances the development of HCC in Japanese patients with or without serum markers for viral hepatitis. Among the 432 HCC cases seen at our hospital between 1995 and 2000, 26 patients had negative serum markers (non-B, non-C) and were heavy drinkers. The mean patient age at the time of HCC diagnosis was 64.2 ± 7.6 years. The mean total ethanol intake was 1617 ± 796 kg. Most of the patients also had liver cirrhosis (LC), although the frequency was significantly higher in non-B, non-C, heavy drinkers HCC cases than in non-B, non-C, non-alcoholic HCC cases. Among the hepatitis C virus (HCV)-positive cases, the mean age at the time of HCC diagnosis was lower in heavy drinkers; this trend was not seen in HBV-positive cases. In HCC cases with heavy drinking, a high frequency of gastrointestinal (oropharynx, esophagus, stomach, colon and anal) cancers was seen. As for the aldehyde dehydrogenase-2 (ALDH2) genotype, the frequency of normal homozygotes was 87.5% in heavy drinkers with HCC and the frequency of heterozygotes was 12.5%; the frequency of heterozygotes was 58.3% in alcoholics with esophageal cancer. More than half of the non-B, non-C, heavy drinkers HCC cases had a normal range of serum alpha-fetoprotein (AFP) levels. These results indicate that heavy drinking enhances HCV-related hepatocarcinogenesis. Whether or not ethanol is directly involved in hepatocarcinogenesis remains controversial, but LC may progress HCC in heavy drinkers even if their serum markers for HBV (including tissue) or HCV are negative. Therefore, close observation, including radiographic examinations, is recommended for non-B, non-C, heavy drinkers with LC. In HCV-positive cases, abstinence or a reduction in daily ethanol intake is recommended.

Keywords: Alcohol; Aldehyde dehydrogenase-2 (ALDH2); Alcoholic liver disease (ALD); Hepatocellular carcinoma (HCC)

1. Introduction

Long-term heavy drinking is known to cause various health problems, such as alcoholic liver dysfunction. The frequency of alcoholic liver disease (ALD) is increasing in Japan, in association with an increase in alcoholic beverage consumption [1,2]. The relationship between alcohol intake and liver dysfunction is well recognized, but the relationship between alcohol intake and the risk of cancer remains unclear. Considerable epidemiological evidence showing that alcoholic beverages were associated with an increased risk of oral cavity, oropharyngolarynx, esophagus and liver cancers and indicating that alcohol was a carcinogen in humans was published by the International Agency for Research of Cancer (IARC) in 1988 [3]. Although a large number of reports on the relationship between alcohol and hepatocellular carcinoma (HCC) have been made, the exact relationship remains controversial.

Previously, HCC was thought to develop as a result of ALD; we now know that many cases are related to hepatitis C virus (HCV) infection. Since widespread serologic surveys of viral hepatitis have been performed, the relationship between alcohol and the hepatitis virus in the development of HCC can be investigated. Some reports have shown that

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HCC and/or liver cirrhosis (LC) developed more rapidly in patients with hepatitis C who were heavy drinkers; consequently, HCC and/or LC was diagnosed at a younger age in heavy drinkers with HCV [4,5]. Furthermore, in patients with HCV cirrhosis, the risk of HCC was larger in heavy drinkers than in either non-drinkers or moderate drinkers [6,7]. On the other hand, the effects of alcohol intake on HBs antigen-positive patients are still controversial. Some reports claim that a habitual high alcohol intake might increase the risk of developing HCC in HBs antigen-positive patients [8,9], while another report showed that alcohol intake was not an independent predictor in HBV cirrhosis [10].

Thus, alcohol may promote carcinogenesis in the liver and may be a risk factor for the progression of chronic viral hepatitis into cirrhosis, which may consequently increase the risk of developing HCC. Since some HCC cases with alcoholic liver fibrosis or cirrhosis and no serologic markers for viral hepatitis have been reported, alcohol seems to be an HCC risk factor that is independent of the hepatitis viral status. Cirrhotic liver caused by long-term heavy drinking may become a precursor to HCC. However, Lieber et al. [11] reported that some alcoholics with HCC did not have cirrhosis, suggesting that ethanol and/or its metabolites may be hepatocarcinogenetic in a manner that is independent of cirrhosis. Carcinogenesis induced developed by chemical agents involves two steps: initiation and promotion. Therefore, ethanol may modulate chemical agent-induced carcinogensis, acting as a tumor promoter. Little is known about the role of ethanol in carcinogenesis, especially in the liver. In the present study, we investigated the characteristics of HCC in heavy drinkers with negative serum markers for viral hepatitis (non-B, non-C) to determine whether ethanol plays a role in the development of HCC independently of the hepatitis virus or enhances hepatocarcinogenesis caused by the hepatitis virus. We also performed aldehyde dehydrogenase-2 (ALDH2) genotyping to determine the relationship between HCC and the ALDH2 genotype in heavy drinkers.

2. Materials and methods

2.1. Subjects with HCC

The subjects consisted of 432 patients with HCC who were consecutively admitted to the Department of Internal Medicine, Keio University Hospital between May 1995 and March 2000. HCC diagnoses were based on histology and/or radiological findings (i.e. abdominal ultrasonography, computed tomography, or angiography). Cirrhosis diagnoses were based on histology and/or laboratory data and the clinical course.

2.2. Definition of negative markers for viral hepatitis

Serum levels HBs Ag, anti-HBs antibodies, anti-HBc antibodies, HBe Ag, anti-HBe antibodies and anti-HCV were determined using commercial EIA kits (Dinabot, Tokyo). Serum HCV-RNA and HBV-DNA levels were measured using Amplicore HCV and the branched DNA probe method (SRL, Tokyo, Japan). All patients were negative for both HBs Ag and anti-HCV antibodies. Although all the markers were not measured in some patients, patients who were positive for even one marker were excluded. Subjects suspected of having autoimmune hepatitis or primary biliary cirrhosis were also excluded.

2.3. Definition of heavy drinkers

The definition of heavy drinkers was based on the Proposed Diagnostic Criteria for Alcoholic Liver Disease [1] which defines a heavy drinker as someone who drinks more than 125 g of alcohol a day continuously for more than 5 years. Information on the drinking profiles of the subjects was obtained by interviewing the patients and/or their families. HBV- and HCV-positive HCC cases were divided into four groups according to their daily ethanol intake: non-drinking group, less than 75 g/day, between 75 and 125 g/day, and more than 125 g/day group.

2.4. Detection of HBx gene in liver tissue

Liver specimens were obtained from six non-B, non-C, heavy drinkers who were HCC patients. HBx gene detection was performed according to method reported by Yotsuyanagi et al. [12]. Briefly, DNA was extracted twice with phenol/chloroform and once with chloroform and then precipitated with ethanol. PCR amplification was performed using a primer set within the X region (5′-CTGGATCCTGCGCGGGACGTCCTT-3′ sense; 5′-GTTCACGGTGGTCTCCAT-3′ antisense). The HBx gene was then detected by Southern blotting using X region-specific probes (5′-GATTCAGCGCCGACGGGGAC-3′).

2.5. Aldehyde dehydrogenase-2 (ALDH2) genotype

Among the 26 non-B, non-C, heavy drinkers HCC cases in our hospital, 14 ALDH2 genotyping was performed. And at the National Institution on Alcoholism (Kurihama National Hospital), where 10 non-B, non-C HCC cases in 2500 alcoholics from 1993 to 2000, 10 ALDH2 genotyping was performed. Overall 24 ALDH2 genotyping was performed. ALDH2 genotyping was performed on lymphocyte DNA samples using polymerase chain reaction/restriction fragment length polymorphism (PCR-RFLP) [13]. Briefly, 100–200 ng of genomic DNA was mixed with 5 pmol of each primer (5′-CAAATTACAGGGTCAACTGCT-3′ sense; 5′-CCACACTCACAGTTTTCTCTT-3′ antisense) to produce a total volume of 50 μl containing 50 μM concentration of each dNTP, 1.5 mM of $MgCl_2$, and 1 U of Taq DNA polymerase (Progema, Madison, WI). Thirty-five cycles of PCR (denaturation at 94 °C for 15 s, anneal-

ing at 58 °C for 1.5 min, and polymerization at 72 °C for 30 s) were performed in a Perkin-Elmer Cetus GeneAmp PCR System 9600. After purification, each PCR product was digested with MboII, electrophoresed on a 20% polyacrilamide gel, stained with ethidium bromide, and viewed.

2.6. Statistical analysis

Data were expressed as the mean ± S.D. or as a percentage. And one-way ANOVA and Scheffe's post hoc test or a χ^2-test were used for the statistical evaluations. Statistical significance was set at $P < 0.05$.

3. Results

Among the 432 cases of HCC examined in this study 296 cases (68.5%) were HCV-positive, 70 cases (16.2%) were HBV-positive, 27 cases (6.3%) were both HCV- and HBV-positive, 13 cases (3.0%) did not have serum markers for viral hepatitis and were not heavy drinkers (non-B, non-C, non-alcoholic), and 26 cases (6.0%) were heavy drinkers who did not have serum markers for viral hepatitis (non-B, non-C, heavy drinkers) (Fig. 1).

All of the non-B, non-C, heavy drinkers HCC cases were negative for HBs Ag and anti-HCV antibodies. Twenty-one of 26 were negative for anti-HBc antibodies. We did not examine the anti-HBc antibodies of five non-B, non-C, heavy drinkers HCC cases whose anti-HBs antibodies were negative.

Twenty-five of the 26 non-B, non-C, heavy drinking HCC cases had cirrhosis. Table 1 shows the clinical aspects of these patients compared with the HCV-positive HCC patients. Twenty-five of the 26 cases were male, and the mean age at the time of HCC diagnosis was 64.2 years. The average total ethanol dose was 1617 kg. All of the male cases had drunk more than 1000 kg of alcohol, but one female case had drunk only 900 kg. All cases showed a higher serum gamma-glutamyl transpeptidase (GGTP) activity than the normal range, with a mean value of 215 IU/l. More than half

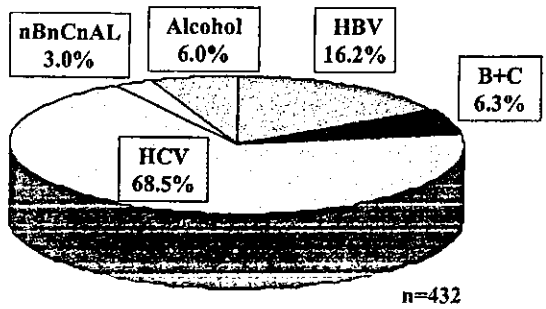

Fig. 1. Etiological trend of HCC. Cases of any HBV markers positive were described as HBV, both HBV and HCV markers positive as B + C, anti-HCV antibody positive as HCV, non-B, non-C, non-drinking as nBnCnAL and heavy drinkers with non-B, non-C as alcohol.

of the cases (64%) had a serum alpha-fetoprotein (AFP) levels that was within the normal range.

Seven cases (26.9%) had other cancers of the gastrointestinal tract. As for their smoking habits, 4% of the subjects smoked between 1 and 10 cigarettes a day, 20% smoked between 11 and 20 cigarettes a day, and 64% smoked more than 21 cigarettes a day. We also investigated the daily intake of alcohol in HCV-positive HCC cases. A higher daily alcohol intake was seen in most of the male population. The mean age at the time of HCC diagnosis was 66.3 years in the non-drinking group, 64.2 years in the less than 75 g/day group, 63.0 years in the 75–125 g/day group and significantly younger (55.7 years) in the more than 125 g/day group. Alcohol consumption did not affect the survival period from the day of HCC diagnosis. The mean total ethanol intake was 1190 kg in the group who drank 75–125 g/day and 1833 kg in the group who drank more than 125 g/day. Sixty-four percent of the non-B, non-C, heavy drinkers group had a normal AFP value, while normal AFP cases in the HCV-positive HCC patients accounted for only 26% in the non-drinking group, 31% in the 75–125 g/day group and 36% in the more than 125 g/day group. The prevalence of normal AFP cases was significantly higher in non-B, non-C, heavy drinkers group than in the HCV-positive, non-drinking group. The prevalence of gastrointestinal cancers was only 6.9% in the non-drinking group and 7.3% in the less than 75 g group, whereas it was 20.0% and 34.8% in the 75–125 g/day and the more than 125 g/day groups, respectively. In the non-drinking group, 72% of the patients did not smoke; however tobacco consumption increased in proportion to the daily alcohol intake. As the daily ethanol intake decreased, the percentage of female cases tended to increase. Although the data for the male cases is shown in parentheses to exclude the effects of gender, the age at the time of diagnosis, the prevalence of gastrointestinal cancer, and smoking habits among the male cases were similar to that observed overall.

Table 2 shows the mean age at the time of HCC diagnosis in the non-B, non-C, heavy drinkers group, the HBV-positive group, the HCV-positive, and the non-B non-C non-alcoholic group. Alcohol consumption did not affect the mean age at the time of HCC diagnosis in the HBV-positive patients. In the non-B, non-C, heavy drinkers group, significantly more HCC cases also had cirrhosis than in the non-B, non-C, non-drinking group. Among the HCV-positive cases, the frequency of cirrhosis showed a tendency to increase with daily alcohol intake in a dose-dependent fashion, but the difference was not significant.

We examined the tissue HBx of six non-B, non-C, heavy drinkers HCC cases (Table 3). They did not have HBx gene in their liver.

Fig. 2 shows the ALDH2 genotypes of HCC patients who were non-B, non-C, heavy drinkers compared to that of healthy cases and alcoholics with esophageal cancer from the Kurihama National Hospital. Twenty-one of the 24 non-B,

Table 1
Characteristics of non-B, non-C, heavy drinkers HCC and HCV-related HCC separated by ethanol intake

	Alcohol ($n = 26$)	HCV > 125 g/day ($n = 23$)	HCV > 75 g/day ($n = 35$)	HCV < 75 g/day ($n = 122$)	HCV only ($n = 116$)
Sex (male/female)	25/1	22/1	33/2	105/17	57/59
Age of diagnosis (years)	64.2 ± 7.7 (63.4 ± 7.1)	55.7 ± 7.0*,**,+ (56.4 ± 6.8*,+)	63.0 ± 5.9 (62.8 ± 6.0)	64.2 ± 6.7 (63.9 ± 6.7)	66.3 ± 7.9 (63.9 ± 8.5)
Total ethanol dose (kg)	1617 ± 796	1833 ± 387	1190 ± 307	–	0
GGTP (IU/l)	215 ± 198*	129 ± 97*	127 ± 119*	113 ± 116	78 ± 92
Normal AFP (%)	64*	36	31	32	26
Gastrointestinal cancer	26.9% (28.0%)	34.8%* (36.4%*)	20.0%* (25.5%*)	7.3% (7.6%)	6.9% (10.5%)
Cigarette					
0/day	12% (8%)	10% (9%)	31% (30%)	47% (44%)	72% (61%)
1–10/day	4% (4%)	0% (0%)	15% (16%)	12% (14%)	3% (6%)
11–20/day	20% (21%)	35% (32%)	37% (36%)	25% (25%)	9% (6%)
>21/day	64% (67%)	55% (59%)	16% (17%)	16% (17%)	16% (27%)

HCV-positive and drinking more than 125 g/day as HCV > 125 g, 75–125 g/day as HCV > 75 g, less than 75/day as HCV < 75 g, and no drinking as HCV only. Data in parentheses are the data of male eases. ANOVA: age of diagnosis, GGTP; χ^2-test:: % of normal AFP; gastrointestinal cancer.

+ $P < 0.01$ vs. Alcohol.

* $P < 0.01$ vs. HCV only.

** $P < 0.01$ vs. HCV > 75 g.

Table 2
Age of HCC diagnosis and % of gastrointestinal cancer and cirrhosis

	Age of diagnosed HCC (years)	Gastrointestinal cancer (%)	Cirrhosis (%)
Non-B non-C heavy drinkers ($n = 26$)	64.2 ± 7.7	26.9	96.5#
HCV non-alcohol ($n = 116$)	66.3 ± 7.9	6.9	87.1
<75 g/day ($n = 122$)	64.2 ± 6.7	7.9	87.7
>75 g/day ($n = 35$)	63.0 ± 5.9	20.0*	88.6
>125 g/day ($n = 23$)	55.7 ± 7.0*,**	34.8*	95.6
HBV non-alcohol ($n = 16$)	56.9 ± 9.2	12.5	68.8
<75 g/day ($n = 28$)	56.0 ± 9.7	10.7	60.7
>75 g/day ($n = 13$)	59.8 ± 7.8	23.1	76.9
>125 g/day ($n = 13$)	58.5 ± 6.3	23.1	69.2
Non-B non-C non-alcoholic ($n = 14$)	60.8 ± 15.4	7.7	35.7

ANOVA: age of diagnosis; χ^2-test: cirrhosis.
* $P < 0.01$ vs. HCV only.
** $P < 0.01$ vs. HCV > 75 g.
# $P < 0.01$ vs. non-B non-C non-alcoholic.

non-C, heavy drinkers HCC cases (87.5%) were ALDH 2 × 1/2 × 1 homozygotes and 3 (12.5%) were ALDH 2 × 1/2 × 2 heterozygotes. Among the entire Japanese population, ALDH 2 × 1/2 × 1 homozygotes account for 58% of the population, ALDH 2 × 1/2 × 2 heterozygotes account for 35%, and 2 × 2/2 × 2 homozygotes account for 7% [14]. The frequency of ALDH 2 × 1/2 × 2 heterozygotes in the non-B, non-C, heavy drinkers group was less than that of overall Japanese population, although the frequency of ALDH 2 × 1/2 × 2 heterozygotes was much higher among alcoholics with esophageal cancer.

4. Discussion

We studied 26 HCC patients, who were heavy drinkers and negative for viral hepatitis markers. The prevalence was 6% of the over all HCC cases. All of them were negative for HBs Ag and anti-HCV antibodies. We excluded cases that had any other serum HBV markers we examined although their HBs Ag was negative. Yotsuyanagi et al. [12] reported that some of the non-B non-C cases had HBV-DNA in cancerous and adjacent non-cancerous liver tissue using RT-PCR and southern blotting method. Thus, HBV-DNA especially the HBx gene may demonstrate hepatocarcinogenesis even if in the cases who were negative for serum HBs Ag. We could not examine all of them. However, all the cases of non-B, non-C, heavy drinkers HCC examined HBx gene in the liver tissue did not have HBx gene. This finding raises a possibility that HCC can develop independently of occult HBV in non-B, non-C, heavy drinkers.

Table 3
HBx gene in the liver tissue of non-B, non-C, heavy drinkers HCC

Number	Sex	Ethanol (kg)	Serum marker			HCC tissue HBx
			HBsAg	HBcAb	HCVAb	
1	M	2000	–	–	–	–
2	M	2000	–	ND	–	–
3	M	1400	–	ND	–	–
4	M	1100	–	–	–	–
5	F	800	–	–	–	–
6	M	1020	–	–	–	–

(–) negative; ND: not done.

Although Lieber et al. [11] reported that cirrhosis was not necessary for the development of HCC in heavy drinkers, 25 of the 26 non-B, non-C, heavy drinkers with HCC also had cirrhosis. Further, the prevalence of cirrhosis was significantly higher in the non-B, non-C, heavy drinkers HCC group than in the non-B, non-C, non-drinking group. These results indicate that alcoholic cirrhosis might progress to HCC in non-B, non-C, heavy drinkers. Non-alcoholic steatohepatitis (NASH) has a histology that closely resembles that of ALD. Recently, the numbers of reports on NASH cases with HCC has been increasing [15,16]. In NASH cases, HCC may develop after a series of clinical events, i.e., steatosis, fibrosis and cirrhosis. The same mechanism may occur in ALD, and cirrhotic liver appears to play an important role in the development of HCC in non-B, non-C cases both in NASH and ALD.

An interesting finding in this study is that more than half of the non-B, non-C, heavy drinkers with HCC had a normal serum AFP level. A few cases had high serum AFP levels, but these cases had huge and/or multiple HCCs. The percentage of HCC patients with a normal AFP value was much higher in the non-B, non-C, heavy drinkers group than in the HCV-positive, non-drinking group. These results suggest that radiographic examinations, such as ultrasonography, computed tomography, etc., may be important in cases with cirrhosis and of heavy drinking, even if the serum tumor marker levels are normal.

Among HCV-positive cases HCC was diagnosed at a younger age in the group that drank more than 125 g/day of alcohol. Some previous papers have demonstrated that high ethanol consumption is a risk factor for HCC in HCV-positive patients [5,6]. Our results support this findings

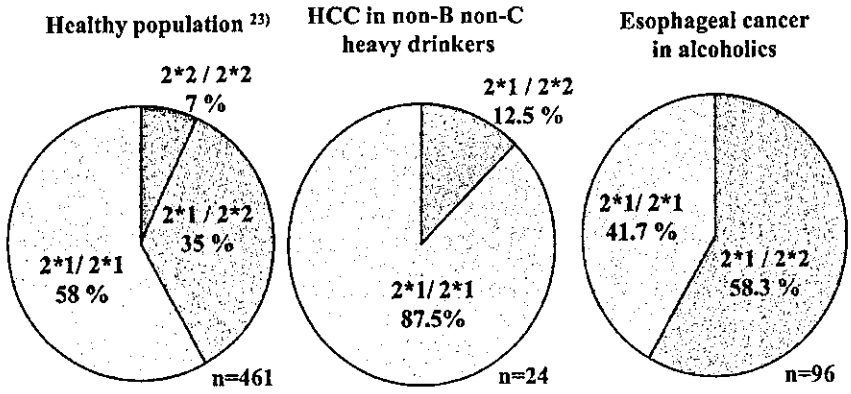

Fig. 2. Aldehyde dehydrogenase-2 genotype of HCC cases in non-B, non-C, heavy drinkers compared to that of healthy population and esophageal cancer in alcoholics.

and suggest that abstinence or a reduced daily ethanol intake should be recommended in HCV-positive cases. Although further studies on HBV-related HCC cases are needed, our results show that large amounts of alcohol consumption did not become an HCC risk factor in HBV-positive cases. Since HBV-related HCC was diagnosed at a younger age than HCV-related HCC, HBV-induced hepatocarcinogenesis may be more severe than HCV-induced hepatocarcinogenesis in heavy drinkers. In non-B, non-C, heavy drinkers with HCC, one of the 26 patients was a woman whose total ethanol dose was less than 1000 kg (900 kg). ALD is more severe in women, even if they have with a lower alcohol intake than men, but little is known regarding gender differences in hepatocarcinogenesis. Although further investigation of female cases is required, our results raise the possibility that women may develop HCC is association with heavy drinking at lower alcohol intakes.

One important characteristic of the non-B, non-C, heavy drinkers with HCC was the high frequency of gastrointestinal (oropharynx, esophagus, stomach, colon, anal) cancers. Seven of the 26 non-B, non-C, heavy drinking HCC cases (26.9%) had gastrointestinal cancers. In the HCV-related HCC cases as well, the frequency of the gastrointestinal cancers increased in accordance with the level of alcohol consumption. These results suggest that large amounts of ethanol consumption may be a risk factor for cancer of the liver and gastrointestinal tract. Since the consumption of cigarettes also increased in parallel with increasing ethanol intake, the effect of smoking may also be involved in the development of gastrointestinal cancers.

The distribution of ALDH2 genotypes differed between heavy drinkers with HCC and esophageal cancer. A higher frequency of ALDH 2 × 1/2 × 1 homozygotes (87.5%) was observed in patients with HCC than in the normal population, while the frequency of ALDH 2 × 1/2 × 2 heterozygotes (58.3%) was higher in patients with esophageal cancers. A mutant allele in the ALDH2 gene, seen in 42% of the Japanese healthy population, markedly diminishes enzyme activity [17]. We have reported that the presence of the ALDH 2 × 2 allele significantly increases the risk of developing these cancers [18]. However, the frequency of ALDH 2 × 1/2 × 2 heterozygotes is lower among HCC patients. These results suggest that the ALDH2 genotype does not affect the development of HCC in heavy drinkers.

Long-term ethanol consumption is known to induce cytochrome P450 2E1 (CYP2E1) [19]. CYP2E1 is an enzyme that metabolizes *N*-nitrosodimethylamine (NMDA), which is present in small quantities in the daily diet, from a procarcinogen to a carcinogen [20]. This metabolic event may enhance hepatocarcinogenesis. Tsutsumi et al. [21] reported that preneoplastic changes in the liver were found only in rats treated with both ethanol and NMDA. These changes were not observed in rats treated with either ethanol or NMDA. Vitamin A levels are known to decrease in the liver after chronic ethanol consumption [22]. This reduction in Vitamin A may enhance hepatic microsomal retinal degradation and contribute to cell differentiation. Despite the above evidence, whether or not ethanol acts as a carcinogen in the liver remains uncertain. Ethanol is unlikely to be an initiator of hepatocarcinogenesis, but it may be a promoter.

Since we did not investigate the presence of hepatitis G virus (HGV) and TT virus (TTV), the possible effects of these viruses on hepatocarcinogenesis cannot be excluded. However, Yotsuyanagi et al. [23] reported that HGV had no association with non-B, non-C HCC. Furthermore, Yamamoto et al. [24] reported that the possibility of TTV-induced hepatocarcinogenesis is low. Although the roles of HGV and TTV in carcinogenesis are unknown, a few reports have demonstrated a relationship between GBV or TTV and hepatocarcinogenesis.

Since ethanol intake in Japan is increasing, the frequency and number of ALD and alcoholic cirrhosis cases are expected to increase. As therapies for viral hepatitis are improved, the frequency of non-B, non-C, alcoholic cases with cirrhosis and HCC may increase. Therefore, in patients with liver cirrhosis whose total ethanol intake is more than 1000 kg, radiographic examination may be necessary to rule-out the possibility of HCC. Moreover, appropriate examinations to detect HCC in heavy drinkers and the

development of specific treatments for HCC will become more important in Japan in the near future.

References

[1] Takada A. A Japanese Study Group for Alcoholic Liver Disease. A new diagnostic criteria of alcoholic liver disease. Acta Hepatologica Japonica 1993;34:888–96 [in Japanese].

[2] Health and Welfare Statistics Association. J Health Welf Stat 1996:102 [in Japanese].

[3] International Agency for Research of Cancer: Alcohol drinking. In: IARC Monographs on the Evaluation of the Carcinogenic Risks to Humans, vol. 44. IARC, Lyon; 1988.

[4] Tsutusmi M, Ishizakki M, Takada A. Relative risk for the development of hepatocellular carcinoma in alcoholic patients with cirrhosis: a multiple logistic-regression coefficient analysis. Alcohol Clin Exp Res 1996;20:758–62.

[5] Tsukuma H, Hiyama T, Yamazaki H, et al. Risk factors for hepatocellular carcinoma among patients with chronic liver disease. N Engl J Med 1993;328:1797–801.

[6] Matsuda Y, Amuro Y, Nakano T, et al. Relation between markers for viral hepatitis and clinical features of Japanese patients with hepatocellular carcinoma: possible role of alcohol in promoting carcinogenesis. Hepatogastroenterology 1995;42:151–4.

[7] Wiley TE, McCarthy M, Breidi L, McCarthy M, Layden TJ. Impact of alcohol on the histological and clinical progression of hepatitis C infection. Hepatology 1998;28:805–9.

[8] Ohnishi K, Iida S, Okuda K, et al. The effect of chronic habitual alcohol intake on the development of liver cirrhosis and hepatocellular carcinoma. Cancer 1982;49:672–7.

[9] Oshima A, Tsukuma H, Tanaka M, et al. Follow-up study of HBsAg-positive blood donors with special reference to effect of drinking and smoking on development of liver cancer. Int J Cancer 1984;34:775–9.

[10] Ikeda K, Saitoh S, Koida I, Arase Y, Tsubota A, Chayama K. A multivariable analysis of risk factors for hepatocellular carcinogenesis: a prospective observation of 795 patients with viral and alcoholic cirrhosis. Hepatology 1993;18:47–53.

[11] Lieber CS, Garro A, Leo MA, Mak KM, Worner T. Alcohol and cancer. Hepatology 1986;6:1005–19.

[12] Yotsuyanagi H, Shintani Y, Koike K, et al. Virologic analysis of non-B, non-C hepatocellular carcinoma in Japan: frequent involvement of hepatitis B virus. J Infect Dis 2000;181:1920–8.

[13] Harada S, Zhang S. New strategy for detection of ALDH2 mutant. Alcohol 1993;28:11–3.

[14] Okamoto K, Murawaki Y, Yuasa I, Kawasaki H. Effect of ALDH2 polymorphism on drinking behavior and alcoholic liver disease in normal Japanese males. Alcohol Biomed Res 1999;19:74–7 [in Japanese].

[15] Bugianesi E, Leone N, Rizzeto M, et al. Expanding the natural history of nonalcoholic steatohepatitis: from cryptogenesis cirrhosis to hepatocellular carcinoma. Gastroenterology 2002;123:134–40.

[16] Shimada M, Hashimoto E, Taniai M, et al. Hepatocellular carcinoma in patients with non-alcoholic steatohepatitis. J Hepatol 2002;37:154–60.

[17] Harada S, Agarwel DP, Goedde HW. Aldehyde dehydrogenase deficiency as a cause of if facial flashing reaction to alcohol in Japanese. Lancet 1981;2:982.

[18] Yokoyama A, Ohmori T, Ishii H, et al. Successful screening for early esophageal cancer using endoscopy and mucosa iodine staining. Cancer 1996;76:919–21.

[19] Takahashi T, Lasker JM, Rosman AS, Liber CS. Induction of cytochrome P-450IIE1 in the human liver by ethanol is caused by a corresponding increase in encoding messenger RNA. Hepatology 1993;17:236–45.

[20] Miyakawa H, Sato C. Alcohol and hepatocellular carcinoma. Clin Gastroenterol 1995;10:1279–86 [in Japanese].

[21] Tsutsumi M, Matsuda Y, Takada A. Role of ethanol-inducible cytochrome P450 2E1 in the development of hepatocellular carcinoma by the chemical carcinogen, *N*-nitrosodimethylamine. Hepatology 1993;18:1483–9.

[22] Leo MA, Lieber CS. Hepatic Vitamin A depletion in alcoholic liver injury. N Engl J Med 1982;307:597–601.

[23] Yotsuyanagi H, Moriya K, Shintani Y, et al. Hepatitis G virus in the liver and serum of patient with hepatocellular carcinoma. Hepatol Res 1997;8:21–7.

[24] Yamamoto T, Kajino K, Hino D, et al. Hepatocellular carcinomas infected with the novel TT DNA virus lack integration. Biochem Biophys Res Commun 1998;25:339–43.

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Review

Recent understanding of immunological aspects in alcoholic hepatitis

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Abstract

Alcoholic hepatitis is a rate-limiting step in the development of alcoholic liver disease into liver cirrhosis, and approximately half of the heavy drinkers with alcoholic hepatitis develop liver cirrhosis within 5 years. Immunologic mechanisms may be involved in the individual differences in the clinical course of this disease. Endotoxin from the intestine seems to play an important role in neutrophil infiltration of the liver, which induces, and at the same time is induced; by cytokines and chemokines. Kupffer cells and monocytes also have a key role in activating other cell types and producing several cytokines, chemokines, and free radicals. Both cytokines and chemokines up-regulate expression of various adhesion molecules, and adhesion molecules accelerate a cell-to-cell contact that stimulates cytotoxic lymphocytes to cause hepatocyte death. Self-antigens and adducts formed as a result of the degenerative effect of ethanol or aldehyde are targets of antibody-dependent cell-mediated cytotoxicity. Oxygen radicals, NF-kB, and AP-1 are key intracellular factors mediating hepatocyte death in alcoholic hepatitis. Viral infections and alcoholic hepatitis exacerbate each other. Integration of both human investigations and accumulated information from various animal models will gradually clarify the immunological mechanism of alcoholic hepatitis in future.

Keywords: Alcoholic hepatitis; Immunological mechanism; Kupffer cell; Neutrophil; Lymphocyte; Oxygen radicals

Contents

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1. Introduction

Alcoholic liver disease (ALD) includes fatty liver, liver fibrosis, alcoholic (steato)hepatitis, and liver cirrhosis. In the United States, about half of the causes of death in liver cirrhosis is alcohol abuse or alcoholism, a situation which is quite different from Japan, where 80–90% of cause of cirrhosis is chronic viral infection. Although, many ALD patients were found to have chronic hepatitis C after discovery of hepatitis C virus (HCV), alcohol intake is an exacerbating factor of chronic hepatitis C [1], and HCV infection is an exacerbating factor of ALD [2]. Eradication of HCV by interferon therapy may result in an increase in the proportion of ALD among chronic liver diseases in Japan in the future.

The rate-limiting step in progression of ALD to liver cirrhosis is the development of alcoholic steatohepatitis, which occurs in 20–30% of heavy drinkers, and steatohepatitis may develop to liver cirrhosis if left untreated. While cytochrome P4502E1 (CYP2E1) is a major microsomal source of oxidative stress and is a candidate for the pathogenesis of alcoholic steatohepatitis [3], approximately 40–50% of the cases of alcoholic steatohepatitis in heavy drinkers have been reported to progress to liver cirrhosis within 5 years. Thus, liver cirrhosis does not occur in all heavy drinkers, and its occurrence is not correlated with the level of alcohol consumption. These observations led to the hypothesis that immunological mechanisms play a role in the development of ALD in addition to individual differences in polymorphisms of CYP2E1 or its expression levels [4], although the precise immunologic mechanisms have not been established.

Animal models are major research tools for understanding the mechanisms of ALD. The initial two reports of animal model were in baboons given a 50% calories alcohol diet [5], and in small animals, rats fed the Lieber–DeCarli diet (36% calories) [6]. Tsukamoto et al. [7] reported continuous intragastric feeding of alcohol to rats and demonstrated that Kupffer cell activation by elevated levels of sinusoidal endotoxin due to increased intestinal permeability to endotoxin caused by alcohol administration was an important event in ALD. Kupffer cells cause oxidative stress following activation of nuclear factor-kappa B (NF-kB), up-regulation of inflammatory cytokines and adhesion molecules, and, finally, inflammatory cell invasion. In rats, these changes are followed by fatty change, patchy necrosis, mild inflammation, and perivenular fibrosis; however, there have been no animal models that are histologically compatible with human ALD, nor have there been any adequate models of viral hepatitis, making it difficult to clarify the pathophysiological mechanisms of ALD.

Immunological mechanisms and immunological abnormalities in ALD have been assessed in terms of both their humoral and cellular aspects. Alcoholic drinking results in two controversial effects on the immunological system. One is that heavy drinking results in a decrease in immunological activity [8], and the other is that an alcohol intake strongly stimulates lymphocytes, leading to inflammation in the liver and a decrease in various immunological markers [9]. There have been many reports about immunological aspects of ALD, but they have not been consistent, because each report has been a reflection of the immunological experimental procedures available when the investigation was performed. For example, various pathogeneses, such as an antibody against Mallory body [10,11], an antibody against lipopolysaccharide [12], an antibody against self-antigen or alcoholic adducts [13–17], cytotoxic T cells [18–21], decrease in cellular immunity [22–24], IgA [25–27], phagocytic activity [28], and cytokines [29–31], have been considered as causes for alcoholic hepatitis in a long research history of alcoholic liver diseases [32]. We review the immunological aspects of alcoholic hepatitis in this article.

2. Role of Kupffer cells in alcoholic hepatitis

Chronic alcohol administration increases intestinal mucosal permeability and the serum lipopolysaccharide (LPS) concentration. LPS binds to LPS-binding protein (LBP), forming an LPS–LBP complex, and this complex binds to the CD14 receptor on the cell membrane of Kupffer cells (KCs). The LSP–CD14 complex reacts with toll-like receptor 4 (Tlr4), which is a membrane-penetration-type receptor, and this stimulates signal transduction and activates nuclear factor-kappa B (NF-kB). Another pathway that activates NF-kB is the oxidative stress caused by LPS itself. NF-kB up-regulates proinflammatory cytokines, tumor necrosis factor (TNF)-α, and cyclooxygenase (Cox)-2, and they induce intrahepatic inflammation. There have been many reports of animal models and human investigations supporting the above scenario as described below.

2.1. Animal models

CD14 and LBP mRNA levels have been demonstrated to correlate well with the extent of liver damage in the Tsukamoto–French model [32]. CD14 has been found to be expressed in KCs and LBP to be expressed in hepatocytes, and alcoholic liver damage has not been induced in CD14-deficient mice or Tlr4-mutant mice [33,34]. NF-kB activation in hepatocytes was demonstrated in the Tsukamoto model [35], but no TNF-α or NF-kB up-regulation or liver damage was observed after ethanol administration to $p47^{Phos}$ knockout (k/o) mice [36]. The $p47^{Phos}$ is a central subunit of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. The findings in this k/o mouse model suggest a scenario, in which the deficiency of NADPH oxidase in KCs cannot induce free-radicals after ethanol administration, and the free-radicals up-regulate NF-kB and TNF-α which then induce liver damage. Intragastric feeding of corn oil and fish oil up-regulated expression of Cox-2 and TNF-α mainly in KCs, that induced necroinflammation in the liver [37]. A significant

role of Cox-2 in hepatic inflammation was also demonstrated in the Cox-2 k/o mice having TNF-α plus galactosamine administration [38].

2.2. Human investigation

Significantly higher levels of TNF-α production has been demonstrated by the monocytes of ALD patients than by the monocytes of healthy controls. The levels of TNF-α, interleukin (IL)-1, and IL-6 in cholangio-endothelial cells and KCs have been found to be significantly higher in ALD patients than in healthy controls [39]. Moreover, monocyte chemoattractant protein-1 and IL-8 are expressed in KCs and may facilitate invasion of the liver by other inflammatory cells.

3. Role of neutrophil invasion

One of the histological characteristics of ALD is neutrophil invasion of the liver. Major neutrophil chemoattractants are CXC chemokines (IL-8, cytokine-induced neutrophil chemoattractant (CINC), macrophage inflammatory protein (MIP)-2, KC, inducible protein (IP)-10, ENA) and RANTES (regulated upon activation, normal T cell expressed and secreted). These chemokines are produced by many different kinds of cells in the liver, including hepatocytes and KCs. On the other hand, several adhesion molecules, such as selectin, which is expressed on vascular endothelium in neutrophil rolling phase, β2 integrin (CD11b, CD18), which is expressed on neutrophils after the rolling phase, and intercellular adhesion molecule (ICAM)-1, which is then expressed on both endothelium and hepatocytes.

TNF-α and chemokine production is increased in both humans with alcoholic hepatitis and animal models [40–43]. Hirano et al. [44] demonstrated that chemokines such as RANTES, are up-regulated by stimulation with TNF-α. Oxidative stress up-regulates redox-reactive transcription factors, such as NF-kB and activator protein (AP)-1, and induces secretion of various cytokines and chemokines [45]. CXC chemokines seem to be produced by KCs, because inactivation of KCs results in attenuation of CXC chemokines and a decrease in liver damage [46]. TNF-α and IL-1 then up-regulate adhesion molecules of neutrophils, hepatocytes, and other cells, and that induces cell-to-cell interaction. The cell-to-cell interactions involve neutrophil-mediated hepatocyte damage [47] or sinusoidal endothelial damage [48,49]. The chronic neutrophil invasion of the liver observed in ALD requires prolonged chemokine production in the liver, because the half-life of neutrophils is short. The cytotoxic effect of neutrophils has been demonstrated in the ischemia–reperfusion model, the endotoxin model, the warm-shock model, the cold-shock model, and a drug toxicity model. Hepatocyte apoptosis also stimulates neutrophil invasion, which then expands inflammation by a positive feedback mechanism [50,51].

Expression of E-cadherin and vascular cell adhesion molecule (VCAM)-1 on the endothelium, in addition to constitutive expression of ICAM-1, is necessary for neutrophil-invasion of the liver parenchyma [52]. Circulating neutrophils are always activated in patients with ALD [53], and neutrophil-activation leads to production of reactive oxygen species (ROS) and Mac-1, followed by increased production of TNF-α and IL-8, leading to hepatocyte toxicity and apoptosis [54]. Serum IL-8 levels have been shown to be correlated with neutrophil-invasion levels [43], which supports the scenario described above.

There is no neutrophil invasion in either the Lieber–DeCarli model; or the Tsukamoto–French model, however, LPS administration in these models was followed by increased expression of CXC chemokines and adhesion molecules and neutrophil invasion of the liver [55–57]. This phenomenon suggests that the most important factor in the establishment of the neutrophil-invasion in the liver seen in ALD is the supply of endotoxin from the intestine.

4. Role of lymphocyte invasion

Circulating lymphocytes in ALD patients may be trapped by the sinusoidal endothelium by adhesion with VCAM-1, ICAM-1, 2, or vascular adhesion protein (VAP)-1 [58], and then they invade into hepatic parenchyma by several CXC chemokines, such as monokine induced by gamma-interferon (KIG), interferon-inducinble T cell alpha chemoattranctant (ITAC) and IL-10, which are up-regulated in hepatitis [40,42,59,60]. The CD31 molecule is thought to be responsible for invasion through inter-endothelial tight junctions [61].

Necroapoptosis in alcoholic hepatitis is thought to be caused by cytokines, such as TNF-α, and free-radicals, such as nitric oxide (NO). The precise role of lymphocytes in ALD, however, is still a matter of controversy. Chedid et al. [62] and Sakai et al. [21] showed increased numbers of intrahepatic CD8+ cells and CD44+ cells, and decreased numbers of B cells and natural killer (NK) cells. NK activity is decreased in animal models [63], but the numbers and activity of CD3–CD56+ cells (compatible with NK cells) in the peripheral blood of ALD patients are increased, while their intrahepatic numbers are decreased [62], in contrast to viral hepatitis. Summarizing the findings in many reports, TNF-α and IL-6 are up-regulated in alcoholic hepatitis patients but IFN-γ is downregulated. T cells that produce TNF-α and IFN-γ are classified as CD57+ cells and are reactive to T helper (Th)1-type cytokines [9], but there are individual differences in the production of these cytokines [8], probably because of promoter polymorphisms [64,65]. It has also been reported that T cells are activated in chronic drinkers who drink more than 80 g/day ethanol, and that the activation persists after they stop drinking [8]. Production of these cytokines has also been demonstrated in animal models.