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# Some Findings on Apoptosis in Hepatocytes

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<http://dx.doi.org/10.5772/51015>

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## 1. Introduction

For development of novel strategy for the treatment of hepatic diseases, in which changes in apoptosis are targeted, it is most important to understand the molecular mechanisms by which apoptotic process is regulated. Rapid and efficient removal of unwanted cells such as aged, damaged, genetically mutated, or virally-infected ones is indispensable in the maintenance of the health of the liver. Means for removal of such cells are provided by nature as apoptosis, a well-controlled and programmed cell death process, which is especially important in the liver where cells are exposed to various toxins and viruses [1]. In a healthy adult body, the number of cells removed by apoptotic process in a certain period of time is comparable to the number of cells that proliferate by mitosis. Proper homeostasis of organs is thus maintained. However, under certain pathophysiological conditions, the balance between the growth and the loss of the cells is often upset, due to the onset of some liver diseases resulting in the loss of tissue homeostasis. Insufficient apoptosis will promote the growth of hepatocarcinoma or biliary carcinoma by failing the removal of cells growing uncontrollably in genetically mutated cells or in chronic or persistent inflammations [2-5]. Persistent stimulation causing apoptosis may be a factor to promote high rate regeneration of cancer cells in the tissue, elevating the risk of errors in mitosis. In contrast, excessive and/or persistent apoptosis may lead to acute damages such as fulminant hepatitis or reperfusion injury [6, 7], or chronic persistent damages such as alcoholic liver diseases, cholestatic liver disease, or viral hepatitis [8-11]. Inhibition of apoptosis in the liver injury or selective killing of malignant cells in tumors will provide strategies for treatment of hepatic diseases. In fact, new drug development is ongoing targeting apoptosis through the understanding of molecular process and pathophysiological role of apoptosis, and such substances are now tested in clinical trial or used as new options for certain human diseases. In this review, we focus the subject on the role of apoptosis in cholestatic liver diseases or alcoholic liver injury in which we carried out some investigations [12-15].

## 2. Cholestasis and hydrophilic or hydrophobic bile acids

It has been demonstrated that hydrophobic bile acids damage cellular functions by affecting intracellular organelle and signaling system at the concentrations 100-500  $\mu\text{M}$ , which are lower than those at which they show cytotoxic or detergent actions. Combettes and his co-workers reported that lithocholic acid (LCA) and taurolithocholic acid (TLCA) induce release of calcium ion ( $\text{Ca}^{2+}$ ) from the endoplasmic reticulum (ER) and the increased level of  $\text{Ca}^{2+}$  within the cell mediates cytotoxicity due to these hydrophobic bile acids [16]. Combettes and his colleagues speculated that this increase in intracellular  $\text{Ca}^{2+}$  levels occurs because LCA activates the inositol (1,4,5)-triphosphate ( $\text{IP}_3$ ) receptor, independent from  $\text{IP}_3$  itself, resulting in the release of  $\text{Ca}^{2+}$  from the intracellular organelle, ER [17]. On the other hand, there is a report that the increase in intracellular  $\text{Ca}^{2+}$  level induced by hydrophobic bile acid depends on extracellular  $\text{Ca}^{2+}$  level [18]. Spivey and his colleagues reported that at 250  $\mu\text{M}$ , glycochenodeoxycholic acid (GCDCA) induces the impairment of mitochondrial function and cellular ATP depletion, followed by a sustained rise in cytosolic  $\text{Ca}^{2+}$  resulting from an influx of extracellular  $\text{Ca}^{2+}$  leading to the death of hepatocytes, and that this cytotoxicity decreases in the order of GCDCA>CDCA>tauro-CDCA [19].

GCDCA is also reported to enhance the mitochondrial membrane permeability and induce cytotoxicity, while ursodeoxycholic acid (UDCA) shows suppression of GCDCA-induced enhancement of mitochondrial membrane permeability and cytotoxicity [20, 21]. Bile acid-induced enhancement of mitochondrial membrane permeability was also shown in the *in vivo* study of cholestasis [22]. Considering the data that increase in reactive oxygen species (ROS) generation in hepatocyte and generation of  $\text{H}_2\text{O}_2$  stimulated by tauro-CDCA (TCDCA) in mitochondria preceded TCDCA-induced hepatocyte necrosis, it was speculated that generation of ROS by hydrophobic bile acid constitutes one of the causative factors of hepatic injury in cholestasis [23].

In cholestatic liver disease, loss of hepatocytes or appearance of apoptotic body in hepatocytes was morphologically observed, and involvement of apoptosis was suggested in the hepatocyte injury in cholestasis [24]. Patel and his colleagues reported for the first time that low concentration of glycodeoxycholic acid induced apoptosis in hepatocytes, and pointed out that bile acids may induce necrosis at higher concentrations and apoptosis at lower concentrations in hepatocytes [25, 26]. Furthermore, Sokol and his colleagues reported that hydrophobic bile acid induced lipid peroxidation and mitochondrial dysfunction via enhancement of mitochondrial membrane permeability [27], and we ourselves also reported mitochondria-mediated time- and concentration-dependent apoptotic cell death and endoplasmic reticulum (ER) stress-mediated apoptosis of hepatocytes induced by GCDCA [28, 29].

Ursodeoxycholic acid (UDCA), a hydrophilic bile acid, has been widely used as a therapeutic agent for primary biliary cirrhosis (PBC) or cholestasis [30]. Suggested mechanism of action of UDCA includes promotion of bile secretion, detoxification metabolism in the liver, and antioxidant stress response, but its molecular mechanism is still

not clarified in detail. In vivo studies showed that UDCA is protecting hepatocytes from hydrophobic bile acid-induced apoptosis [31]. Furthermore, tauro-conjugated form of UDCA, tauro-UDCA (TUDCA), was shown to protect hepatocytes in ischemia-reperfusion injury in rats [32], and ethanol-fed rats [33]. When toxic bile acid is given to rats, apoptosis is induced in the liver, but when UDCA is given in combination, apoptosis is suppressed by inhibition of translocation of pro-apoptotic protein Bax from cytosol to mitochondria and ROS generation. This suppression is observed in cells other than hepatocytes, and UDCA was shown to act on classic mitochondrial-mediated pathway in different types of cells [34]. Tauroursodeoxycholic acid (TUDCA), a substance in which UDCA is conjugated with taurine, was shown to play an important role in various disorders including some liver diseases, type-II diabetes and metabolic syndrome [35, 36] possibly by its actions shown in isolated mitochondria to stabilize mitochondrial membrane directly through affecting channel formation by Bax [37], and bringing changes in ER stress-mediated pathways by decreasing caspase-12 activity and decrease in  $Ca^{2+}$  releases.

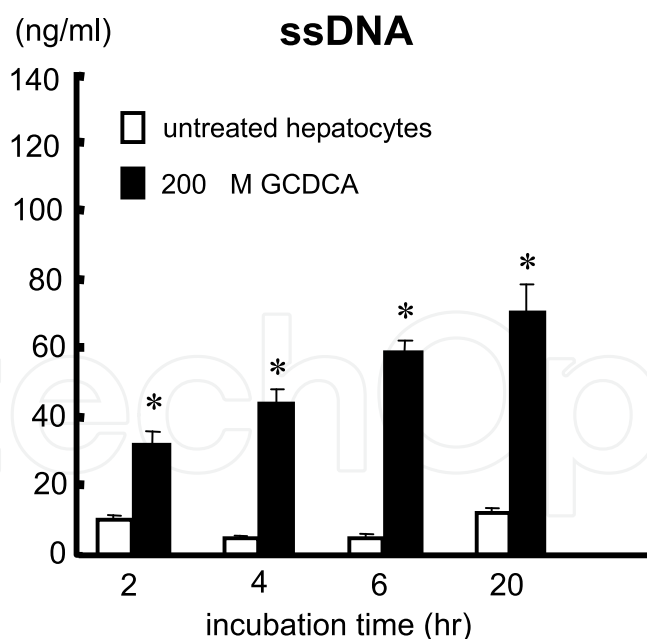
However, in a clinical trial for reevaluation of effectiveness in PBC patients, the effectiveness of UDCA was not acknowledged [38]. In rat isolated primary cultured hepatocytes, UDCA, given in combination with hydrophobic bile acids, was shown to be cytotoxic [39] and activates the pro-apoptotic pathway in some condition [40].

### 3. Apoptosis induced by hydrophobic bile acids in rat hepatocytes

Bile acids are synthesized from cholesterol in the liver, and act as surfactants that help digestion and absorption of lipids, and lipid-soluble vitamins. Major bile acids found in human bile are cholic and chenodeoxycholic acids, and they are secreted into bile as conjugates with taurine or glycine, via amide-bond. Most of bile acids secreted into the duodenum are reabsorbed by active transport in the terminal ileum, and returned to the liver. Bile acids are not so potently toxic as to injure hepatocytes in healthy subjects, but if the bile acid levels in the liver are too high or the ratio of hydrophobic bile acids to hydrophilic bile acids increases, as in the cases when there is some abnormality in bile acid synthesis, they induce apoptosis or necrosis [41]. The potency of their hepatotoxicity is, in the decreasing order, LCA > deoxycholic acid (DCA) > CDCA > CA > UDCA > dehydrocholic acid. The total bile acid in the liver tissue in normal subjects is not more than 10  $\mu$ M when determined as serum bile acid level, but in patients with cholestasis, CDCA level in the liver tissue increases to about 20 times higher than in the normal case, and the serum bile acid level elevated to 10 to 30 times (100-300  $\mu$ M) higher than normal, of which hydrophobic bile acid accounted for about 50-60 % [42, 19]. Cholestatic liver diseases are associated with bile duct obstruction by the formation of biliary stones, genetic defects, hepatotoxicity, hepatobiliary tumors [43]. Acute and chronic cholestasis induces hepatocellular injury, biliary dilatation, hepatic fibrosis, cirrhosis, and ultimately hepatic failure [44]. Decrease in bile flow or total obstruction of bile duct upon cholestasis is induced by the stasis of metabolized products such as cholesterol and bile acids in the liver which are normally eliminated into bile. Especially some hydrophobic bile acids induce cytotoxicity in

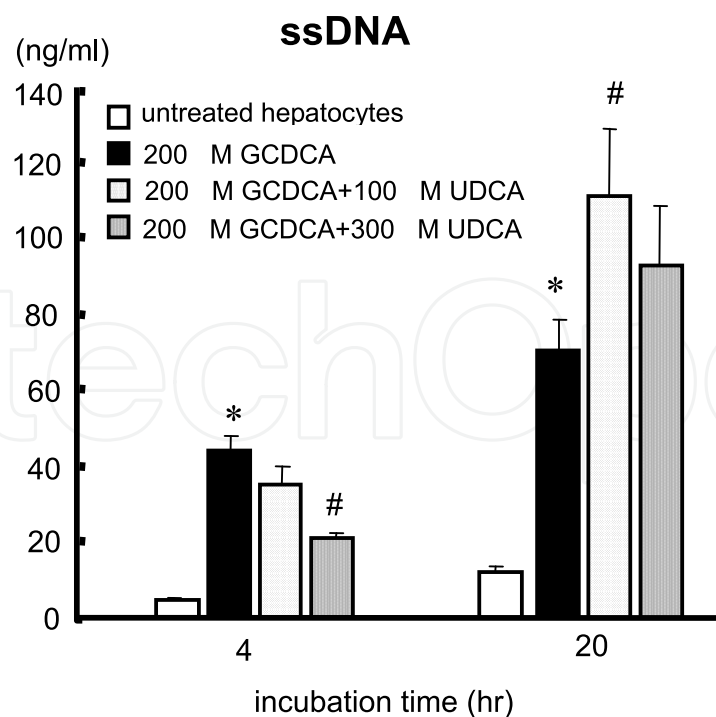
cultured hepatocytes [45]. In a major hypothesis on the mechanisms of hepatocyte injury, bile acids accumulated in the hepatocytes are regarded to be the major cause of cell death [23]. While bile acids such as tauro lithocholic acid (TLCA), deoxycholic acid (DCA), glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid (GCDCA) are known to induce necrosis of hepatocytes via increase in oxidative stress mediated by hydroperoxide generation by mitochondria [23], attention is recently focused on apoptotic cell death associated with these bile acids [45, 46].

The mechanism and pathways of bile acid-induced apoptosis in hepatocytes are reported to include death receptor-mediated pathway [9] and increase in ROS generation [31]. We ourselves reported that, in primary cultured hepatocytes isolated from healthy rats, apoptosis is induced by a hydrophobic bile acid in a concentration- and time-dependent manner [28]. When hepatocyte was cultured with 200  $\mu$ M of GCDCA for 6 hours, ssDNA and caspase-3 activity which are measures of apoptosis increased about 10 times higher than in the untreated cultured hepatocytes (Fig. 1). Also, in hepatocytes treated with 200  $\mu$ M GCDCA for 4 hours, the level of mRNA of Fas and activities of caspase-8 and caspase-3 were significantly increased compared with those in untreated hepatocytes. Further, mitochondrial membrane potential difference of 200  $\mu$ M GCDCA-treated hepatocytes was decreased by 60% compared with the same in untreated hepatocytes. GCDCA, a hydrophobic bile acid, was demonstrated to induce an increase in Fas death receptors located in cell membrane to activate apoptotic pathway.



**Figure 1.** The effect of GCDCA on detection of apoptotic cells. Isolated hepatocytes ( $1 \times 10^6$  cells/well) were treated with GCDCA at 37 °C. Formamide-denaturable DNA was detected in apoptotic hepatocytes. The ssDNA was stained with the primary antibody (anti-ssDNA-mAb) and peroxidase-labelled secondary antibody for color development. Each value represents the mean+SEM of 6-12 samples. Statistically significant changes are indicated as \* $p < 0.05$  compared with the untreated hepatocytes.

On the other hand, oriental traditional pharmacognosy has utilized bear bile as a medicine for improving gastrointestinal symptoms. The effective ingredient of the bear bile is UDCA, which has been used widely as a choleric or calculolytic drug for cholesterol gallstone. UDCA is now used for a wide range of liver disease treatment, and the evidence for its effectiveness has been reported one after another. The indications of UDCA include primary biliary sclerosis (PBC), calculolysis, prevention of gallstone formation and UDCA is demonstrated to be effective in most of these. However, there has been a report that in a clinical trial for reevaluation of effectiveness in PBC patients, the effectiveness of UDCA was not acknowledged [47]. In rat isolated primary cultured hepatocytes, when given in combination with hydrophobic bile acids, UDCA was reported to be cytotoxic [48] and activates the proapoptotic pathway [49]. We ourselves examined the action of UDCA on GCDCA-induced apoptosis in rat primary cultured hepatocytes [28]. When hepatocytes were incubated in the co-presence of UDCA and GCDCA, UDCA significantly inhibited GCDCA-induced apoptosis in a short incubation (4 hours), but a prolonged incubation (20-hour incubation) potentiated the apoptosis (Fig. 2). In the study of decrease in mitochondrial membrane potential difference, a further decrease in the potential was observed in co-incubation of hepatocytes with UDCA and GCDCA even in a short incubation. It was suggested that when cholestatic condition is severe and hepatotoxicity of bile acids is more potent, UDCA might potentiate the toxicity and we pointed out the need for attention in the clinical use [28].



**Figure 2.** Effect of UDCA on ssDNA in GCDCA-induced apoptotic hepatocytes. Each value represents the mean+SEM of 5-15 samples. Other culture conditions were the same as in Fig. 1. \* $p < 0.05$ : significant difference from untreated cells, #  $p < 0.05$ : significant difference from GCDCA-treated cells.



#### 4. Endoplasmic reticulum involvement in hydrophobic bile acid-induced apoptosis in rat hepatocytes

In addition to the major two intracellular apoptotic pathways, that is, death receptor-mediated and mitochondria-mediated pathways, attention has recently been focused on the ER stress-mediated pathway. The response against the accumulation of unfolded proteins in ER is called unfolded protein response (UPR) and this is featured by activation of three different signaling pathways, inositol-requiring (IRE)-1<sup>Ⓢ</sup>, protein kinase RNA-activated (PKR)-like ER kinase (PERK), activating transcription factor (ATF)-6<sup>Ⓢ</sup>. Changes in ER functions are induced by various stimulations, extrinsic chemicals administered pharmacologically, or increase in physiologically secreted proteins, and these upsets are called ER stress, which can be detected by the UPR transducer activation. ER stress is observed in many liver diseases, and UPR activity is correlated with hepatic resistance against insulin in obesity and fatty liver. Chronic viral B and C hepatitis, alcohol-induced liver injury, ischemic re-perfusion damages, and cholestatic liver injury are also correlated with UPR activity. Prolonged or potent ER stress induces apoptosis.

Just like an anti-apoptotic protein Bcl-2, pro-apoptotic proteins Bax and Bak are localized in membranes of ER, and regulate homeostasis of Ca<sup>2+</sup> within the cell. Release of Ca<sup>2+</sup> from ER activates calpain, which in turn activates caspase-12 (in human cells, caspase-4), and initiates apoptosis. Ca<sup>2+</sup> intake by mitochondria leads to enhancement of mitochondrial membrane permeability, and cytochrome c is released from mitochondria. Membranes of mitochondria and ER are connected through protein junctions [50]. These junctions seem to facilitate transports of Ca<sup>2+</sup> and phospholipids, and are possibly associated with apoptosis. However, the exact signaling pathway that mediates ER stress-induced apoptosis in hepatocytes is not clarified yet. UPR is activated for restoration of ER homeostasis. The upset of ER homeostasis induced by damages or activations of UPR sensors is observed in some liver diseases, and ER stress is observed in various liver diseases. Drugs that are hepatotoxic activate several intracellular stress responses (for example, lysosome disorder, increased permeability of mitochondrial membrane, oxidative stress, inflammation and so on) in hepatocytes.

Correlations have been observed between these responses and ER dysfunction. Steatosis occurs in the liver after an acute ER stress mediated by a transcription factor for lipid regulation, sterol regulatory element-binding protein (SREBP)-1c and SREBP-2c. Activation of nuclear factor kappa B (NF-κB) occurs in the downstream of ER upset in alpha-1 antitrypsin (AAT) deficiency disease. Chronic viral hepatitis (hepatitis C virus HCV, hepatitis B virus HBV) accompanies ER dysfunction. Prolonged ER stress leads to apoptosis by activation of C/enhancer binding protein (EBP) homologous protein (CHOP), change in Ca<sup>2+</sup> homeostasis and premature resuming of mRNA translation.

In cholestasis, toxic hydrophobic bile acid/salts are retained in the liver due to impaired biliary excretion. Sodium deoxycholate (DC) induced expressions of UPR genes BIP and

CHOP in vitro [51]. When hepatocytes of CHOP-null mice were incubated with toxic GCDCA, cell death was decreased [52]. In a hereditary model of intrahepatic cholestasis, accumulation of bile acids in the liver was correlated with ER stress [53]. We ourselves studied the effects of GCDCA treatment in hepatocytes, and found that when the culture medium contained  $\text{Ca}^{2+}$ , persistent increase in intracellular  $\text{Ca}^{2+}$  was observed while only transient increase in intracellular  $\text{Ca}^{2+}$  was observed when cells were cultured in  $\text{Ca}^{2+}$  free medium, showing that GCDCA promotes  $\text{Ca}^{2+}$  influx from extracellular matrix and release of  $\text{Ca}^{2+}$  from ER. Activations of calpain and caspase-12 due to the increase in intracellular  $\text{Ca}^{2+}$  were also observed, and we reported that GCDCA induces apoptosis mediated by ER stress [29]. Furthermore, we reported the correlation between ER stress associated with GCDCA and caspase-8 activation. GCDCA activates caspase-8 via Fas death receptor, but when hepatocytes were pretreated with a caspase-8 inhibitor, z-IETD-FMK, expressions of BIP, an ER chaperone molecule, and CHOP, an ER stress response transcription factor, were suppressed. From these, we speculated that caspase-8 activated by GCDCA regulates ER stress [54].

## 5. Detailed mechanism of hydrophobic bile acid-induced apoptosis in HepG2 cells

A hydrophobic bile acid GCDCA activates caspase-8 in hepatocytes via Fas death receptor in the cell membrane. Activated caspase-8 enhances mitochondrial membrane permeability to facilitate the release of cytochrome c, a pro-apoptotic protein, activates caspase-9 and caspase-3 to induce apoptosis. Caspase-8 activated by GCDCA cleaves BAP31 protein in ER membrane and may possibly be associated with  $\text{Ca}^{2+}$  release from mitochondria from ER. BAP31 is cleaved to be BAP20 which is an activated form. As shown in Fig. 3, intact (uncleaved) BAP31 protein is observed in ER in untreated hepatic cells, but in GCDCA-treated hepatic cells, the content of intact BAP31 protein is decreased. The decrease in intact BAP31 observed in GCDCA-treated hepatocytes was suppressed by pretreatment with z-IETD-FMK. When recombinant active caspase-8 was added to hepatocytes, decrease in intact BAP31 was observed. GCDCA-induced increased intracellular  $\text{Ca}^{2+}$  interacted with mitochondria and caused its dysfunction, followed by increase in mitochondrial membrane permeability and release of pro-apoptotic factors from mitochondria. Also, calpain was activated by the increase in  $\text{Ca}^{2+}$  release followed by activation of caspases. Furthermore, ER chaperone BIP is usually bound to an ER stress sensor located within the lumen of ER, but GCDCA treatment causes the increase in unfolded protein, and promotes the release of BIP from the stress sensor and increases the expression of CHOP, an ER stress-related transcription factor that works in the downstream of PERK, an ER stress sensor (Fig. 4). CHOP induces the transcription of Bim, a pro-apoptotic BH3 only protein [55]. Membranes of mitochondria and ER are connected by complexes composed of tethering proteins [50].

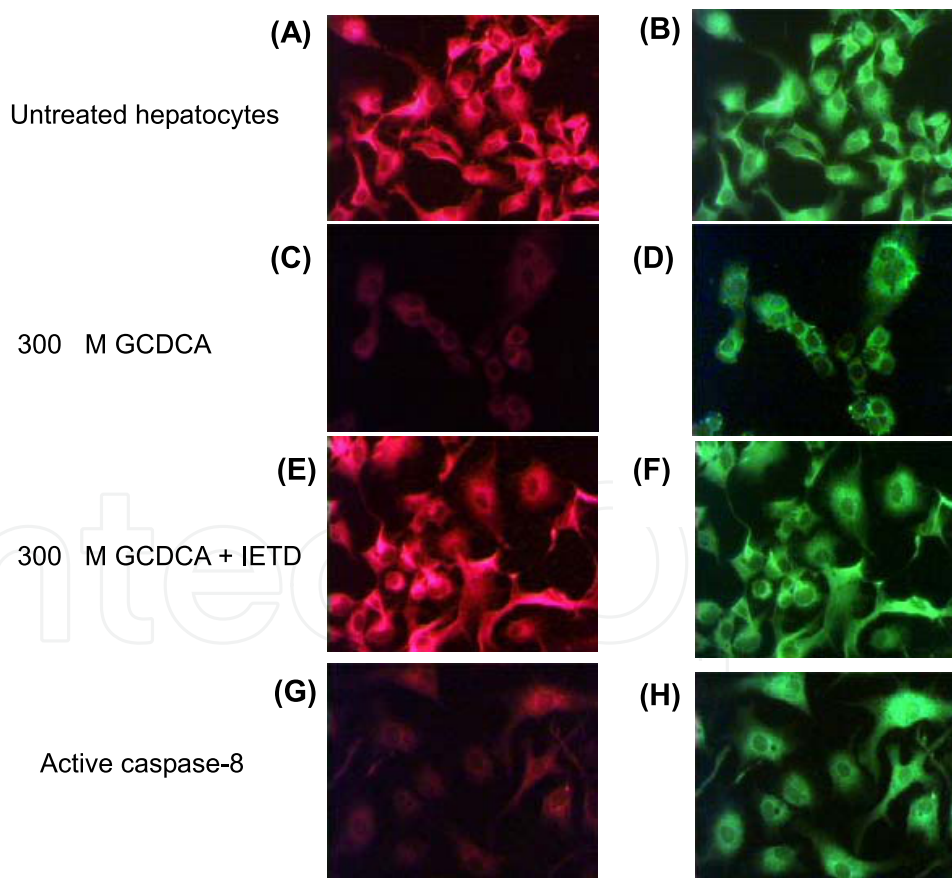
These complexes facilitate the transport of calcium and phospholipids and possibly are associated with apoptosis. GCDCA is known to induce apoptosis via mitochondria- and ER



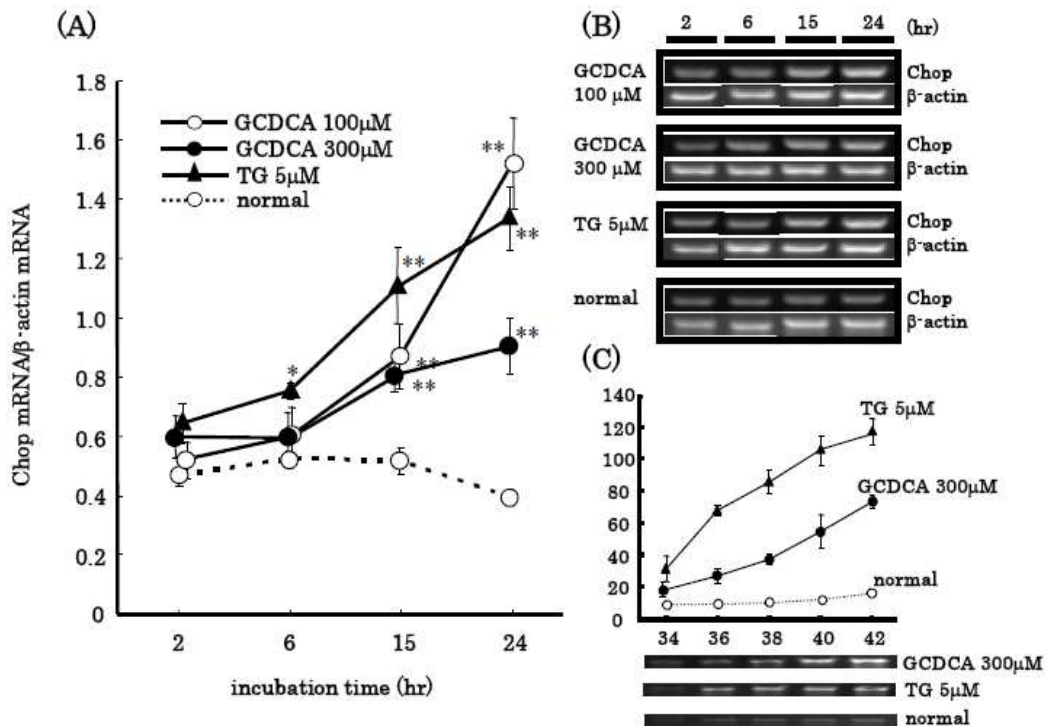
stress-mediated pathways via death receptor, but mitochondria and ER themselves seem to be interrelated to each other.

## 6. Some new findings with effects of ethanol-induced oxidative stress on apoptosis in SK-Hep1 cells

Overconsumption of alcohol is associated with deaths of about 2 million people per year throughout the world. In its early stage, fatty liver is induced, which may progress to liver disorders accompanied with hepatocyte death, inflammation, and fibrosis, further to cirrhosis and hepatocarcinoma [56, 57]. Rate of incidences of acute alcohol intoxication and heavy alcohol drinking indifferent to one's health is globally increasing and acute liver injury caused by alcohol is attracting attention [58]. Suggested molecular mechanisms for liver disorders induced by alcohol include increase of ROS and changes in various signaling pathways, but the molecular mechanism for hepatocyte death was not clarified yet. In experimental studies using model animals of ethanol-induced liver injury, it was emphasized that apoptosis is playing an important role in the pathogenesis of alcoholic



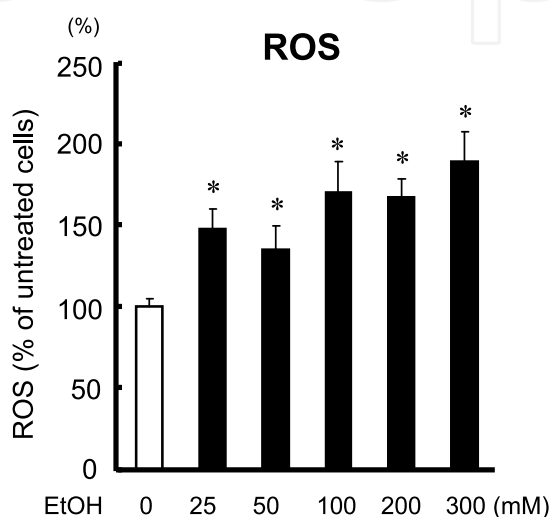
**Figure 3.** The effect of GCDCA on BAP31 in HepG2 cells. HepG2 cells treated with GCDCA (300  $\mu$ M for 24 hours) were double-labeled for BAP31 with rat anti-BAP31 antibody (A, C, E, G) and for ER with Alexa Fluor 488 (B, D, F, H). Original magnification  $\times$  200. (untreated hepatocytes (A, B), hepatocytes treated with 300  $\mu$ M GCDCA (C, D), 300  $\mu$ M GCDCA + Z-IETD-FMK (E, F), recombinant active caspase-8 (G, H))



**Figure 4.** The effect of GCDCA on the CHOP mRNA expression in isolated hepatocytes. (A) CHOP mRNA expression was determined by semiquantitative PCR. Line graphs representation of the observed fold induction normalized to  $\beta$ -actin for each time point. (B) RT-PCR analysis of CHOP and  $\beta$ -actin expression at each incubation time after treatment with GCDCA. (C) Reaction cycles-PCR product yield curves of each reaction mixture were plotted. The intensity of fluorescence was fitted to the data in the linear portion of curves. The resulting CHOP mRNA /  $\beta$ -actin mRNA ratio is represented as the mean+SEM of 8-15 samples. \* $p<0.05$  and \*\* $p<0.01$ : significant difference from untreated hepatocytes (control).

hepatitis or alcoholic liver cirrhosis [3, 59]. Clinical studies suggested similar findings. Various factors including cytotoxicity by alcohol and its metabolites, changes in metabolizing enzymes, invasion of inflammatory cells, reactive oxygen species, cytokines, hepatic microcirculation, nutritional factors, etc. are involved in the onset of apoptosis. Among others, correlation between ROS or oxidative stress and hepatocyte apoptosis is attracting attention. Orally fed ethanol is absorbed in the upper digestive organs, mainly small intestine, and 90% of it is metabolized in the liver. Upon metabolization, ROS is generated in the liver by alcohol dehydrogenase, microsomal ethanol oxidizing system (MEOS) via cytochrome P450, and NADPH oxidase (NOX) in the cell membrane, which is a center for ROS generation (Fig. 5). In general, oxidative stress inflicts cytotoxicity when excessive ROS is generated in the cells. Antioxidant was shown to reduce hepatocyte apoptosis in acute ethanol-addicted rats [60]. On the other hand, several death receptors and their ligands (especially Fas/FasL) are over-expressed in the liver cells of alcoholic hepatitis patients compared with those in healthy subjects. The levels of Fas and FasL are increased in serious alcoholic hepatitis patients, but there are still many uncertainties about the details of their mediators and biological importance [61]. The increase in FasL might be mediated by ROS or increase in NF- $\kappa$ B which increases the transcription of Fas and FasL genes based on

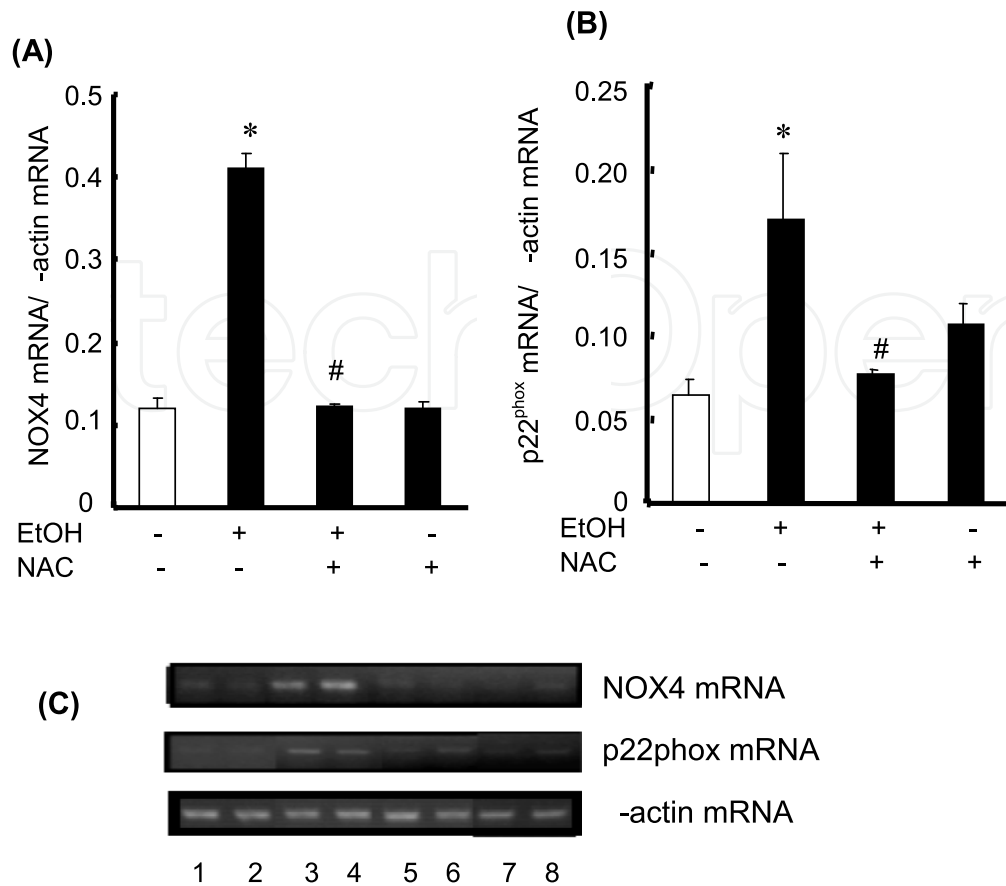
TNF- $\alpha$  inducing activity [62]. In fact, serum TNF- $\alpha$  level increases also in alcoholic hepatitis patients, and plays an important role in inflicting hepatotoxicity [63]. Chronic ethanol administration increases expressions of TNF-R in hepatocytes [64], and during the exposure to ethanol, hepatocytes underwent apoptosis induced by TNF- $\alpha$ . TNF- $\alpha$ / TNF-R1 system seems to be required in the cell death mediated by Fas. In fact, recent studies showed that in TNF-R1/TNF-R2 double knockout mice, apoptosis mediated by TNF- $\alpha$  did not occur, and the mice showed resistance against induced fulminant hepatic failure [65]. The activation of TNF- $\alpha$ /TNF-R1 complex may be co-working with the signaling conveyed by Fas for inducing hepatocyte apoptosis.



**Figure 5.** The effect of ethanol on ROS generation in SK-HEP1 cells. SK-HEP1 cells ( $1 \times 10^6$  cells/ well) were treated with ethanol (25-300 mM) for 5 hours at  $37^\circ\text{C}$ . The generation of ROS in SK-HEP1 treated with ethanol was analyzed spectrofluorometrically. Each value represents the mean  $\pm$  S.E.M. of 6-12 samples. \*:  $p < 0.05$ : significant difference from untreated SK-HEP-1 cells.

Histologically, nick-end label positive apoptotic cells were abundantly found around Mallory body in biopsied tissue from patients with alcoholic liver injury [66]. In *in vivo* animal studies, hepatocyte apoptosis is increased in animals fed with alcohol-containing feed, and long-term alcohol administration is suggested to induce hepatocyte death [67]. Apoptotic cells are observed even in normal cells in some part around the central vein, but in rats fed with alcohol for a long term, many apoptotic cells were observed in various parts around the central vein.

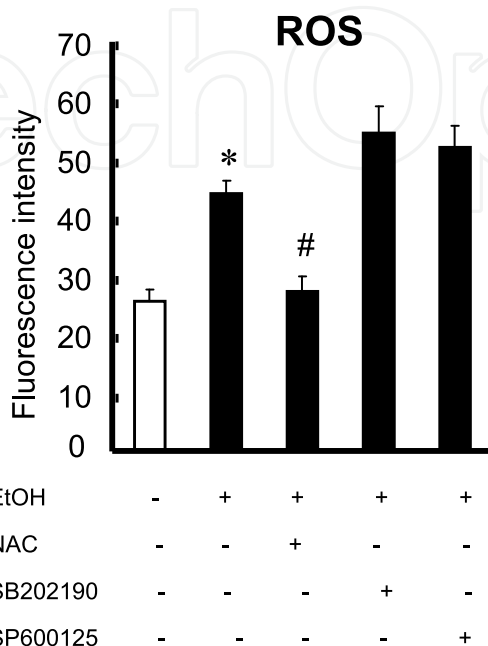
We ourselves reported the ethanol-induced apoptosis in cultured hepatocytes, and showed that apoptosis is induced *in vitro* in the presence of alcohol. We also found that at ethanol concentrations lower than those that induce apoptosis, significant increase in ROS generation was observed. It was suggested that in the process of apoptosis induced by ethanol, ROS generation by NOX was important as inducer of apoptosis [68]. As shown in Fig. 6, in the presence of  $200 \mu\text{M}$  ethanol, the mRNA expression of p22<sup>phox</sup>, which is a protein constitutively bound to NOX to enhance the action of NOX4, was found significantly increased and the increase of the expression was suppressed by the pretreatment with N-acetyl-L-cysteine (NAC), a precursor of glutathione and an antioxidant.



**Figure 6.** The effect of ethanol on NOX-4 and p22<sup>phox</sup> mRNA in SK-HEP-1 cells. (A): The expression of NOX-4 mRNA in SK-HEP1 treated with ethanol (200 mM) and NAC (10 mM). (B): The expression of p22<sup>phox</sup> mRNA in SK-HEP1 treated with ethanol and NAC. The resulting NOX-4 or p22<sup>phox</sup> mRNA/ $\beta$ -actin mRNA ratio is represented as the mean  $\pm$  S.E.M. of 8-12 samples. (C): RT-PCR analysis of NOX-4 or p22<sup>phox</sup> and  $\beta$ -actin expression after treatment with ethanol or ethanol+NAC. Lane1, 2: untreated SK-HEP1 cells, Lane3, 4: 200 mM ethanol, Lane5, 6: ethanol+NAC, Lane7, 8: 10 mM NAC. \* $p < 0.05$ : significant difference from untreated cells, #  $p < 0.05$ : significant difference from 200 mM ethanol-treated cells.

In a recent study on the actions of ethanol on hepatocytes, the importance of mitogen-activated protein kinases (MAPKs) (mainly, ERK1/2, p38 and JNK1/2) and histone modification (acetylation, methylation, or phosphorylation) is emphasized. MAPK pathway is correlated with many signaling pathways including tyrosine, serine/threonine kinase, G protein and calcium signals [69]. MAPKs are a family of protein kinases of which main members are ERK1 and ERK2, p38MAPK, and c-Jun-N-terminal kinase/stress-activated protein kinase (JNK/SAPK). MAPKs regulate various biological processes including cell growth, proliferation, movement, inflammation, fatty degeneration, necrosis and apoptosis [70]. In a primary culture of rat hepatocytes, ethanol showed modest activation of ERK1/2 and notable activation of JNK [71]. In rat cultured hepatocytes, when ERK1/2 phosphorylation was inhibited by U-0126 (a MEK1/2 inhibitor), phosphorylation of JNK by ethanol was increased [72]. In previous studies on neurons, ethanol was found to activate MAPK cascade and increase ROS generation via p38MAPK pathway [73, 74].

We found that exposure of cultured hepatocytes to ethanol increase generation of ROS and MAPK (p38MAPK and JNK) phosphorylation activity. However, ROS generation was not significantly affected when hepatocytes were pretreated with MAPK inhibitors (SB202190 for p38MAPK, and SP600125 for JNK) [75]. These results suggest that ROS may be generated by the upstream effector of p38 MAPK (Fig. 7).



**Figure 7. The effect of MAPK inhibitors on ROS generation in ethanol-induced apoptotic SK-Hep1 cells.** Generation of ROS in SK-Hep1 cells treated with 200 mM ethanol or MAPK inhibitors (SB202190: p38 inhibitor, SP600125: JNK inhibitor) + 200 mM ethanol was analyzed spectrofluorometrically. \* $p < 0.05$ : significant difference from untreated cells, #  $p < 0.05$ : significant difference from 200 mM ethanol-treated cells.

Furthermore, as described above, overconsumption of alcohol induces various pathological stress responses and a part of them is endoplasmic reticulum (ER) stress response. ER stress is associated with alcoholic injury in such organs as the liver, pancreas, heart and brain. The possible mechanism for triggering alcoholic ER stress response is directly or indirectly correlated with alcohol metabolism, which, in turn, is correlated with toxic acetaldehyde and homocysteine, oxidative stress, upset of calcium or iron homeostasis, decrease in the ratio of S-adenosylmethionine/S-adenosyl-homocysteine and abnormal epigenetic modifications. Inhibition of triggering process of ER stress could hopefully be beneficial in the treatment of alcoholic diseases.

In the study of genetic expression in ethanol-fed mice, remarkable increase in caspase-12 mRNA and BIP, a ER chaperone, and CHOP mRNA [76]. When the protein levels were examined, BIP, CHOP and caspase-12 were increased. When CHOP null mice was fed with ethanol, apoptosis of hepatocyte was found to be dependent on CHOP [77], showing that ethanol-induced hepatic injury was associated with hepatocyte apoptosis mediated by CHOP. Furthermore, when micropigs were fed with ethanol, fatty liver and apoptosis are



found to be correlated with the increase in mRNAs of CYP2E1, GRP78, SREBP-1c and also increase in protein levels of CYP2E1, GRP78, nuclear SREBP-1c and caspase-12 activity [78].

We found that when human hepatocarcinoma cell line, SK-Hep1, were exposed to ethanol, expressions of mRNAs of BIP, CHOP, and sXBP-1 were increased. The ethanol-induced increase in expressions were suppressed by NAC, an antioxidant, and we speculated the increase to be associated with oxidative stress induced by ethanol. We also observed a transient increase in intracellular  $\text{Ca}^{2+}$  and calpain activity, but they were not suppressed by NAC, and we believe these were independent from ethanol-induced oxidative stress. It was suggested, therefore, there are two pathways of ethanol-induced apoptosis mediated by ER stress, that is, ethanol-induced oxidative stress-dependent and independent pathways [79].

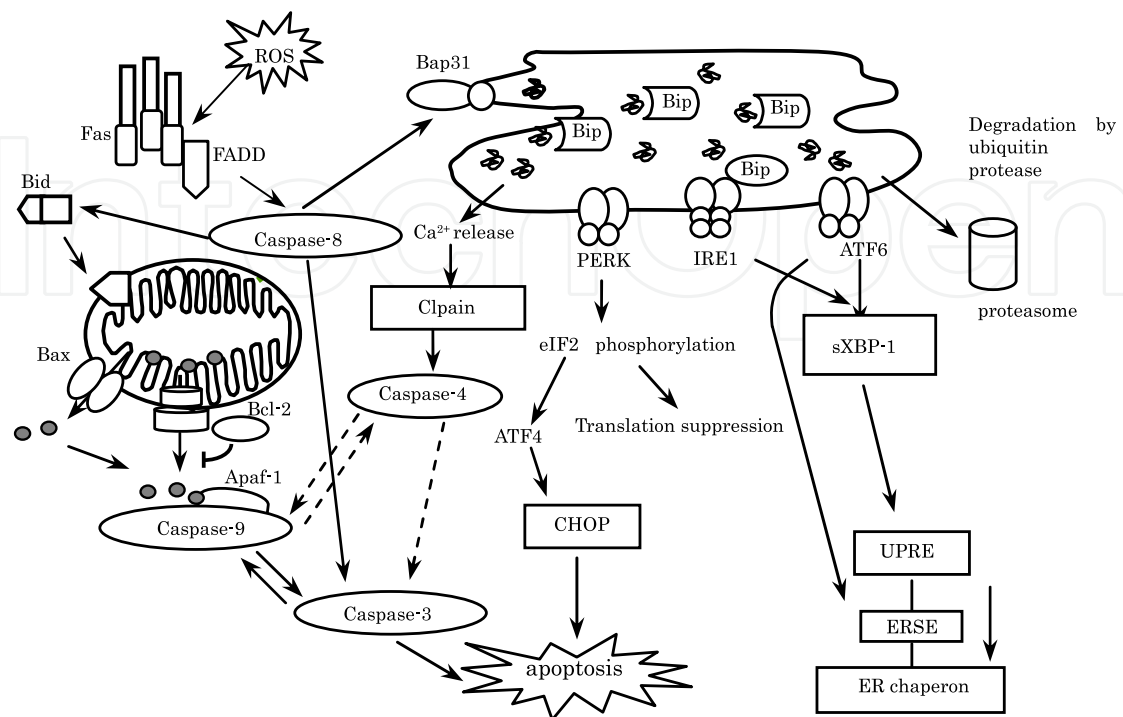
Possible approaches for the treatment of ER stress induced by ethanol include decreasing homocysteine and increase in SAM by betain or folic acid [80-82], improvement of protein folding using chemical chaperone, PBA(sodium phenylbutyrate) and TUDCA [81, 83, 84], inhibition of dephosphorylation of eukaryotic initiation factor-2 $\alpha$  (eIF2 $\alpha$ ) using its inhibitor salubrinal [85], and reducing oxidative stress by decreasing ROS generation from oxidized protein using antioxidants. However, the results of clinical study cannot be obtained. Mechanism of ethanol-induced ER stress is too complex, and the approaches for treatment in human may not be so simple or universal. It may be necessary to employ properly combined therapy of every known beneficial medication.

## 7. Proposed conceptual diagram of apoptosis process in hepatocytes

Apoptosis is an indispensable process in line with cell proliferation for maintenance of tissue homeostasis and health by removal of injured and/or aged cells. This is especially important in the liver where cells are exposed to toxins and viruses [1]. Any loss of balance between cell death and proliferation due to excessive or insufficient apoptosis always leads to pathologic conditions due to unstable state. In the liver, massive hepatocyte apoptosis is observed in acute hepatic failure, and persistent hepatocyte apoptosis is associated with fibrilization, chronic dysfunction and cancerous transformation of the liver [86]. Apoptosis is induced by various intracellular and extracellular stimuli. In all types of hepatocytes, death receptors (especially Fas) are universally expressed [87], and hepatocyte apoptosis is usually transmitted by external pathways. Especially, activations of Fas and TNF-R1 are correlated with hepatocyte apoptosis in various liver diseases including viral hepatitis, fulminant hepatitis, cholestatic liver disease, alcoholic hepatitis, non-alcoholic fatty-liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH), Wilson's disease, and ischemia-reperfusion injury [88]. In the study of cholestasis, because no liver injury was observed after ligation of the common bile duct (a model of extrahepatic cholestasis) in Fas-knockout mice, high levels of toxic intracellular bile salts were speculated to increase Fas in the cell membrane resulting in the activation of the receptor [89, 10]. Furthermore, apoptosis is induced via ER stress. Caspase-8 activated via death receptor may regulate ER stress mediated by BAP31 on ER [54]. In alcoholic liver injury, ROS generation is enhanced via NOX, and the excessive ROS interact with Fas death receptor [8] to induce mitochondria-



mediated and ER stress-mediated apoptosis. Taken together, we propose a diagram showing the mechanism of hepatocyte apoptosis as seen in Fig. 8.



**Figure 8.** Schematic diagram of apoptotic pathway.

## 8. Conclusion

Apoptosis is involved in various diseases, and affects a wide variety of organs including the liver, kidney, central nervous system, and heart. This field of research has been increasingly active in both basic medical sciences and clinical levels, and in the future, when novel findings are obtained on how changes in the process of apoptosis lead to aggravation or improvement of diseases, an innovative strategy for more effective therapy will be designed. Progress of elucidation of apoptotic process is greatly sought after.

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## Acknowledgement

This work has been supported by Special subsidy for promotion of higher level education & research in private universities from Japanese Ministry of Education, Culture, Sports, Science & Technology and Showa University Joint Research grant. The authors thank the graduate students and fellows who contributed to the studies.

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