Differential Induction of Transglutaminase 2-mediated Hepatic Apoptosis in ASH and NASH

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**Differential induction of transglutaminase 2-mediated hepatic apoptosis in ASH and NASH**

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### ABBREVIATIONS

<table>
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<tbody>
<tr>
<td>AIF</td>
<td>apoptosis inducing factor</td>
</tr>
<tr>
<td>ASH</td>
<td>alcoholic steatohepatitis</td>
</tr>
<tr>
<td>CHOP</td>
<td>CCAAT/enhancer-binding protein homologous protein</td>
</tr>
<tr>
<td>CLSp1</td>
<td>crosslinked Sp1</td>
</tr>
<tr>
<td>eIF2α</td>
<td>eukaryotic initiation factor-2 alpha</td>
</tr>
<tr>
<td>ER stress</td>
<td>endoplasmic reticulum stress</td>
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<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>FFAs</td>
<td>free fatty acids</td>
</tr>
<tr>
<td>GRP78</td>
<td>78-kDa glucose-regulated protein</td>
</tr>
<tr>
<td>NASH</td>
<td>non-alcoholic steatohepatitis</td>
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<tr>
<td>NFκB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl D-aspartate receptor</td>
</tr>
<tr>
<td>PERK</td>
<td>pancreatic endoplasmic reticulum kinase</td>
</tr>
<tr>
<td>pIκB</td>
<td>phosphorylated inhibitor of kappa B</td>
</tr>
<tr>
<td>RAR</td>
<td>retinoic acid receptor</td>
</tr>
<tr>
<td>TG2</td>
<td>transglutaminase 2</td>
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<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
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GENERAL INTRODUCTION

The liver plays a major role in metabolism and has a number of functions, including energy storage, decomposition of red blood cells, plasma protein synthesis, hormone production, and detoxification. The liver shoulders a heavy workload for the body, however, it shows no noticeable symptoms upon injuries. Though the liver possesses the ability to regenerate by itself, still it should not be taken for granted. Certain symptoms such as fatty liver diseases and steatohepatitis, is frequently uncovered during a routine physical examination and may eventually lead to permanent liver damage [1].

The term “steatohepatitis” is composed by steato- and hepatitis, referring to fat accumulation and inflammation of the liver, respectively. Heavy alcohol consumption leads to fatty liver and inflammation, whose status is usually called alcoholic steatohepatitis (ASH). Steatohepatitis also occurs in people who seldom or never drink alcohol. This status is called nonalcoholic steatohepatitis (NASH) [2]. NASH can often be found in patients with obesity or certain metabolic syndromes, such as diabetes or insulin resistance. Both ASH and NASH can lead to liver cirrhosis and once cirrhosis has developed, 30% to 40% of patients will suffer liver-related death [2, 3]. The development progression of the fatty liver to NASH or cirrhosis is shown as Fig. 1A [3]. ASH and NASH are extremely similar or even identical in histology (Fig. 1B) [4]. Worldwide agreement on the histological criteria for distinguishing ASH and NASH has not been achieved.

Transglutaminase 2 (TG2) (EC 2.3.2.13) is a member of the transglutaminase family and expressed abundantly and ubiquitously. TG2 is involved in three major enzymatic conversions as shown in Fig. 2A: (i)
posttranslational modification of specific glutamines of protein substrates by covalent linkage to other proteins (or potentially to themselves) via a reaction termed transamidation, (ii) deamidation of glutamines on proteins to form glutamic acid, and (iii) covalent linkage to soluble amines such as polyamines [5-7]. TG2 also exerts multiple catalytic activities under different microenvironments. One classic example is its GTP binding/GTPase activities reciprocally regulated against transamidation activity (Fig. 2B) [8]. In normal cells where intracellular Ca\(^{2+}\) concentration is as low as 10-20 nM and/or when the concentration of GDP is high, TG2/GDP complexes are formed (shown as a closed conformation) and TG2 acts as a GTPase and participates in transmembrane signaling [5]. On the other hand, at high concentration of intracellular Ca\(^{2+}\) exceeding 700-800 nM, TG2 forms complexes with Ca\(^{2+}\) as an open conformation and catalyzes transamidation reaction. Without doubt, TG2 plays important physiologic roles and inappropriate or impaired functions of TG2 gives rise to pathologic situations.

TG2 is highly expressed in liver cells and contributes to many symptoms shared by ASH and NASH, including Mallory-Denk body formation [9], hepatic apoptosis [10], and fibrosis [11]. Thus TG2 might be involved in some common or unique pathological mechanisms between ASH and NASH.

In my study, I confirmed the elevation of nuclear TG2, cross-linked Sp1 (CLSp1), and hepatic apoptosis in NASH patients, as had been demonstrated before in ASH patients [10]. I further investigated the induction mechanisms of nuclear TG2 in cellular systems that mimic ASH and NASH respectively. By applying different inhibitors and antagonists, I found that ethanol and FFAs induce nuclear TG2 through distinct pathways: FFAs increase nuclear TG2 mainly via ER stress-stimulated PERK pathways, while retinoid signaling
contributes much to the ethanol-induced nuclear TG2. These findings suggest potential applications against ASH or NASH by targeting nuclear TG2 but also provide new insights to distinguish ASH and NASH.
Figure 1. Alcoholic steatohepatitis (ASH) and non-alcoholic steatohepatitis (NASH). (A) The development progression of the fatty liver to cirrhosis. Modified from Méndez-Sánchez N et al. [3] (B) The hematoxylin and eosin (HE) staining of liver tissues from ASH (left panel) and NASH (right panel) patients. Modified from Sanderson S and Smyrk T [4].
Figure 2. Functions and structure of transglutaminase 2 (TG2). (A) TG2 is involved in 3 major enzymatic conversions: (i) posttranslational modification of specific glutamines of protein substrates by covalent linkage to other proteins (or potentially to themselves) via a reaction termed transamidation, (ii) deamidation of glutamines on proteins to form glutamic acid, and (iii) covalent linkage to soluble amines such as polyamines. Modified from Strnad P and Omary [5]. (B) TG2 is reciprocally regulated as a GTPase (left, closed conformation) or a transaminase (right, open conformation. Modified from Pinkas et al. [8]
REFERENCES


Differential induction of transglutaminase 2-mediated hepatic apoptosis in ASH and NASH
SUMMARY

Non-alcoholic steatohepatitis (NASH), a progressive form of fatty liver, shares histological similarities with alcoholic steatohepatitis (ASH), including accumulated fat, hepatic apoptosis and fibrous tissues in the liver, but the molecular mechanisms responsible for hepatic apoptosis remain unclear. It was reported that transglutaminase 2 (TG2), induced in the nuclei of ethanol treated hepatocytes, crosslinks and inactivates the transcription factor Sp1, leading to hepatic apoptosis. Here, I investigated whether a similar change is involved in NASH, and if so, how TG2 and crosslinked Sp1 (CLSp1) are induced. Elevated nuclear TG2 and CLSp1 formation were demonstrated in NASH patients, as well as increased activation of apoptosis inducing factor (AIF) and release of cytochrome c. In Hc human normal hepatocytes and HepG2 cells treated with free fatty acids (FFAs), biochemical analyses revealed that ethanol and FFAs provoked fat accumulation, endoplasmic reticulum (ER) stress, increased nuclear factor kappa B (NFκB) and nuclear TG2. Salubrinal, a selective inhibitor of the ER stress-induced pancreatic ER kinase (PERK) signaling pathway, inhibited NFκB activation, nuclear TG2 expression and apoptosis only if it was induced by FFAs, but not by ethanol. Reversely, CD2665, a retinoid receptor antagonist, inhibited ethanol-induced but not FFAs-induced nuclear TG2, suggesting different induction mechanisms of nuclear TG2 in ASH and NASH. These results not only provide us new insights to ASH and NASH but also imply potential applications by targeting the ER stress-NFκB-nuclear TG2 axis in NASH.
INTRODUCTION

Non-alcoholic steatohepatitis (NASH) represents progressive liver damage with inflammation and fat infiltration, and shares histological similarities with alcoholic steatohepatitis (ASH) [1-3]. ASH results from an excessive intake of alcohol while NASH does not involve alcohol abuse but is often associated with elevated free fatty acids (FFAs) in the blood. Moreover, NASH appears to be related to certain metabolic syndromes, including obesity and insulin resistance [1-3]. Steatohepatitis is known to develop into cirrhosis, liver dysfunction due to hepatic apoptosis, and the prevalence is increasing world-wide [1-3]. With the exception of the nutritional status of patients, ASH and NASH are similar in terms of pathology, but the underlying mechanisms, particularly those responsible for hepatic apoptosis, are poorly understood.

Transglutaminase 2 (TG2, EC 2.3.2.13) belongs to the transglutaminase family of proteins, the primary catalytic activity of which is Ca^{2+}-dependent crosslinking of the ε-amino group of a lysine residue to the γ-carboxamide group of a glutamine residue [4]. TG2 is multifunctional and ubiquitous in cells [4-6] and can serve as a guanosine triphosphatase (GTPase) or protein disulfide isomerase (PDI) in different microenvironments (4-7). Therefore, TG2 has been implicated in the regulation of cell growth, differentiation, metastasis and apoptosis (4-9). It was demonstrated that TG2 is induced in the nuclei of ethanol (EtOH)-treated hepatocytes, with crosslinking and inactivation of a general transcription factor, Sp1 [10]. This results in reduced expression of c-Met, a functional receptor for the most important survival factor for hepatocytes, leading to caspase-independent hepatic apoptosis as shown in Fig. 1 [4, 6, 10]. Since NASH shares several histological similarities with ASH, I
investigated whether a similar change would be observed in NASH, and if so, how TG2 is induced in NASH.

Hepatocytes are rich in endoplasmic reticulum (ER), which is essential for many cellular processes including calcium homeostasis, lipid synthesis, protein metabolism and the stress response [11, 12]. Conditions that interfere with ER functions are generally known as ER stress, and are typically associated with accumulation of misfolded or unfolded proteins in the ER lumen (referred to as the unfolded protein response, UPR), which is mediated by three ER-resident sensors: inositol-requiring kinase 1 (IRE1), pancreatic ER kinase (PERK) and activating transcription factor 6 (ATF6) [11, 12]. The 78-kDa glucose-regulated protein (GRP78 or Bip) resides in the ER lumen and its expression is regulated by these three sensors, so it serves as a marker of ER stress [11-13]. Overloading with ER stress activates these three pathways and eventually drives cells to apoptosis by an ER-stress apoptotic mediator, CCAAT/enhancer-binding protein homologous protein (CHOP)/GADD153 [11, 13-15]. Accumulating evidence suggests close relationships between ER stress and ASH/NASH [11, 16, 17]. Elevated ER stress is observed after free fatty acid treatment of hepatocytes [16, 17]. Excessive ER stress promotes apoptosis and lipid accumulation. Furthermore, it activates pro-inflammatory pathways such as nuclear factor kappa B (NFκB), which is thought to be important in the pathogenesis of NASH [11, 16]. However, it is as yet unclear how ER stress affects TG2 expression in hepatocytes during pathogenesis of NASH.

In my study, the induction of nuclear TG2 and CLSp1 in NASH patients was investigated, and the involvement of ER stress and NFκB in this phenomenon was explored using a cell culture model. Furthermore, by employing salubrinal,
a selective inhibitor of the dephosphorylation of eukaryotic initiation factor-2α (eIF2α), which is mandatory in the ER stress-induced PERK signal pathway [18], I demonstrated that the PERK pathway-dependent induction of TG2 is essential for FFAs-induced, but not EtOH-induced, apoptosis. In addition, knockdown of TG2 could greatly reverse cell death caused by FFAs treatment.
MATERIALS AND METHODS

Materials

Hoechst 33258 and 5-(biotinamido) pentylamine (5-BAPA), a biotinylated primary amine substrate for TG2, were from Calbiochem-Novabiochem (La Jolla, CA) and Pierce (Rockford, IL), respectively. Salubrinal and CD2665 were purchased from Tocris Bioscience (Ellisville, MO). Bay117085 and some other general chemicals were from Sigma Aldrich, Inc. (St. Louis, MO). Antibodies against NFκB, GRP78/Bip, CHOP/GADD153, lamin B, cytochrome c and AIF were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); p-eIF2α from Cell Signaling Technology, Inc. (Danvers, MA); GAPDH from Chemicon International (Temecula, CA), and TG2 from Lab Vision (Fermont, CA). A polyclonal antibody against CLSp1 was produced in rabbits and purified as described in Tatsukawa et al. [10]

Immunohistochemistry

Five μm sections of paraffin-embedded tissues were deparaffinized and rehydrated by routine processes. Slides were soaked in 3% hydrogen peroxide for 15 min, washed with PBS twice, blocked with serum blocking solution for 30 min and then incubated with primary antibodies for 1 h at room temperature. Staining was enhanced using ABC kits (Vector Laboratories, Inc., Burlingame, CA) and developed with DAB substrate. Apoptotic cells in the liver sections were detected following standard manufacturer’s instructions of DeadEnd Colorimetric TUNEL System (Promega Corporation, Madison, WI). Sections were afterward counterstained with hematoxylin and mounted.
Cell Culture

Hc cells, human normal hepatocytes, were purchased from Applied Cell Biology Research Institute (Kirkland, WA). HepG2 cells overexpressing alcohol dehydrogenase were generous gifts from Prof. Tsukamoto (USC, CA) [19]. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Equitech-Bio, Inc., Kerrville, TX) and 1% antibiotics (Sigma Aldrich, Inc., St. Louis, MO).

Cell Counting

Transfected Hc cells were seeded in a number of $2 \times 10^4$ in 6-cm dishes. After 0-48 h treatment, Hc cells were collected and viable cells were counted by a hemocytometer calculator.

Treatment with Free Fatty Acids (FFAs)

Treatment of Hc or HepG2 cells with free fatty acids was based on the method described in a previous study [20]. Briefly, oleic acid, palmitic acid, stearic acid, and linoleic acid (Sigma Aldrich, Inc., St. Louis, MO) were mixed in isopropanol at 80 mM each. Cells were incubated in fresh DMEM including 100-200 µM FFAs and 1% bovine serum albumin from 18 to 48 h (final isopropanol concentration was 0.25%, which was confirmed not to be toxic to cells). The combination of these FFAs at this concentration has been shown to mimic the situation in NASH patients’ sera [1, 21].

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from HepG2 cells using the RNeasy kit (Qiagen, Hilden, Germany) and was reverse transcribed by avian myeloblastosis virus
(AMV) reverse transcription (RT). The resultant cDNA was used as a template for polymerase chain reaction (RT-PCR) performed with a combination of specific nucleotide primers as shown in Table 1. Amplified products were analyzed in 2% agarose gels stained with ethidium bromide.

**Oil-Red O Staining**

FFAs-treated cells were stained with Oil-red O solution as previously described [22]. Briefly, FFAs-treated cells were fixed in 4% paraformaldehyde for 1 h after a wash with phosphate buffered saline. They were then washed with 60% triethyl phosphate and incubated for 15 min in Oil-Red O working solution, which was freshly made by mixing 30 ml of 0.5% Oil-Red O stock solution and 20 ml of distilled water immediately prior to use. After rinsing with 60% triethyl phosphate for 2-3 s, the cells were washed twice with distilled water, counterstained with hematoxylin and mounted.

**Immunofluorescence Staining**

Equal numbers of cells were seeded on glass cover slips and allowed to attach overnight in serum-free DMEM medium. On the following day they were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. After blocking in 2.5% bovine serum albumin for 30 min, the cells were incubated with the primary antibody for 1 h followed by another 1 h with fluorescent isothiocyanate–conjugated anti-rabbit secondary antibodies at room temperature. They were mounted in Fluomount solution (Diagnostic BioSystems, Pleasanton, CA). Images were captured using a Leica fluorescence scanning microscope (Leica Microsystems, Wetzler, Germany).
**Transglutaminase 2 (TG2) Activity**

In situ TG2 activity was detected by incorporation of 5-BAPA into HepG2 cells. The cells were incubated with 0.2 mM 5-BAPA and 100 µM aminoguanidine for 12 h, then fixed, permeabilized, and stained with tetramethylrhodamine isothiocyanate (TRITC)-conjugated streptavidin as described above.

**Immunoblotting**

Isolation of cytosolic and nuclear proteins and Western blotting were performed as previously described [10]. The dilution of primary antibodies is NFκB (1:500), GRP78/Bip (1:1000), CHOP/GADD153 (1:200), p-eIF2α (1:1000), p-IκB (1:2000), TG2 (1:1000), caapase 3 (1:1000), caspase 9 (1:1000), lamin B (1:10000), and GAPDH (1:10000).

**MitoCapture Assay**

Mitochondrial membrane potential was detected by MitoCapture detection kit (BioVision. Inc, CA) following standard manuscript supplied by manufacturer.

**Lentivirus-based SiRNA Knockdown of TG2**

Lentiviral particles of TG2 siRNA or control (scrambled sequences) were purchased from Santa Cruz, Biotechnology (Santa Cruz, CA). Transfection and puromycin selection were assayed according to standard protocols provided by Santa Cruz.
**Human Resources**

Human liver specimens from ASH, NASH were obtained after informed consent and approval by the Ethics Committee for Biomedical Research, Jikei University School of Medicine (Tokyo, Japan) as well as RIKEN. Normal control specimens were from noncancerous regions of the liver from a patient with uterine cancer with hepatic metastasis.

**Statistics**

The *paired student’s t test* was used to evaluate differences between two groups. *P*<0.05 (*) and *P*<0.01 (**) were considered statistically significant.
RESULTS

Elevation of nuclear TG2, CLSp1 and apoptosis in hepatic cells in ASH and NASH

Immunohistochemical staining showed induction of TG2, CLSp1, and significant TUNEL positivity in several cell nuclei from the liver in both ASH and NASH patients (n=8 for each staining) (Fig. 2A, arrows). Other apoptotic molecules such as apoptosis inducing factor (AIF) and cytochrome c reported to be up-regulated upon TG2 induction [5, 23] were also observed in ASH and NASH patients (n=5 for each staining)

Elevation of nuclear TG2, CLSp1 and fat accumulation was reproduced in a cellular system mimicking NASH

I further established a cellular system following treatment with increasing concentrations of the FFAs mixture which based on previous report [20] to mimic the high FFAs found in the sera of NASH patients [1]. By using a specific primer set against human TG2 (Table 1), increased TG2 mRNA was determined in the HepG2 cell cultures (Fig. 3A). In parallel with fat accumulation as detected with Oil-Red O staining (Fig. 3B, column 1), similar induction of nuclear TG2 (column 3), TG2 activity (column 4), and CLSp1 (column 5) was also observed.

Increased ER stress, nuclear TG2 and NFκB activation by EtOH and FFAs

The molecular mechanisms underlying induction of nuclear TG2 was investigated using Hc human normal hepaocytes (Fig 4) and HepG2 cells (Fig 5) after treatment with EtOH and FFAs. Western blotting revealed that EtOH
(Fig. 4, lanes 1-4) and FFAs (Fig. 4, lanes 5-8) dose-dependently increased nuclear NFκB and TG2, accompanied by a marked increase in cytosolic GRP78 and CHOP, two reliable ER stress markers. Cytosolic TG2 levels were not significantly altered. There was also a noticeable increase in cytosolic phosphorylated IκB (pIκB), suggesting that NFκB was activated by both EtOH and FFAs treatments in a dose-dependent manner. Similar results were reproduced in HepG2 cells as shown in Fig. 5.

**NFκB activation-dependent induction of nuclear TG2 by FFAs, but not by EtOH**

I continuously analyzed how NFκB is involved in TG2 induction by EtOH and FFAs. Bay117085, which suppresses the activation of NFκB by inhibiting phosphorylation of IκB [24], did not alter the induction of nuclear TG2 by EtOH, but significantly inhibited the induction by FFAs in Hc cells (Fig. 6A) as well as HepG2 cells (Fig. 6B). This suggests that while EtOH-activated NFκB does not affect nuclear TG2, FFAs-activated NFκB plays an important role in the induction of nuclear TG2. Bay117085 did not affect cytosolic or nuclear TG2 levels when being treated alone (data not shown). Similar results were also confirmed by immunofluorescence staining (Fig 7), showing significant reduction of FFAs-induced TG2 but not EtOH-induced TG2. These results strongly support the NFκB-activation-dependent induction of nuclear TG2 by FFAs but not EtOH.

**Induction of nuclear NFκB and TG2 by FFAs but not by EtOH is reduced by salubrinal, a PERK-pathway inhibitor**

I then explored the relationships among TG2, NFκB and ER stress. NFκB
activation is involved in the PERK signaling, one of three ER stress sensors, and contributes to the inflammatory responses following ER stress [12, 25]. To see the activation of PERK pathway, I measured the levels of phosphorylated eIF2α (p-eIF2α), which is the downstream substrate of PERK. I observed a significant increase in the levels of p-eIF2α in both HepG2 cells and Hc cells after treatment with 100 µM FFAs, while relatively lower induction was found after 50 mM EtOH treatment (Fig. 8).

To further investigate the involvement of the PERK pathway in NFκB-dependent nuclear TG2 induction after FFAs stimulation, salubrinal, an inhibitor which selectively inhibits the dephosphorylation of eIF2α and thus blocks ER stress-induced PERK signaling (Fig. 9), was utilized [18, 26]. Salubrinal markedly inhibited the induction of nuclear NFκB and TG2 by FFAs but not by EtOH in Hc cells (Fig. 10A) and in HepG2 cells (Fig. 10B), suggesting that EtOH and FFAs promote nuclear TG2 through distinct pathways, and that ER stress induced by FFAs predominantly triggers the PERK signaling pathway, including NFκB activation and induction of nuclear TG2. Immunostaining results also confirmed that FFAs-induced nuclear TG2 expression and activity were sensitive to salubrinal, while EtOH-induced expression and activity were not (Fig. 11).

**FFAs-induced hepatic cell apoptosis is sensitive to salubrinal**

Significant apoptosis was present in the liver tissues of NASH patients as detected by TUNEL staining (Fig. 1, arrows). EtOH- and FFAs-induced apoptosis was observed in Hc cells (Fig. 12, left columns). Administration of salubrinal strongly inhibited FFAs-induced apoptosis, which decreased from 89% to 31%, but was less effective against EtOH-induced apoptosis (from
Mitochondrial transmembrane potential was monitored by a MitoCapture assay kit. In healthy cells, MitoCapture dye aggregates in the mitochondria and releases a red fluorescence as demonstrated in the control of Fig. 13. In apoptotic cells, altered mitochondrial membrane potential prevents aggregation of the dye and it remains in a monomer form in the cytosol, resulting in a green fluorescence (Fig. 13, EtOH- or FFAs-treated cells).

Salubrinal moderately decreased apoptosis induced by FFAs but had no effect on apoptosis induced by EtOH (Fig. 13, right columns). Taken together, these results suggest the involvement of PERK pathway-mediated TG2 elevation in FFAs-induced apoptosis but not in EtOH-induced apoptosis.

**TG2 plays a key role in FFAs-induced hepatic apoptosis**

Direct effects of TG2 on FFAs-induced hepatic apoptosis were further verified by lentivirus-based siRNA knockdown of TG2 in Hc hepatocytes. The knockdown efficiency was evaluated by Western blotting (Fig. 14A), which demonstrated an approximate 80% reduction in total TG2 in Hc cells but no effect on NFκB levels. After TG2 knockdown, Hc cells treated with FFAs recovered noticeable cell growth compared with control-infected cells (Fig. 14B). *In situ* TUNEL staining also showed reduced apoptosis by FFAs after TG2 knockdown (Fig. 15), once again strengthening an essential role for TG2 in FFAs-induced hepatic apoptosis.

I also measured activated caspase 3 and caspase 9, which represent classic apoptotic signals, in Hc cells treated with EtOH or FFAs. To my surprise, activated caspase 3 and caspase 9 levels didn’t show much difference among each treatment (Fig. 16), suggesting that the FFAs-induced TG2-dependent
apoptosis may be independent to traditional caspase signaling.

**Induction of nuclear NFκB and TG2 by EtOH but not by FFAs is reduced by CD2665, a retinoic acid receptor (RAR) β/γ antagonist**

After determining the dependence of NFκB and PERK pathway activation in FFAs-induced nuclear TG2, I wondered how EtOH induces TG2. Recently, Currò et al demonstrated that homocysteine would increases TG2 expression in Neuro2a cells [27], and homocysteine is well known to be increased after uptaking EtOH [28]. Moreover, Mukhopadyay et al. [29] revealed that retinoic acid mediates the transcriptional activation of cannabinoid receptor-1 in ASH, and retinoic acid is the most potent inducer of TG2 [30].

Inspired by these reports, I first examined the relationship between homocysteine and TG2. Homocysteine mainly provokes N-methyl D-aspartate receptor (NMDAR) signaling and can by inhibited by antagonists such as MK801 (against glutamate binding site) or ACPC (against glycine binding site) (Fig. 17A) [31]. As depicted in Fig. 17B, nuclear TG2 increased upon homocystenine treatment and this induction could be moderately blocked by both MK801 and ACPC in Hc cells. However, in Hc cells treated with EtOH, NMDAR antagonists didn’t show any reduction in nuclear TG2, while CD2665, a RAR β/γ antagonist that blocks retinoids signaling [32], strongly inhibited EtOH-induced nuclear TG2 (Fig. 18B). Moreover, results in Fig 19 show that CD2665 significantly inhibited nuclear TG2 induced by EtOH but not by FFAs both in Hc and HepG2 cells. Results of immunofluorescence data indicated decreased nuclear TG2 expression and activity by CD2665 (Fig. 20), supporting the results of Western blotting. These results suggest the involvement of retinoid signals in EtOH-induced nuclear TG2 in the liver.
In conclusion, I demonstrated that though both EtOH and FFAs induce nuclear TG2, the induction mechanisms are different. FFAs induce nuclear TG2 via ER stress-mediated PERK and this induction is dependent on NF\(\kappa\)B activation, while EtOH-induced TG2 is mostly via RAR signaling activation. Upregulation of nuclear TG2 would result in hepatic apoptosis accompanying CLSp1, AIF and cytochrome c. (Fig. 21).
DISCUSSION AND PERSPECTIVE

TG2 has been implicated in many liver injuries and diseases such as ASH-associated cirrhosis for decades [33, 34]. It has also been implicated in the formation of Mallory-Denk bodies [35] in steatohepatitis, but its role in NASH has yet to be elucidated.

Previously, with my help in immunostaining, Tatsukawa et al. published a novel apoptotic mechanism in EtOH-injured hepatocytes involving the crosslinking and inactivation of the transcription factor Sp1 by nuclear TG2, leading to cell death [10]. Here, elevation of nuclear TG2 and CLSp1 in FFAs-treated HepG2 cells was confirmed. Furthermore, such elevation was demonstrated in NASH patients. The induction of TG2 begins when ER stress is induced and PERK signaling and NFκB are activated (Fig. 6). Accumulated cell death and hepatocyte malfunction gradually drives fatty liver disease to severe liver injuries such as cirrhosis and hepatocellular carcinoma [16]. Now we are still trying to elucidate the relationships among NFκB and CLSp1, AIF or cytochrome c.

ASH and NASH share many pathological similarities, but the reason remains unknown [1, 16]. Combining the results obtained in the current and previous studies [10] suggests that although ASH and NASH provoke ER stress, NFκB activation and TG2-dependent hepatic apoptosis, the underlying mechanisms are different. The FFAs-induced ER stress-NFκB activation-TG2-mediated apoptosis axis was sensitive to salubrinal, while the EtOH-induced TG2-mediated apoptosis axis was not and partially dependent on retinoid signaling. Moreover, Ji et al. [11] addressed an importance of the PERK pathway as well as IRE1 and ATF6 pathways in
homocysteine-mediated ER stress observed in ASH. It is possible that two other ER stress sensors, IRE1 and ATF6 are activated by EtOH.

Other studies have reported the involvement of NFκB in the transcriptional activation of TG2 in ASH [33, 34]. In this study, however, Bay117085 only slightly affected nuclear TG2 induction in response to EtOH. We cannot explain this discrepancy in terms of experimental conditions. However, TG2 has been shown to interact mutually with NFκB under different conditions; e.g. NFκB transcriptionally promotes TG2 expression by binding to its consensus sequence within the TG2 gene promoter [7, 35] while TG2 activates NKκB by polymerizing and inactivating IκB [36]. Besides, as FFAs treatment induced TG2 about 3 folds at transcription level, thus combination of siTG2 and FFAs should also enhanced TG2 to relative levels of 0.6 fold. But I observed a rather great reduction in nuclear TG2 with the treatment of siTG2 plus FFAs. I don’t have a good answer to this yet, but FFAs might affect the inhibitory action of siRNA. I will try to disclose this question through further investigation.

I have confirmed the importance of nuclear TG2 in hepatic apoptosis in NASH, but it remains largely unknown how TG2 is transported into the nucleus despite for the implication that importin α is involved [10]. Dardik and Inbal reported that TG2 could be involved in modulating the cellular response to VEGF by forming an intracellular complex with VEGFR-2 and mediating its translocation into the nucleus [37], but detailed mechanisms are still under investigation.

Besides, during investigation of nuclear TG2, I discovered a short form TG2 in FFAs-treated cells, especially in the nucleus, whose molecular weight was smaller (50-60 kD) compared to full length TG2 (70 kD) (Fig. 22). This short form TG2 (designated as TG2-S) was ever reported in neuron cells [38] but
never in hepatocytes before and still remains very mysterious in its emergence and functions. TG2-S is shown in other reports to be the product of alternative splicing [39, 40]. Consistently, my preliminary data showed that TG2-S levels could be reduced by treatment of cells with spliceostatin A (SSA), an inhibitor of alternative splicing [41], suggesting that TG2-S is also to be at least in part the product of alternative splicing from full length TG2 in hepatocytes (Fig. 23). However, further investigation is needed to verify the exact role of TG2-S and how it is involved in cellular processes.

Findings presented herein will probably be applied generally to diseases related to ER stress, but as hepatocytes contain relatively abundant ER and TG2, the liver is an appropriate model for monitoring such pathways. This study has provided the first evidence that ER stress-induced nuclear TG2 is the major pathway responsible for hepatic apoptosis in NASH, implying potential applications by targeting the ER stress-NFκB-nuclear TG2 axis in this disease. Detection of this axis will be a unique marker distinguishing NASH from ASH. Identification of this mechanism provides new insights toward ASH and NASH but also provokes new questions such as how EtOH or FFAs induce ER stress. I believe disclosure of related issues will deepen our understanding to these two diseases and benefit much to those patients as well.
REFERENCES


Homocysteine-induced toxicity increases TG2 expression in Neuro2a cells. Amino acids. 36(4):725-730


Table 1. Primers for RT-PCR experiments

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Nucleotide no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human TG2</td>
<td>Sense AACCCCAAGTTCTGAAG</td>
<td>597-614</td>
</tr>
<tr>
<td></td>
<td>Antisense AGGTTGCTTTCTGGTC</td>
<td>916-932</td>
</tr>
<tr>
<td>Human GAPDH</td>
<td>Sense GCAGGGGGGAGCCAAAAGGG</td>
<td>395-414</td>
</tr>
<tr>
<td></td>
<td>Antisense TGCCAGCCCCAGCGTCAAAG</td>
<td>942-961</td>
</tr>
</tbody>
</table>
Figure 1. TG2-induced apoptotic mechanism in ethanol (EtOH)-injured hepatocytes. TG2 is induced in the nuclei of EtOH-treated hepatocytes, with crosslinking and inactivation of a general transcription factor, Sp1. This results in reduced expression of c-Met, a functional receptor for the most important survival factor for hepatocytes, leading to caspase-independent hepatic apoptosis. Modified from Tatsukawa et al. [10]
Figure 2. Induction of TG2, CLSp1 and apoptosis in ASH and NASH. Liver sections from patients with ASH and NASH were stained with antibodies against TG2, crosslinked Sp1 (CLSp1), TUNEL, apoptosis inducing factor (AIF), and cytochrome c (shown as Cyt. C in the figure). Noncancerous regions of liver from a patient with uterine cancer and hepatic metastasis were used as a normal control. Arrows indicate representative signals (for TG2, CLSp1 and TUNEL, n=8 for each staining; for AIF and cytichrome c, n=5 for each staining). Scale bar = 50 µm. Representative results from three independent experiments are shown.
Figure 3. Induction of TG2 and CLSp1 in ASH and NASH. (A) After treatment of HepG2 cells with EtOH (100-200 mM) or FFAs (100-200 µM) for 18 h, total RNA extraction and RT-PCR were carried out to examine transcriptional levels of TG2. C stands for control HepG2 cells and GAPDH was the loading control. (B) HepG2 cells treated with 100 or 200 µM free fatty acid mixtures (FFAs) for 18 h were fixed and stained with Oil-red O solution (lane 1), Hoechst 33258 (lane 2), anti-TG2 antibody (lane 3), tetramethylrhodamine isothiocyanate (TRITC)-conjugated streptavidin (lane 4) and anti-CLSp1 antibody (lane 5). HepG2 cells treated with 0.25% isopropanol were used as a control. Scale bar = 50 µm. Representative results from three independent experiments are shown.
Figure 4. Induction of ER stress, nuclear NFκB and TG2 by EtOH and FFAs in Hc human normal hepatocytes. After treatment of Hc cells with EtOH (50-200 mM, lanes 1-4) or FFAs (50-200 µM, lanes 5-8) for 18 h, cytosolic and nuclear extracts were prepared and protein levels of NFκB, pIkB, TG2, GRP78, CHOP, GAPDH (cytosolic loading control) and lamin B (nuclear loading control) in each fraction were estimated using Western blotting. Relative changes in the densitometric profiles of nuclear NFκB and TG2 levels are presented under corresponding bands after normalizing to the changes in lamin B and comparing to each control. Representative results from three independent experiments are shown.
Figure 5. Induction of ER stress, nuclear NFκB and TG2 by EtOH and FFAs in HepG2 cells. After treatment of HepG2 cells with EtOH (50-200 mM, lanes 1-4) or FFAs (50-200 µM, lanes 5-8) for 18 h, cytosolic and nuclear extracts were prepared and protein levels of NFκB, pIκB, TG2, GRP78, CHOP, GAPDH (cytosolic loading control) and lamin B (nuclear loading control) in each fraction were estimated using Western blotting. Relative changes in the densitometric profiles of nuclear NFκB and TG2 levels are presented under corresponding bands after normalizing to the changes in lamin B and comparing to each control. Representative results from three independent experiments are shown.
Figure 6. NFκB activation-dependent induction of nuclear TG2 by FFAs, but not by EtOH. After treatment of Hc cells (A) or HepG2 cells (B) with 50 mM EtOH or 100 µM FFAs for 18 h in the absence or presence of 20 µM Bay117085, an NFκB inhibitor, nuclear extracts were prepared, and protein levels of NFκB, TG2 and lamin B (nuclear loading control) in each extract were estimated by Western blotting. Relative changes in the densitometric profiles of nuclear NFκB and TG2 levels are presented under corresponding bands after normalizing to the changes in lamin B and comparing to each control. Representative results from three independent experiments are shown.
Figure 7. NFκB activation-dependent induction of nuclear TG2 by FFAs, but not by EtOH. After treatment of Hc cells with 50 mM EtOH or 100 µM FFAs for 18 h in the absence or presence of 20 µM Bay117085, an NFκB inhibitor, cells were fixed and stained with Hoechst 33258 (left columns), anti-TG2 antibody (middle columns), TRITC-conjugated streptavidin (right columns). Scale bar = 50 µm. Representative results from three independent experiments are shown.
Figure 8. Activation of PERK pathway by EtOH and FFAs. Activated levels of PERK pathway was measured by phosphorylated eIF2α (p-eIF2α) using Western blotting in Hc and HepG2 cells after treatment with 50 mM EtOH or 100 µM FFAs for 18 h. Relative changes in the densitometric profiles are presented under corresponding bands after normalizing to the changes in GAPDH to each control. Representative results from three independent experiments are shown.
Figure 9. Salubrinal specifically inhibits phosphatase 1 (PP1) so as to inhibit the dephosphorylation of eIF2\(\alpha\). Physiological or experimentally induced ER stress leads to the activation of PERK and, eventually, the GADD34/PP1 phosphatase complex dephosphorylates eIF2\(\alpha\), promoting apoptosis. Salubrinal that selectively inhibits PP1, resulting in phosphorylation of eIF2\(\alpha\) and protects cells from ER stress-induced apoptosis. Modified from Boyce M and Yuan. [26].
Figure 10. Induction of nuclear NFκB and TG2 by FFAs but not by EtOH is blocked with salubrinal, an ER stress inhibitor. After treatment of Hc cells (A) or HepG2 cells (B) with 50 mM EtOH or 100 µM FFAs for 18 h in the absence or presence of 50 µM salubrinal, cytosolic and nuclear extracts were prepared and protein levels of NFκB, TG2, GAPDH (cytosolic loading control) and lamin B (nuclear loading control) in each fraction were estimated by Western blotting. C stands for control cells, EtOH stands for 50 mM EtOH, and FFAs stands for 100 µM FFAs. Relative changes in the densitometric profiles of nuclear NFκB and TG2 levels are presented under corresponding bands after normalizing to the changes in lamin B and comparing to each control. Representative results from three independent experiments that gave similar results are shown.
Figure 11. Induction of nuclear TG2 by FFAs but not by EtOH is blocked with salubrinal, an ER stress inhibitor. Immunofluorescence staining was performed after treatment of Hc cells with 50 mM EtOH or 100 µM FFAs for 18 h in the absence or presence of 50 µM salubrinal. Cells were fixed and stained with Hoechst 33258 (left columns), anti-TG2 antibody (middle columns), TRITC-conjugated streptavidin (right columns). Scale bar = 50 µm. Representative results from three independent experiments are shown.
Figure 12. FFAs-induced hepatic apoptosis is blocked by salubrinal. In situ TUNEL staining of apoptotic Hc cells after treatment with 50 mM EtOH or 100 µM FFAs in the absence (left columns) or presence (right columns) of 50 µM salubrinal. C represents untreated control Hc cells. Each averaged percentage of apoptotic cells was counted from four different fields of view and shown in the squares. Scale bar = 50 µm. Representative results from three independent experiments are shown.
Figure 13. FFAs-induced change in mitochondrial membrane potential is blocked by salubrinal. Mitochondrial membrane potential was investigated using a MitoCapture assay, showing healthy cells (red) and apoptotic cells (green). Scale bar = 50 µm. Representative results from three independent experiments are shown.
Figure 14. TG2 is revealed to be essential in FFAs-induced apoptosis by siTG2 knockdown in Hc cells. (A) Lentiviral particles of TG2 siRNA (siTG2) or scrambled sequences (control) were transfected into Hc cells and selected by puromycin. The knockdown efficiency was evaluated by Western blotting. N stands for non-treated Hc cells, C stands for control (vector with scrambled sequences), and Si stands for siTG2. (B) Cell counting of control-transfected Hc cells (upper) and siTG2-transfected Hc cells (lower) after treatment with FFAs at different doses (0-200 µM) for 48 h. Representative results from three independent experiments are shown.
Figure 15. TG2 is revealed to be partially involved in FFAs-induced apoptosis by siTG2 knockdown in Hc cells. *In situ* TUNEL staining of apoptotic cells after treatment with 100 µM FFAs of control-transfected Hc cells (left) and siTG2-transfected Hc cells (right). Each averaged percentage of apoptotic cells was counted from four different fields of view and shown in the squares. Scale bar = 50 µm. Representative results from three independent experiments are shown.
Figure 16. Caspase 3 and caspase 9 were slightly inhibited by salubrinal. After treatment of Hc cells with 50 mM EtOH or 100 µM FFAs for 18 h in the absence or presence of 50 µM salubrinal, cytosolic extracts was prepared and protein levels of activated caspase 3, caspase 9, and GAPDH (cytosolic loading control) were estimated by Western blotting. C stands for control cells, E stands for 50 mM EtOH, FFAs stands for 100 µM FFAs and sal stands for 50 µM salubrinal. Representative results from three independent experiments that gave similar results are shown.
Figure 17. Homocysteine induced nuclear TG2 through NMDAR signaling.

(A) Homocysteine induces NMDAR signaling pathway. Modified from Kim AH et al.[31] (B) After treatment of Hc cells with 50 µM homocysteine in the absence or presence of MK (MK801) or ACPC, cytosolic and nuclear extracts were prepared and protein levels of nuclear TG2, NFκB, GAPDH (cytosolic loading control), and lamin B (nuclear loading control) were estimated by Western blotting. C stands for control cells. Representative results from three independent experiments that gave similar results are shown.
Figure 18. EtOH-induced nuclear TG2 is blocked by a RAR β/γ antagonist CD2665. (A) Retinoic acid receptor (RAR) signaling pathway. Modified from Malcolm Maden [32]. (B) After treatment of Hc cells with 50 mM EtOH in the absence or presence of NMDAR antagonists or CD2665, a RAR β/γ antagonist, cytosolic and nuclear extracts were prepared and protein levels of nuclear TG2, NFκB, GAPDH (cytosolic loading control) and lamin B (nuclear loading control) were estimated by Western blotting. C stands for control cells. Representative results from three independent experiments that gave similar results are shown.
Figure 19. A RAR $\beta/\gamma$ antagonist, CD2665, strongly inhibited EtOH-induced, but not FFAs-induced nuclear TG2 in Hc and HepG2 cells. Cytosolic and nuclear extracts were prepared after treatment of HepG2 and Hc cells with 50 mM EtOH or 100 $\mu$M FFAs for 18 h in the absence or presence of 10 $\mu$M CD2665. TG2, GAPDH (cytosolic loading control) and lamin B (nuclear loading control) levels were estimated using Western blotting. Relative changes in the densitometric profiles of nuclear TG2 levels are presented under corresponding bands after normalizing to the changes in lamin B and comparing to each control. C stands for control cells, E stands for EtOH, and CD stands for CD2665. Representative results from three independent experiments are shown.
Figure 20. CD2665 strongly inhibited EtOH-induced TG2 in Hc cells. Immunofluorescence stainings were performed after treatment of Hc cells with 50 mM EtOH in the absence or presence of 10 µM CD2665. Cells were fixed and stained with Hoechst 33258 (left columns), anti-TG2 antibody (middle columns), TRITC-conjugated streptavidin (right columns). Scale bar = 50 µm. Representative results from three independent experiments are shown.
Figure 21. Schematic diagram showing how FFAs induce nuclear TG2 via ER stress-mediated PERK and NFκB activation, resulting in hepatic apoptosis accompanying CLSp1, AIF and cytochrome c.
Figure 22. Detection of short form TG2 (TG2-S) using different TG2 antibodies. Cytosolic and nuclear extracts were prepared after treatment of Hc cells with 100 µM FFAs for 18 h. Purified TG2 from mice (P), cytosolic fraction (C) and nuclear fraction (N) were probed by different TG2 antibodies: home-made polyclonal antibody (Polyclonal), Fermont TG100 (TG100), Fermont 7402 (7402) and Cell Signaling Technology (CST). M stands for the protein marker. Black arrows indicate the full length TG2 (TG2-L) and blue arrows indicate TG2-S. Estimated molecular weight to each band was noted in the brackets. Representative results from three independent experiments that gave similar results are shown.
Figure 23. TG2-S appears to be the product from alternative splicing.
Cytosolic and nuclear extracts were prepared after treatment of Hc cells with 100 µM FFAs in the absence or presence of spliceostatin A (SSA) for 18 h. Acetylated SSA (AcSSA) represents inactivated analogue of SSA and served as control. Antibodies from Fermont 7402 (7402) and Cell Signaling Technology (CST) were used to detect TG2-S. Black arrows indicate the TG2-S. Representative results from three independent experiments that gave similar results are shown.
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